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Chemical regulation of body feather microbiota in a wild bird

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Abstract

The microbiota has a broad range of impacts on host physiology and behaviour, pointing out the need to improve our comprehension of the drivers of host microbiota composition. Of particular interest is whether the microbiota is acquired passively, or whether and to what extent hosts themselves shape the acquisition and maintenance of their microbiota. In birds, the uropygial gland produces oily secretions used to coat feathers that have been suggested to act as an antimicrobial defence mechanism regulating body feather microbiota. However, our comprehension of this process is still limited. In this study, we for the first time coupled high-throughput sequencing of the microbiota of both body feathers and the direct environment (i.e. the nest) in great tits with chemical analyses of the composition of uropygial gland secretions to examine whether host chemicals have either specific effects on some bacteria or non-specific broad-spectrum effects on the body feather microbiota. Using a network approach investigating the patterns of co-occurrence or co-exclusions between chemicals

and bacteria within the body feather microbiota, we found no evidence for specific pro or anti-microbial effects of uropygial gland chemicals. However, we found that one group of chemicals was negatively correlated to bacterial richness on body feathers, and a higher production of these chemicals was associated with a poorer body feather bacterial richness compared to the nest microbiota. Our study provides evidence that chemicals produced by the host might function as a non-specific broad-spectrum antimicrobial defence mechanism limiting colonization and/or maintenance of bacteria on body feathers, providing new insight about the divers of the host's microbiota composition in wild organisms.

Introduction

The microbiota, that is the diverse microbial communities inhabiting eukaryotic hosts, provides multiple essential functions for the host, from digestion and nutrient synthesis to protection against pathogens and can even mediate behaviour (Fukuda *et al.* 2011; Ezenwa *et al.* 2012; Yatsunenko *et al.* 2012; Jacob *et al.* 2014b; Roggenbuck *et al.* 2014; Jacob *et al.* 2015; Evans *et al.* 2017). It is also, however, associated with various diseases such as infections, obesity or diabetes (Ley *et al.* 2005; Wen *et al.* 2008; Turnbaugh & Gordon 2009; Evans *et al.* 2017), making our understanding of the drivers of microbiota composition of general importance. Numerous studies have documented the extensive variability in microbiota composition in animal hosts, both among individuals and among body parts of a given individual (Spor *et al.* 2011; Ding & Schloss 2014; Roggenbuck *et al.* 2014; Edwards *et al.* 2015). This variability can be attributed to factors such as host diet and genotype (Benson *et al.* 2010; Spor *et al.* 2011), with a significant proportion of the microbiota being acquired from the environment or diet and depend on the microbial colonization history of the host (Tannock 2007; Benson *et al.* 2010; Morgan *et al.* 2013; Roggenbuck *et al.* 2014). However, it remains unclear to what extent and by which mechanisms a host can control the acquisition and maintenance of its microbiota.

The microbiota of host body surfaces is particularly important since such surfaces are the first point of contact between the host and the environmental microbial pool, acting as an interface between the host and its environment (Kulkarni & Heeb 2007; Schommer & Gallo 2013). Unsurprisingly, body surfaces are home to abundant and diverse microbial communities (Schommer & Gallo 2013) and are suggested to play significant roles in host health (Fredricks 2001; Muletz *et al.* 2012; Schommer & Gallo 2013; Jacob *et al.* 2014a, 2015). Skin microbiota disturbance (i.e. dysbiosis), for example, has been linked with various diseases (Fredricks 2001; Hubálek 2004; Benskin *et al.* 2009; Schommer & Gallo 2013). Some studies have suggested interactive effects of host traits and environmental factors in driving skin microbiota (*reviewed in* Schommer & Gallo 2013). However, the factors responsible for microbiota richness and composition on a host's body surfaces remain poorly understood (Schommer & Gallo 2013), and still biased towards humans or laboratory animals. Given the relevance of the microbiota for the ecology and evolutionary biology of hosts (Ezenwa *et al.* 2012; McFall-Ngai *et al.* 2013; Jacob *et al.* 2015; Bestion *et al.* 2017; Evans *et al.* 2017; Leclaire *et al.* 2017), our comprehension of the drivers of microbiota composition must extend to non-model wild organisms to improve our understanding of the occurrence and mechanisms underlying such processes.

Almost all bird species possess an external gland, the uropygial gland, which produces oily secretions used to coat feathers. Well known for their waterproofing properties (Jacob & Ziswiler 1982) and their role in plumage signalling and communication (Piersma *et al.* 1999; Piault *et al.* 2008; Lopez-Rull *et al.* 2010), these secretions have also been suggested to function as an antimicrobial defence mechanism used to regulate bacterial communities on feathers (Shawkey *et al.* 2003; Martín-Vivaldi *et al.* 2009; Møller *et al.* 2009; Czirjak *et al.* 2013; Jacob *et al.* 2014b; Leclaire *et al.* 2014; Fülöp *et al.* 2016). Accordingly, by experimentally manipulating great tit (*Parus* major) microbiota, Jacob *et al.* (2014b) demonstrated that individuals modify the quantity and chemical composition of uropygial secretions produced depending on their exposure to bacteria. Similarly, captive feral pigeons (*Columba livia*) increase the quantity of secretions produced and time spent preening when exposed to experimentally increased feather bacterial loads (Leclaire *et al.* 2014). Furthermore, house

finch (*Carpodacus mexicanus*) secretions have been found to inhibit the *in vitro* growth of several isolated bacterial strains (Shawkey *et al.* 2003), but evidence for such inhibition effects on feather bacteria is mixed and mostly limited to *in vitro* studies (Czirjak *et al.* 2013; Giraudeau *et al.* 2013).

Given their suspected anti-microbial activity, secretions are expected to contain antimicrobial peptides, acids or alcohols, either directly produced by the birds or indirectly through a symbiosis with specific bacteria living in the gland (Soler *et al.* 2008; Martin-Vivaldi *et al.* 2010; Soler *et al.* 2010). However, most bird species produce secretions that are mainly composed of esters (Jacob & Ziswiler 1982; Reneerkens *et al.* 2002; Whittaker *et al.* 2010; Leclaire *et al.* 2011, 2012). As with all lipids, esters are energy stores that might be used by some microorganisms for growth (Shelley *et al.* 1953; Ara *et al.* 2006). Therefore, coating feathers with uropygial secretions could favour rather than inhibit the colonisation and maintenance of some microorganisms on feathers. Such a pro-microbial effect could help saturate the microbial niche on feathers by favouring commensal or mutualistic microorganisms and result in a limited colonisation or reduced activity of pathogenic microorganisms, or instead have negative consequences for the hosts if pathogenic bacteria exploit these esters to colonize and grow (Currie *et al.* 1999; Davis *et al.* 2007; Soler *et al.* 2010; Jacob *et al.* 2014b). Alternatively, these oily secretions might have non-specific broad-spectrum effects, acting as a physical barrier limiting colonization ability of any bacteria on feathers. Because body feathers are potentially exposed to a large diversity of environmental bacteria, selection might indeed favour the evolution of non-specific antimicrobial activities. Altogether, these studies suggest that uropygial secretions potentially function to control colonisation of feathers by environmental microorganisms (Shawkey *et al.* 2003; Martín-Vivaldi *et al.* 2009; Møller *et al.* 2009; Czirjak *et al.* 2013; Jacob *et al.* 2014b; Fülöp *et al.* 2016). However, our comprehension of the mechanisms behind this regulation remains in its infancy.

Here we aimed at advancing our understanding of the mechanisms underlying body surface microbiota regulation by investigating the relationships between host chemical production and their associated microbiota in a wild bird (great tits; *Parus major*), following previous work experimentally demonstrating that several phenotypic and fitness traits depend on the microbiota (Jacob *et al.* 2014b,

2015). To do so we coupled high-throughput sequencing of body feather and environmental (i.e. nest) bacterial communities with chemical analyses of the composition of uropygial gland secretions. We first compared the composition of body feather and nest bacterial communities. Birds indeed spend a significant amount of time in their nests during breeding. Bacteria from the nests thus constitute an important source of bacterial colonization of bird feathers (Jacob *et al.* 2014b). We then investigated the relationships between chemical compounds produced by the host uropygial gland and feather bacterial community composition and examined two, non-exclusive, hypotheses. First, gland chemicals might have specific effects on certain bacterial taxa, either favouring commensals or hindering pathogens. Under this hypothesis, we expected significant co-occurrence or co-exclusions between chemicals and certain bacteria within the body feather microbiota. Second, uropygial gland chemicals might have non-specific broad-spectrum effects, affecting the colonization or maintenance of any bacteria on feathers. For this second hypothesis, we tested for correlations between chemicals and the richness and composition of body feather bacterial communities.

We further investigated whether these chemicals could be involved in the acquisition of microbes from the environment by testing whether chemicals correlate with the differences in bacterial richness and composition between body feathers and the nest. Finally, males and females usually differ in their microbiota: female great tits spend more time in the nest, host higher bacterial loads and richness compared to males (Jacob *et al.* 2014b), and show higher investment in gland secretions (Jacob *et al.* 2014b). Here we thus investigated whether the sexes differ in their body feather bacterial communities, composition of gland secretions and in their chemical-microbiota relationships.

Methods

Studied populations

The study was performed during the 2013 reproductive period on two great tit populations breeding in woodcrete nest boxes in South-West France (Lauragais: 43° 39' N, 1° 54' E; Moulis: 42°58' N, 1°05' E). Nest boxes were checked at least twice a week from mid-March to detect the onset of nest construction, then everyday from its completion, allowing accurate determination of lay date. Adults were captured on the nest between 8 and 13 days after hatching, and were measured (wing to the nearest mm, tarsus to the nearest 0.1 mm and mass to the nearest 0.05 g) under permits to A.S. Chaine from the French bird ringing office (CRBPO; n°13619) and animal care permits from the state of Ariège (Préfecture de l'Ariège, Protection des Populations, n°A09-4) and the Région Midi-Pyrenées (DIREN, n°2012-07).

Microbiota sampling and analysis

Nest bacterial communities were sampled at day 3 post hatching using sterilized tweezers. Following Jacob et al. (2014), samples were taken from a standardized position in the centre of the nest cup. Each sample was placed in a separate sterile Eppendorf tube filled with 1ml Phosphate Buffer Saline (PBS), and stored at -20°C until lab analyses. During adult trapping in the nests, feather bacterial communities were sampled by collecting ~20 ventral feathers from each individual at a standardized position close to the left leg (Jacob *et al.* 2014b). As with nest samples, feathers from each bird were placed in separate 1ml PBS and stored at -20°C. All sampling and manipulations were made after systematically washing hands and materials with 70% ethanol to avoid cross contaminations.

Nest and feather samples were sonicated and vortexed to detach microorganisms from nest material and feathers (Møller *et al.* 2009; Czirják *et al.* 2010; Jacob *et al.* 2014b). Bacterial DNA was then extracted using the Promega extraction protocol (Promega, Fitchburg, WI, USA; for details see Carriconde *et al.* 2008). PCR reactions were performed in 30 µL volumes containing 3 µL of 1/10

diluted DNA extract, 1U of AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA), 2.5 mM of $MgCl_2$, 1x of Taq Buffer, 0.2mM of each dNTP and 4ng of bovine serum albumin (Promega, Fitchburg, WI, USA). PCR conditions consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation (95°C for 30 s), annealing (57°C for 30 s) and elongation (72°C for 30 s). The universal primer pair used specifically amplifies the v5-6 region (ca 295 bp length) of the bacterial 16S rRNA gene (BACTB-F: GGATTAGATACCCTGGTAGT; and BACTB-R: CACGACACGAGCTGACG; Fliegerova *et al.* 2014). To discriminate samples after sequencing, both forward and reverse primers were labelled at the 5' end with a combination of two different 8 bp tags. PCR products were purified using the QIAquick PCR purification Kit (Qiagen GmbH, Hilden, Germany) and pooled. Amplicon multiplex was prepared with the METAfast method and sequenced with an Illumina MiSeq platform using the 2x250 bp protocol (Fasteris SA, Plan-les-Ouates, Switzerland). PCR blank controls were included in the sequenced multiplex to detect and withdraw potential reagent contaminants.

The sequence reads were analyzed as recommended by Taberlet *et al.* (2012) with some adjustments using the OBITools package (Boyer *et al.* 2015). Briefly, after paired-end reads assembly, reads were assigned to their respective samples (respectively 0 and 2 mismatches allowed on tag and primer sites). Reads with low assembly scores or containing ambiguous bases (i.e. "N") were excluded. Strictly identical reads were dereplicated, and singletons (i.e. one single occurrence over the entire dataset) were removed. Potential PCR/sequencing errors were detected and removed using the *obiclean* algorithm (Boyer *et al.* 2015). The remaining sequences were then clustered into OTUs (Operational Taxonomic Units) based on their similarity using the *sumaclust* algorithm (Mercier *et al.* 2013; Kopylova *et al.* 2016), with a 97% similarity threshold. Next, we removed all OTUs with a total read abundance < 10 reads or detected in only one sample (Taberlet *et al.* 2012; Zinger *et al.* 2016). Finally, we used the PCR blank controls to remove contaminant OTUs from the data set (241 low-abundance OTUs removed out of 6413 OTUs). The most abundant sequences of each OTU were then taxonomically assigned using the RDPII classifier (Wang *et al.* 2007) with the RDPII database release 11 (May 2015; Table S2 & S3). We here considered a taxonomic assignment

as reliable when its probability (provided at each taxonomic level) was > 0.8. A total of 65 samples from adult great tits (43 females and 22 males) and 48 samples from nests have been sequenced in this study.

Gland chemical sampling and analysis

Uropygial gland secretions were sampled during adult trapping by draining the gland papilla with a glass capillary. Filled capillaries were then immediately transferred in 2ml sealed glass vials and stored at -20°C until extraction of organic compounds within 6 months. Following previously developed procedures (Martin-Vivaldi *et al.* 2010; Leclaire *et al.* 2011; Jacob *et al.* 2014b; Leclaire *et al.* 2017), samples were diluted in 500µl hexane, evaporated and then diluted in 150µl of dichloromethane and vortexed for 1min to extract organic chemical compounds. Analyses were performed on a mass spectrometer quadrupole detector (ISQ QD) coupled to a Trace 1300 gas chromatography (Thermo Fisher Scientific Inc) with a capillary column (Restek RTX-5MS 30 m × 0.25 mm, 0.25 µm film thickness, 5 % diphenyl and 95 % dimethylpolysiloxane) and a splitless injector (300 °C). Ionization was done by electron impact (70 eV, source temperature 250 °C). Helium was the carrier gas (1.2 ml/min). The oven temperature was initiated at 50 °C for 1 min. After the injection of the sample (1 µl), the oven was programmed to increase 10 °C/min to 240 °C, then at 5 °C/min to 300 °C, and held for 40min. The scan range of the mass spectrometer was 60 to 500 m/z. Blanks were regularly interspersed throughout the sample analyses. To generate composition matrices, resulting profiles were analysed using the *runGC* function (*metaMS* R-package; Wehrens *et al.* 2014), with 43 samples analysed manually and blindly using Xcalibur software to verify the resulting composition matrix. Since we cannot standardize the quantity of secretions sampled by the GC-MS, we used a matrix of intra-individual relative quantity of compounds in all analyses. A standard mixture of alkanes from n-C12 to n-C60 (Supelco, Sigma-Aldrich, 0.01% w/w for each component) was used as a reference for computing Kovats retention indices (KI). Then, compound identification was performed based on mass spectral fragmentation patterns and comparison with the NIST mass spectral library (Table S4).

Statistical analyses

All analyses were performed using R software (version 3.2.2; R Development Core Team 2008). We first used linear mixed models to compare bacterial and chemical richness between host sexes, along phenotypic traits (tarsus length, body mass, scaled mass index; Peig & Green 2009) and with lay date and populations defined as fixed effects, and with nest identity as a random factor (*lme*, *nlme* R-package). Then to investigate the role of uropygial gland chemicals for body feather microbiota, we constructed an association network (*igraph* R-package). This approach consists of exploring the connections between items (i.e. proteins, individuals, species) by drawing the association structure of these items based on their presence/absence or abundances in a series of samples (Barberán *et al.* 2012; Faust *et al.* 2012; Faust & Raes 2012). This approach is now often used in microbiota research and helps investigate the potential interactions between microbial taxa from large microbial datasets generated by high-throughput sequencing (Barberán *et al.* 2012; Faust *et al.* 2012; Faust & Raes 2012). Here we constructed a network comprising both bacterial OTUs and feather chemical compounds to explore the potential interactions between chemicals produced by the uropygial gland and the bacteria present on bird body feathers. Following previous key studies using this approach, pairwise associations were inferred from Spearman's rank correlations between bacteria and chemical relative abundances. We excluded correlation coefficients with absolute values lower than 0.6 as these are often reported to be unreliable (Barberán *et al.* 2012). Correlations were obtained from bacteria and chemical relative abundances across samples, which were expressed as the percent abundance of OTUs/chemicals in each sample.

Chemicals from the uropygial gland might act individually or synergistically on the microbiota. To assess this potential synergistic effect we constructed a second network comprising only gland chemicals to define groups of chemicals co-occurring in the samples based on relative abundances. Modules of chemical compounds were obtained with the walktrap algorithm, a community detection algorithm that allows definition of densely connected subgraphs through random walks (*igraph* R-package; Pons & Latapy 2006), with Spearman correlations >0.6 (Barberán *et al.* 2012). Negative correlations between chemical relative abundances were considered equivalent to no correlation in

this analysis, allowing us to split chemicals that negatively co-occur in different modules. Because our aim was to test for correlations between these groups of chemicals and the richness and composition of the bacterial communities (see below), we discarded chemical modules present in less than 30% of samples, as these may lead to spurious associations (Faust *et al.* 2012).

To investigate whether uropygial gland chemicals have broad-spectrum effects on body feather bacterial communities, we tested for correlations between the modules of uropygial gland chemicals and body feather bacterial richness (i.e. number of OTUs), with modules of chemicals calculated as the summed relative abundance of all chemical compounds included in a given module. We used linear mixed models followed by a backward selection procedure. We defined log-transformed bacterial richness (i.e. the number of OTUs in a sample) as the dependent variable, modules of chemicals as explanatory variables, and included host phenotype (i.e. body mass and tarsus length), sex, lay date and population to control for host and environmental particularities. Nest identity was defined as a random factor (*lme*, *nlme* R-package). Models were checked for normality and homoscedasticity of residuals. We additionally performed these analyses using the Scaled Mass Index as a measure of bird phenotype (Peig & Green 2009).

We then investigated whether host chemicals might act as a filter between environmental bacterial communities and body feathers. To do so, we computed the difference between bacterial richness in nests and body feathers as $Bacterial\ richness_{nest} - Bacterial\ richness_{feather}$, expressed in number of OTUs. The values of this metric hence increase when bacterial richness in the nests increase compared to the feathers, meaning a reduced proportion of environmental bacterial colonizing body feathers. We used this difference as a dependent variable in linear mixed models, with modules of chemicals as explanatory variables, host phenotype, sex, lay date and population as covariates/cofactors, and nest identity as a random factor. Models were checked for normality and homoscedasticity of residuals. Finally, to test for general differences in bacterial community composition between body feather and nests, and for effects of chemicals on body feather bacterial

community composition, we used PERMANOVA (i.e. non-parametric multivariate analysis of variance; *adonis, vegan* R-package) using relative abundance based Bray-Curtis dissimilarity and 999 permutations, with nests as a *strata* argument within which to constrain permutations.

Results

Body feather and environmental microbiota

Females hosted higher bacterial richness on their body feathers than males (females: mean ± SE = 262.02 ± 18.72 OTUs; males: 184.73 ± 19.01; estimate ± SE = 0.32 ± 0.11; $t_{1,65} = 2.94$; $p = 0.009$), and both sexes were home to lower bacterial richness compared to the nest microbiota (333.60 ± 23.22 OTUs in nests; estimate ± SE = -0.34 ± 0.08; $t_{1,113} = 4.20$; $p < 0.001$). We found no significant correlation between bird phenotype and body feather bacterial richness (tarsus length: estimate ± SE = 15.89 ± 23.85; $t_{1,65} = 0.67$; $p = 0.52$; body mass: estimate ± SE = -2.05 ± 12.86; $t_{1,65} = -0.16$; $p = 0.88$; scaled mass index: estimate ± SE = -3.96 ± 11.44; $t_{1,65} = -0.35$; $p = 0.74$). As expected since females spend more time in nests than males, the difference in bacterial richness between nests and body feathers was higher in males (sex*location interaction: estimate ± SE = 0.35 ± 0.12; $t_{1,111} = 2.89$; $p = 0.005$; females: estimate ± SE = -0.23 ± 0.09; $t_{1,43} = 2.59$; $p = 0.013$; males: estimate ± SE = -0.58 ± 0.10; $t_{1,22} = 6.06$; $p < 0.001$).

Bacterial richness in the nests significantly increased with lay date (estimate ± SE = 8.48 ± 3.7; $t_{1,65} = 2.29$; $p = 0.026$) and differed between populations (estimate ± SE = 200.80 ± 43.30; $t_{1,65} = 4.64$; $p < 0.001$), but appeared unaffected by bird phenotype (tarsus length: estimate ± SE = 16.34 ± 31.13; $t_{1,65} = 0.53$; $p = 0.60$; body mass: estimate ± SE = -16.47 ± 19.89; $t_{1,65} = -0.83$; $p = 0.41$; scaled mass index: estimate ± SE = -1.56 ± 8.17; $t_{1,65} = -0.19$; $p = 0.85$).

Male and female body feather microbiota composition differed from nest microbiota (Fig 1; $F_{1,112} = 13.20$; $p = 0.001$), and body feather microbiota composition differed between sexes ($F_{1,64} = 2.32$; $p = 0.006$). This difference in composition between nest and body feathers differed between sexes

slightly but not significantly ($F_{1,112}$ = 1.32; p = 0.080). Both nest and body feather microbiota were mainly composed of Actinobacteria, Proteobacteria, Bacteroidetes, Firmicutes and unidentified OTUs (Fig 2), but body feathers host more γ-Proteobacteria and unidentified Proteobacteria than nests, and fewer Actinobacteria and unidentified OTUs (Fig 2).

Chemical composition of the uropygial gland secretions

Our analyses revealed the presence of 121 chemical compounds in great tit uropygial gland secretions, with individuals showing a mean chemical richness of 34.95 ± 0.68 compounds. There was no significant difference in chemical richness (i.e. the number of chemical compounds in a sample) between sexes (estimate ± SE = 1.92 ± 1.63; $t_{1,65}$ = 1.18; p = 0.25) nor by phenotypic traits (tarsus length: estimate ± SE = -0.89 ± 1.48; $t_{1,65}$ = -0.60; p = 0.56; body mass: estimate ± SE = 0.26 ± 0.80; $t_{1,65}$ = 0.32; p = 0.75; scaled mass index: estimate ± SE = 0.007 ± 0.023; $t_{1,65}$ = 0.29; p = 0.77), but chemical richness significantly differ between populations (5.00 ± 1.79; $t_{1,65}$ = 2.79; p = 0.008). Among these chemicals, 76.99 ± 1.51 % (mean ± SE) were esters, 3.16 ± 0.52 % were acids, 0.46 ± 0.12 % were alcohols, and 19.37 ± 1.52 could not be identified. Females showed a higher proportion of acids than males (estimate ± SE = 0.03 ± 0.01; $t_{1,65}$ = 3.16; p = 0.005; p>0.05 for all other types of compounds).

Specific effects of chemicals?

We used a network approach (Barberán *et al.* 2012; Faust *et al.* 2012; Faust & Raes 2012) to explore specific relationships between chemical compounds and feather bacteria. Drawing a network including Spearman rho correlation coefficients of absolute values higher than 0.6, we found no evidence for specific relationships between gland chemicals and specific bacteria (Fig 3). First, we did not find any negative correlations between pairs of chemicals and bacteria. Second, only some chemical compounds co-occurred with bacteria, but they were among the rarest compounds in the

dataset (Fig 3). These results suggest that great tits do not produce antimicrobial compounds with specific effects on certain bacteria, either favouring commensals or hindering pathogens in their uropygial secretions.

Broad-spectrum effects of chemicals?

Using a network approach to summarize the chemical compounds into modules of chemicals that co-occur in the samples, we retained 10 modules with >30% prevalence in the dataset (Fig S1). Testing for correlations between these groups of compounds and body feather microbiota, we found that one group was negatively correlated to bacterial richness (estimate ± SE = -0.87 ± 0.25; $t_{1,65}$ = -3.55; p = 0.003; Fig 4). We did not find a significant interaction between this group of compounds and sex on feather bacterial richness ($t_{2,65}$ = -0.32; p = 0.75), but male secretions contained significantly more of these compounds than female secretions relative to other compounds (estimate ± SE = 0.23 ± 0.05; $t_{1,65}$ = 4.67; p < 0.001). Moreover, this negative correlation occurred in both studied populations, and was stronger in the population where body feather bacterial richness was higher (module * area interaction: $F_{2,65}$ = 9.24; p = 0.008; Lauragais: estimate ± SE = -436.08 ± 103.85; $t_{1,26}$ = -4.20; p = 0.004; Moulis: estimate ± SE = -123.23 ± 46.06; $t_{1,39}$ = -2.68; p = 0.025).

This module contained 18 chemicals (16 esters, 1 acids and 1 unidentified compounds; *highlighted in grey in* Fig S1; Tab S4), and post-hoc analyses reveal that it was negatively correlated with OTU richness of each major bacterial phylum (linear mixed model: module * phylum interaction: $F_{8,585}$ = 1.59; p = 0.12). We further tested whether the negative relationship with these chemicals came from an indirect effect of host condition, as condition might itself affect both feather microbiota and investment in gland compounds. We did not find significant effects of phenotypic traits on the relative abundance of these chemicals (linear mixed model: tarsus length: $t_{1,65}$ = 2.61; p = 0.13; body mass: $t_{1,65}$ = 1.98; p = 0.18; scaled mass index: $t_{1,65}$ = 0.29; p = 0.77), nor differences between populations ($t_{1,65}$ = 0.53; p = 0.60). Finally, chemical modules did not significantly correlate with body feather microbiota composition (PERMANOVA; all p>0.1).

Gland chemicals and the relationship between feather and environmental microbiota

We then tested whether uropygial gland secretions are involved in the acquisition of bacteria from nest microbiota. First, we found that the relative abundance of the module of chemicals linked to lower body feather bacterial richness was also positively correlated with the difference between nest and body feather bacterial richness (estimate ± SE = 289.55 ± 75.91; $t_{1,65}$ = -3.81; p = 0.001; Fig 5; no significant effect for other modules, all p>0.05). This module was negatively correlated to OTU richness in each major bacterial phylum (Table S1), suggesting a potential broad-spectrum effect as found for body feather microbiota. This means that the more birds invested in the production of these chemical compounds, the lower was the bacterial richness on feathers was compared to their nests (Fig 5). This correlation appeared stronger in the population where body feather bacterial richness was higher (module * area interaction: $F_{2,65}$ = 8.25; p = 0.011; Lauragais: estimate ± SE = 531.67 ± 144.73; $t_{1,26}$ = 3.67; p = 0.008; Moulis: estimate ± SE = 122.89 ± 64.37; $t_{1,39}$ = 1.91; p = 0.089), despite no significant difference between populations in investment in this module (see above). Finally, we found no significant correlation between chemical modules and the difference of microbiota composition between nests and feathers (abundance-based Bray-Curtis dissimilarity; all p > 0.10). Importantly, bacterial richness in the nests did not significantly correlate with the relative abundance of gland chemical modules (all p > 0.1), suggesting that these chemicals play a role in determining body feather microbiota with no further direct or indirect effect on environmental microbiota.

Discussion

In this study, we coupled high-throughput sequencing of both host body surface and nest bacterial communities with chemical analyses of the composition of great tit uropygial gland secretions to examine the role host chemicals might play in the regulation of feather microbiota. In this species, the time spent nest building, incubating and nestling rearing exposes adults to the dense and diverse nest microbiota (Saag *et al.* 2011; Kilgas *et al.* 2012; Jacob *et al.* 2014b), which in turn is expected to

colonize body feathers and potentially affect health and reproduction (Jacob *et al.* 2015). Indeed, experimental modifications of the nest microbiota have been shown to lead to changes of feather microbiota (Jacob *et al.* 2014b), and had significant consequences for host phenotype and fitness (Jacob *et al.* 2015). Coating feathers with secretions from the uropygial gland might allow a bird to control colonisation of body feathers by environmental microorganisms to some degree (Shawkey *et al.* 2003; Møller *et al.* 2009; Ruiz-Rodriguez *et al.* 2009b; Czirjak *et al.* 2013; Jacob *et al.* 2014b; Fülöp *et al.* 2016).

Oily uropygial secretions could contain antimicrobial chemicals inhibiting the growth of specific microorganisms, or pro-microbial compounds favouring some commensal or mutualistic bacteria (Shawkey *et al.* 2003; Møller *et al.* 2009; Soler *et al.* 2010). However, we found no evidence for specific effects of uropygial gland chemicals favouring commensals or hindering pathogens. First, we did not find any significant negative correlations between pairs of chemicals and bacteria (Fig 3), suggesting that great tits do not produce narrow-spectrum antimicrobial compounds in their uropygial secretions. Second, only some chemical compounds co-occurred with bacteria. These were among the rarest compounds in the secretions (Fig 3) and were in turn correlated with rare and relatively isolated bacteria in the network (Fig 3). We further cannot rule out that these correlations only appeared by chance given the large number of correlations considered in network approaches (Barberán *et al.* 2012; Faust *et al.* 2012; Faust & Raes 2012). The presence of specific bacteria on the hosts might also induce the production of these compounds by the birds, or could be the result of the degradation of more complex chemical compounds into sub products by these bacteria, which would explain these rare co-occurrences (Jacob *et al.* 2014b).

Chemicals coated on feathers might otherwise have broad-spectrum effects, either by favouring the maintenance of a diverse microbiota or on the contrary by acting as a physical barrier limiting colonization ability of any bacteria on feathers. Indeed, because body feathers are potentially exposed to a large diversity of environmental bacteria, selection might indeed favour the evolution of non-specific antimicrobial actions. In this species for instance, a recent experimental study showed that the overall bacterial load in nests, but not microbiota composition, affects parental investment, costs of

reproduction and nestling condition (Jacob *et al.* 2015). After summarizing the chemical community data into modules of chemicals that co-occur in the samples, we found that one module of chemicals was negatively correlated to bacterial richness on body feathers (Fig 4). Furthermore, the more birds invested in these chemicals, the poorer body feather bacterial richness was compared to the nest microbiota (Fig 5), a relationship observed within all major bacterial phyla. Our results suggest that these chemical compounds produced by the birds might function as a non-specific broad-spectrum antimicrobial defence mechanism limiting colonization and/or maintenance of bacteria on body feathers (Shawkey *et al.* 2003; Møller *et al.* 2009; Ruiz-Rodriguez *et al.* 2009b; Czirjak *et al.* 2013; Jacob *et al.* 2014b; Fülöp *et al.* 2016).

Among the 18 chemicals included in the anti-microbial module, 14 were esters, 2 were acids and 2 remained unidentified. Esters are oily substances that are unlikely to have a direct antimicrobial effect, but could form an oily physical barrier limiting colonization of bacteria from the environment. Alternatively, the presence of acidic compounds in the uropygial gland secretions might explain the broad-spectrum antimicrobial action observed. In humans, differences between men and women in skin acidity has been suggested to be a potential explanation for the higher skin microbiota diversity on women (Fredricks 2001; Giacomoni *et al.* 2009; Schommer & Gallo 2013). Furthermore, men have higher sebum production than women, a substance that consists mostly of acids and esters (Giacomoni *et al.* 2009). Here we found that female great tits showed a higher proportion of acidic compounds in their uropygial gland secretions compared to males in addition to a previously found higher production of secretions (Jacob *et al.* 2014b). However, the module of chemicals we identified to be negatively correlated with body feather bacterial richness represented a larger fraction of secreted chemicals in males than in females. Such sex-specific investment in coating body feathers with gland secretions might thus explain the observed bacterial richness differences between males and females. Sexes indeed significantly differed in the richness and composition of their microbiota. Furthermore, males and females usually differ in their investment in reproduction, a physiologically demanding activity that can increase oxidative stress and is associated with increased susceptibility to infection by parasites (Sheldon & Verhulst 1996; Monaghan *et al.* 2009; Metcalfe & Monaghan

2013). Interestingly, such differences in reproductive strategies and associated costs might in part result from sex differences in microbiota richness and composition. For instance, the microbiota has been found responsible for a significant part of the oxidative costs of reproduction in this species, and sexes differ in their investment in antimicrobial defence mechanisms (Jacob *et al.* 2014b, 2015). Deciphering the relative role of the microbiota in sex-specific reproductive investment might thus help explain the evolution of sexual dimorphism and sex-specific reproductive strategies.

So far we have assumed that uropygial secretions influence the microbiota, but an alternative hypothesis is that changes in environmental or feather microbiota resulted in modification of the chemical composition of uropygial secretions produced (Jacob *et al.* 2014b). However, we found no significant correlation between uropygial gland chemicals and the nest microbiota as would be expected under this hypothesis. Although experimentally investigating how these gland chemicals affect the colonization and maintenance of feather microbiota is an important next step, our results strongly suggest that great tit preening behaviour plays a role in the colonization of feathers by environmental microorganisms.

Interestingly, our study revealed that both body feather bacterial richness and uropygial gland chemical richness differ between the two populations we studied. Furthermore, the relationship between increased investment in specific chemical modules and lower body feather bacterial richness compared to the nest was stronger in the population where bacterial richness was the highest. Empirical studies have provided evidence for variability of microbiota composition along environmental gradients, such as for instance along temperature, altitude, vegetation diversity and cover (Fierer 2017; Thompson *et al.* 2017; Delgado-Baquerizo *et al.* 2018), and in response to climate warming (Bestion *et al.* 2017). When facing such differences in microbial exposure, hosts are expected to adjust their investment in antimicrobial defences, as experimentally demonstrated in this species (Jacob *et al.* 2014b). Contrasts between populations in uropygial gland chemicals might thus be one way in which hosts deal with different microbial environments. Although our work was limited to two populations, this result points out the potential importance of breeding habitat characteristics in shaping the microbiota and host-microbiota interactions (Lucas & Heeb 2005; Ruiz-

Rodriguez *et al.* 2009a), and urge the need for experimental approaches to shed light on how hosts and their microbiota will respond to environmental changes (Jacob *et al.* 2015; Bestion *et al.* 2017).

Developing our knowledge of the drivers of a host's microbiota composition is of major importance for the comprehension of host-microbe interactions and their consequences for the hosts' ecology and evolution (Benson *et al.* 2010; Fukuda *et al.* 2011; Spor *et al.* 2011; Ezenwa *et al.* 2012; Sullam *et al.* 2012; Yatsunenko *et al.* 2012). Here, we found no evidence for specific effects of chemical compounds on body feather microbiota, but rather that chemicals produced by the host uropygial gland might function as a broad-spectrum antimicrobial defence mechanism limiting colonization of feathers by environmental bacteria. Given the known consequences (positive or negative) of the microbiota for the host (Fukuda *et al.* 2011; Ezenwa *et al.* 2012; Gilbert *et al.* 2012; Yatsunenko *et al.* 2012), including in this species (Jacob *et al.* 2015), further studies should investigate the detailed mechanisms underlying this defence mechanism, its potential plasticity (Whittaker *et al.* 2011; Jacob *et al.* 2014b) and costs and benefits of such a broad-spectrum microbial regulation.

Author contribution

SJ, LS, ASC, and PH defined the research project and set up the sampling protocol. LS, ASC, AR and PH performed the fieldwork and sampling. SJ and LZ performed molecular and bioinformatics analyses. SJ, CD and LB performed the chemical analyses. SJ analysed the data, with the help LZ. SJ wrote the first draft of the manuscript, and all authors contributed to manuscript editing.

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Data availability

The data supporting the findings of this study are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.k36t400

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Figure 1: Male and female body feather microbiota composition differ from nest microbiota (NMDS analysis using relative abundance based Bray-Curtis dissimilarity; stress value 0.18; PERMANOVA: $F_{1,112} = 13.20$; $p = 0.001$). Circles represent scatter diagrams (*s.class* function, *ade4* R-package; inertia ellipse size coefficient = 1).

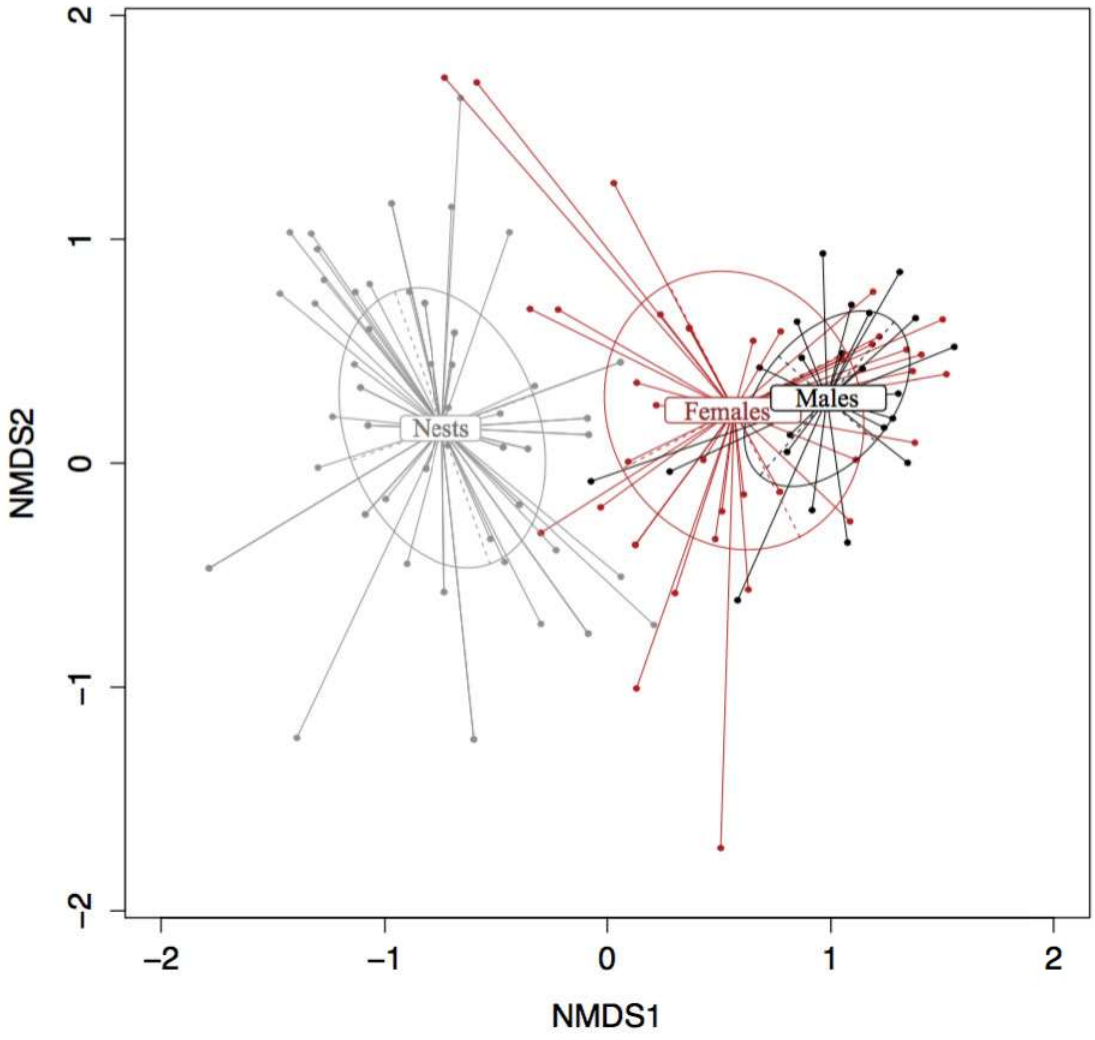

Figure 2: Bacterial composition of nests and body feather microbiota. Nest and body feather microbiota are mainly composed of Actinobacteria, Proteobacteria, Bacteroidetes, Firmicutes and unidentified OTUs, but body feathers host more γ-Proteobacteria and unidentified Proteobacteria and less Actinobacteria and unidentified OTUs than the nests. Mean ± SE of the relative abundance of the major phyla are shown. Proteobacteria are split in subphyla, and unidentified OTUs are included as a separate group. Letters indicates when phylum relative abundance significantly differs between categories (linear mixed models with nest as random factor and Bonferroni multiple testing correction of p values).

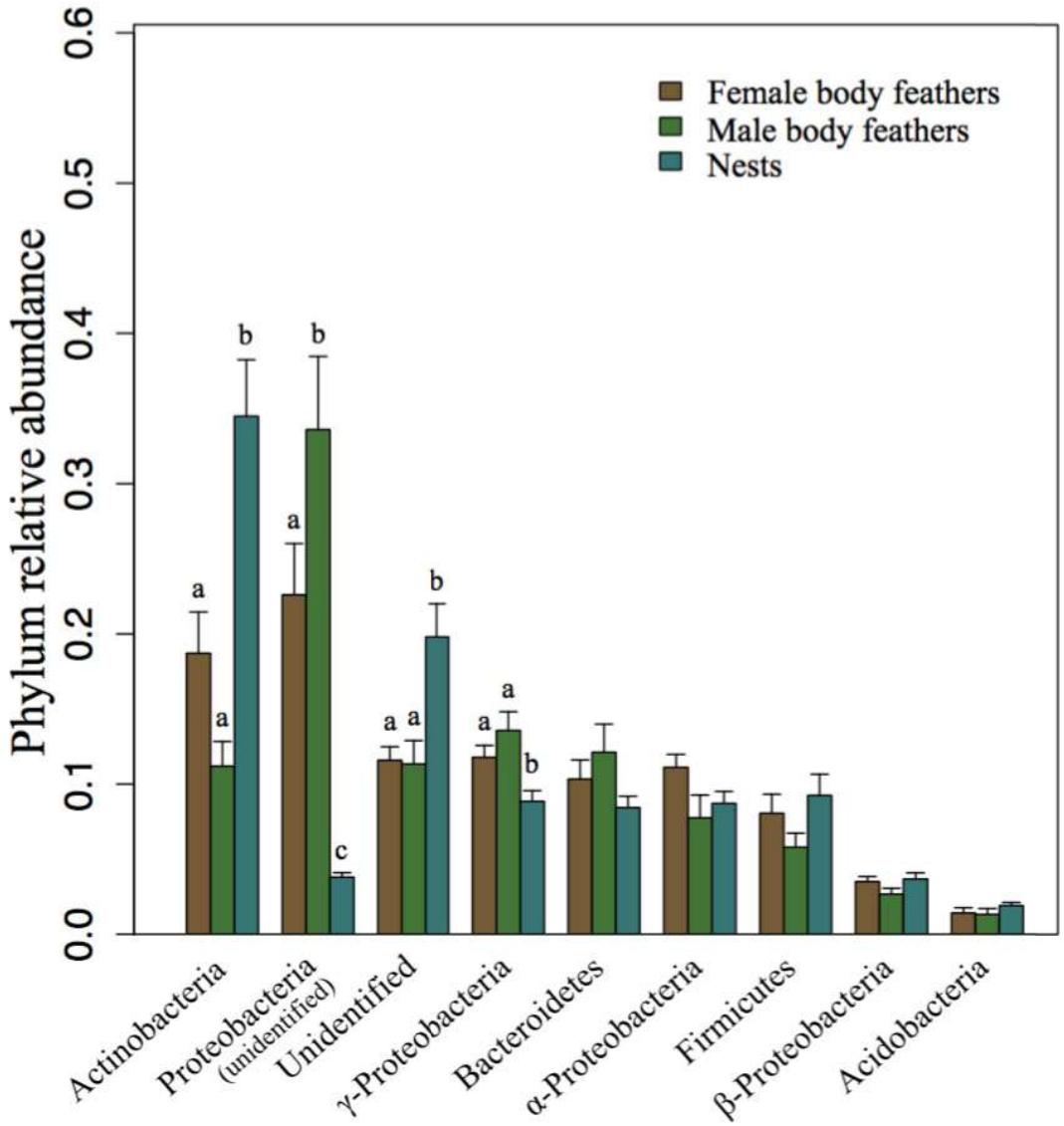

Figure 3: Co-occurrence network of uropygial gland chemicals (*dark grey*) and feather bacterial OTUs (*light grey*). Connections represent Spearman correlations >|0.6|. Strong positive co-occurrences appear in grey, negative co-occurrences in red (only in the upper left, between chemical compounds). Network graph was built using Fruchterman-Reingold layout (igraph R-package; iterations = 500, maximum change = number of OTUs/chemicals, cooling exponent = 3; Barberán *et al.* 2012). Node size is proportional to bacteria or chemical prevalence in the dataset.

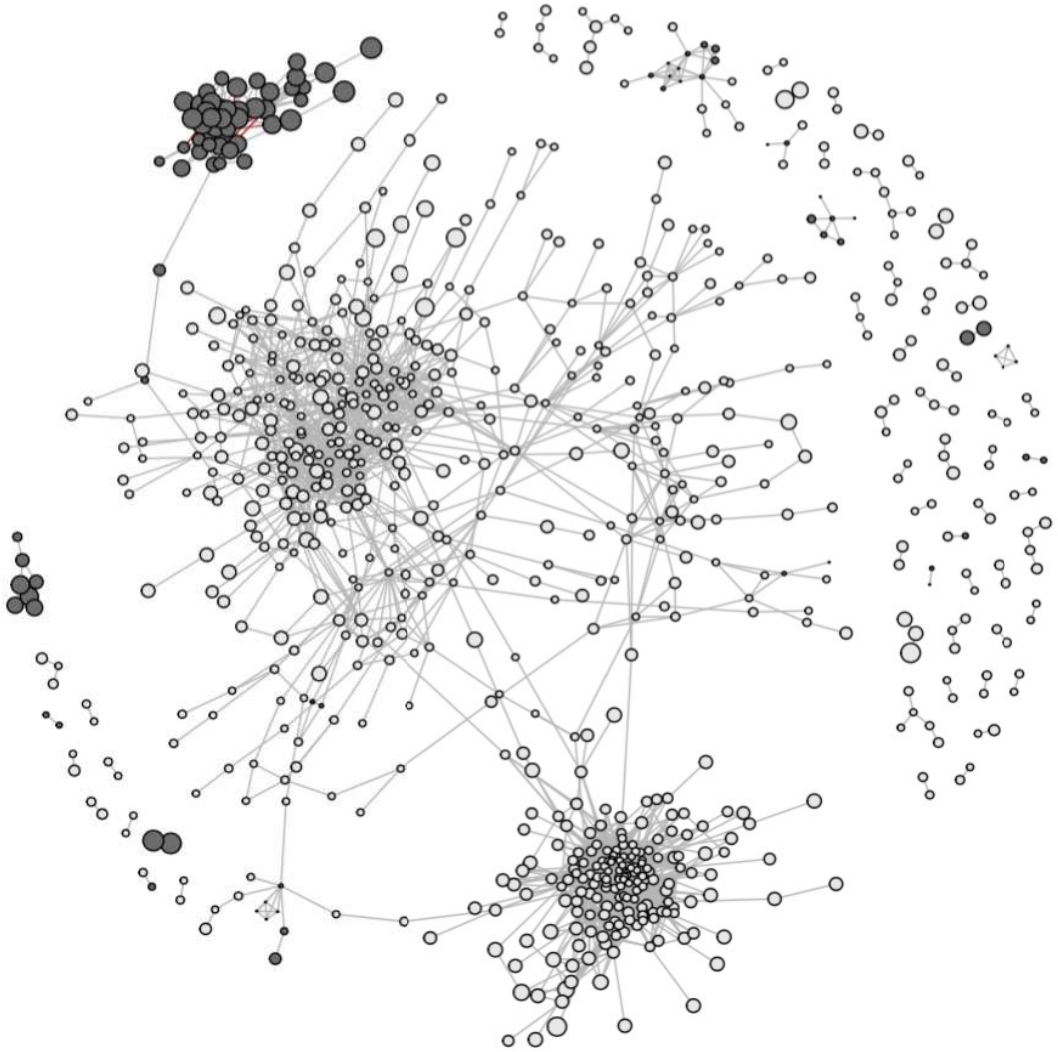

Figure 4: Bacterial richness on great tit body feathers is negatively correlated to a module of chemical compounds produced by the uropygial gland. The line shows predicted values from a linear mixed model including module abundance as explanatory variable and nest identity as a random effect; the grey area represents the standard error of model predictions.

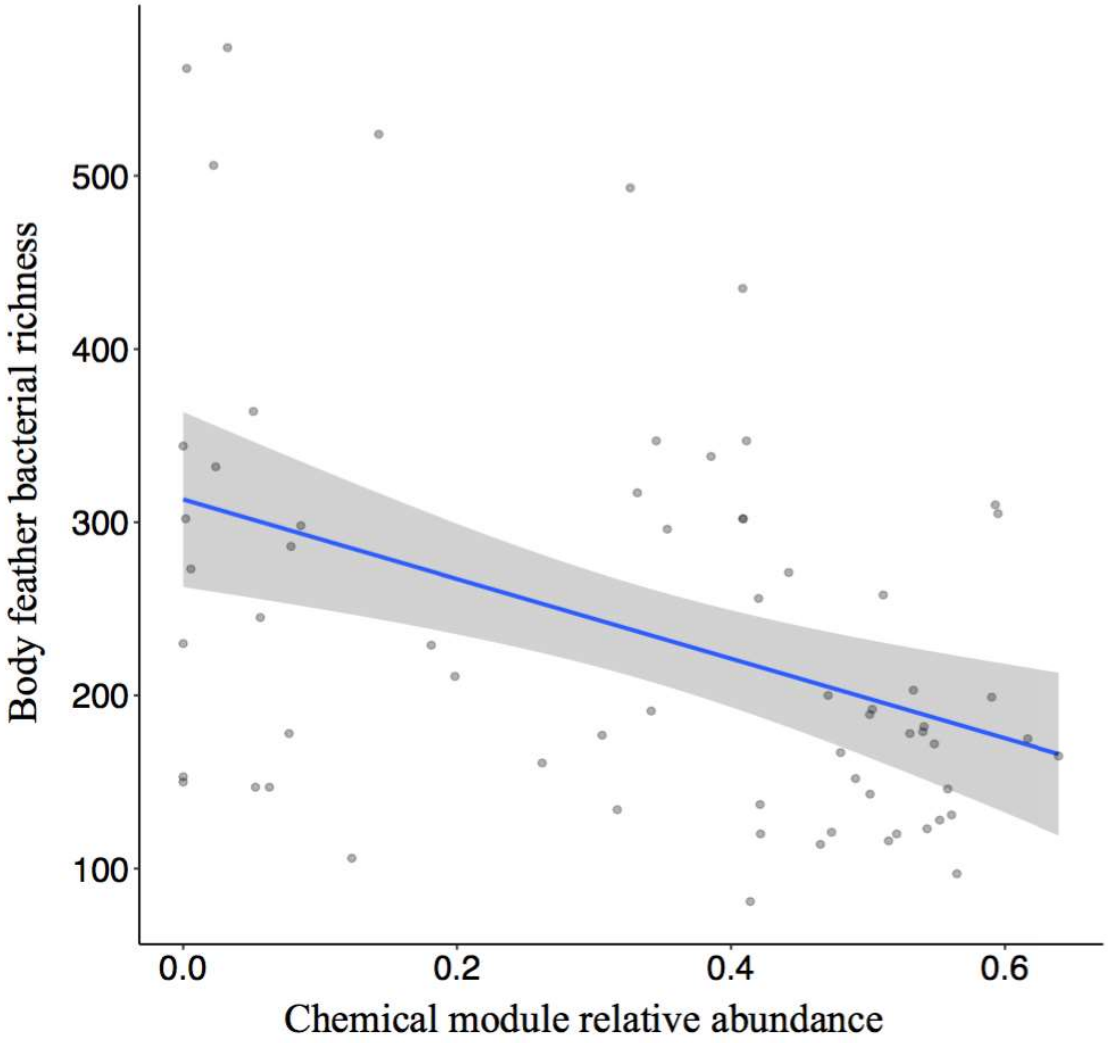

Figure 5: The difference in bacterial richness between nest and body feathers increases with the abundance of a module of chemical compounds produced by the uropygial gland. The line shows predicted values from a linear mixed model including cocktail abundance as explanatory variable and nest identity as a random effect; the grey area represents the standard error of model predictions. The dashed line represents no difference between nest and feathers in bacterial richness, and feathers host fewer bacteria than nests when points are above this line.