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# Preen secretions encode information on MHC similarity in certain sex-dyads in a monogamous seabird

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**Animals are known to select mates to maximize the genetic diversity of their offspring in order to achieve immunity against a broader range of pathogens. Although several bird species preferentially mate with partners that are dissimilar at the major histocompatibility complex (MHC), it remains unknown whether they can use olfactory cues to assess MHC similarity with potential partners. Here we combined gas chromatography data with genetic similarity indices based on MHC to test whether similarity in preen secretion chemicals correlated with MHC relatedness in the black-legged kittiwake (*Rissa tridactyla*), a species that preferentially mates with genetically dissimilar partners. We found that similarity in preen secretion chemicals was positively correlated with MHC relatedness in male-male and male-female dyads. This study provides the first evidence that preen secretion chemicals can encode information on MHC relatedness and suggests that odor-based mechanisms of MHC-related mate choice may occur in birds.**

The major histocompatibility complex (MHC) is an extraordinarily diverse cluster of genes that play a major role in vertebrate adaptive immunity. MHC heterozygosity offers several fitness advantages, including increased disease resistance and survival[1,2]. Given that MHC-dissimilar parents are more likely to produce MHC heterozygous offspring, mate choice of MHC-disparate partners is a common strategy in taxa as diverse as mammals, fish, lizards and birds[3–7].

In numerous species, MHC similarity is assessed through olfactory cues[8]. For example, rodents, humans and lizards prefer the odour of MHC-dissimilar individuals[4,9,10]. However, while several birds preferentially reproduce with MHC-dissimilar partners[7,11,12], it remains unknown whether birds can assess MHC relatedness via odour cues. The few studies that have shown that birds can discriminate between their relatives and non-relatives based on odour cues have relied on pedigree relatedness[13–15]. Although pedigree is a reliable estimator of genetic relatedness and is useful when investigating inbreeding avoidance[16], MHC relatedness may be a more direct link between odor and genotype that has not yet been explored in birds.

In a previous study, we had shown that preen secretion chemicals can reflect genetic relatedness at microsatellite loci in the black-legged kittiwake (*Rissa tridactyla*)[17], a species that preferentially mates with genetically dissimilar individuals[18]. In contrast to MHC, microsatellites, being neutral markers, are not under selection for high levels of polymorphism. Microsatellite diversity is therefore usually lower than MHC diversity across populations and correlation between microsatellites and MHC may be weak[19–21]. Showing that odour is linked to MHC is therefore an important step in the study of odour-based mate choice and immunology in birds. Here, we tested whether the chemical composition of kittiwake scent secretion was related to variation at the MHC, by combining gas chromatography data with indices of relatedness based on MHC.

## Results

**MHC characteristics.** We isolated a maximum of four MHC alleles per individual (range: 2–4 alleles; mean = 3.2 ± 0.7 SD alleles), indicating that we amplified duplicated MHC Class II DRB loci. These two loci were highly polymorphic with 23 alleles being isolated from the 39 individuals (Table 1). The amino acid sequences contained

Table 1 | MHC Class II DRB alleles isolated in this study and their corresponding Genbank accession numbers. Alleles have more than one accession number in cases where they are identical to multiple previously published sequences within the 258 bp fragment used in this study. In these cases, the previously published sequences only differ from each other outside the exon 2 fragment characterised here

| Allele ID | Accession number | Study |
|---|---|---|
| Ritr_MHC01 | EU326254 | Mulard *et al.*, unpublished |
| | HQ822398 | Serbielle *et al.*, unpublished |
| | HQ822408 | Serbielle *et al.*, unpublished |
| | HQ822414 | Serbielle *et al.*, unpublished |
| Ritr_MHC02 | HQ822399 | Serbielle *et al.*, unpublished |
| Ritr_MHC03 | KJ210580 | This study |
| Ritr_MHC04 | KJ210581 | This study |
| Ritr_MHC05 | HQ822402 | Serbielle *et al.*, unpublished |
| Ritr_MHC06 | EU326259 | Mulard *et al.*, unpublished |
| | HQ822439 | Serbielle *et al.*, unpublished |
| Ritr_MHC07 | KJ210582 | This study |
| Ritr_MHC09 | KJ210583 | This study |
| Ritr_MHC10 | HQ822419 | Serbielle *et al.*, unpublished |
| Ritr_MHC11 | HQ822460 | Serbielle *et al.*, unpublished |
| | HQ822472 | Serbielle *et al.*, unpublished |
| Ritr_MHC18 | HQ822413 | Serbielle *et al.*, unpublished |
| Ritr_MHC23 | HQ822406 | Serbielle *et al.*, unpublished |
| | HQ822443 | Serbielle *et al.*, unpublished |
| Ritr_MHC24 | HQ822442 | Serbielle *et al.*, unpublished |
| Ritr_MHC25 | KJ210586 | This study |
| Ritr_MHC27 | HQ822446 | Serbielle *et al.*, unpublished |
| | HQ822459 | Serbielle *et al.*, unpublished |
| Ritr_MHC28 | HQ822423 | Serbielle *et al.*, unpublished |
| Ritr_MHC30 | KJ210587 | This study |
| Ritr_MHC31 | KJ210588 | This study |
| Ritr_MHC32 | KJ210589 | This study |
| Ritr_MHC33 | HQ822440 | Serbielle *et al.*, unpublished |
| | HQ822462 | Serbielle *et al.*, unpublished |
| Ritr_MHC34 | KJ210590 | This study |
| Ritr_MHC35 | HQ822430 | Serbielle *et al.*, unpublished |
| Ritr_MHC36 | KJ210591 | This study |

characteristic features of functional class II molecules including conserved residues and putative peptide binding regions (Fig. 1). We found no stop codons or frame shift mutations in any allele. The putative peptide binding regions contained 47 segregating sites and a nucleotide diversity ($\pi$) of 0.18. The putative non-peptide binding regions contained 24 segregating sites and a nucleotide diversity of 0.05. Codons that were located within putative peptide binding regions had an excess of non-synonymous substitutions ($dN = 0.277 \pm 0.054$, $dS = 0.074 \pm 0.059$; $Z = 2.960$, $p = 0.002$), indicating positive selection. This was not the case for codons outside the putative peptide binding regions ($dN = 0.058 \pm 0.018$, $dS = 0.041 \pm 0.017$; $Z = 0.665$, $p = 0.254$). Up to three cDNA sequences were isolated per individual confirming that both loci were transcribed in blood cells.

**MHC and preen secretions.** When chemical distances were mainly influenced by the most abundant compounds, they increased significantly with MHC amino acid distances in male-male dyads (Mantel test: $r = 0.22$, $P = 0.009$, $n = 210$ dyads; Fig. 2a) and male-female dyads (Spearman's correlation permutation test: $r = 0.13$, $P = 0.007$, $n = 378$ dyads; Fig. 2b), while being unrelated to MHC distances in female-female dyads (Mantel test: $r = 0.05$, $P = 0.31$, $n = 153$ dyads; Fig. 2c) and showing a trend when all kittiwake dyads were pooled (Mantel test: $r = 0.13$, $P = 0.051$, $n = 741$ dyads). When chemical distances were calculated when considering all chemical compounds equally, they were not related to MHC distances in any of the dyad groups (all $P > 0.38$). These results suggest that the relationship between preen secretion and MHC similarities is mainly linked to the most abundant chemical compounds. This is supported by the finding that when analyses were conducted with only the 22 most abundant chemical compounds (i.e., compounds with an average abundance $> 1\%$), the correlations between MHC distances and chemical distances were significant in male-male, male-female and all dyads ($r = 0.20$, $P = 0.015$, $r = 0.12$, $P = 0.024$, and $r = 0.20$, $P = 0.012$ respectively), but not in female-female dyads: $r = 0.08$, $P = 0.25$. In contrast, none of the correlations were significant when excluding these 22 compounds (all $P > 0.35$).

## Discussion

Our study provides the first evidence that preen secretion chemicals can encode information on MHC relatedness in birds. The findings suggest that odour cues present in preen secretion may be recognized by birds and allow them to pair MHC-disassortatively. The relationship between preen secretion and MHC dissimilarities in cross-sex dyads and male-male dyads suggests that kittiwakes may recognize related individuals by self-referent or known-kin matching, with individuals avoiding breeding with partners that have scent signatures similar to their own or their known-kin.

A positive correlation between preen secretion and MHC dissimilarities was detected in male-male dyads, but not in female-female dyads. This finding confirms our previous results using a different dataset, which showed that preen secretion and microsatellite similarity correlated in male-male dyads only[17]. Similar sex-differences in the correlation between chemical and genetic similarities were detected in giant pandas (*Ailuropoda melanoleuca*)[22], but not in ring-tailed lemurs (*Lemur catta*)[23,24] and mandrills (*Mandrillus sphinx*)[25]. In kittiwakes, philopatry and intense competition for securing a nesting site is common in males[26]. Kin recognition may therefore be favoured in males because it may reduce competition between related males thereby increasing inclusive fitness. In contrast, females being the dispersing sex are much less likely to be surrounded by kin and social interactions amongst females are rare. Kin selection amongst females may thus be under lower selection than amongst males.

How odours are influenced by MHC genes remains largely unknown[8]. Recent studies suggest that in humans and zebrafish (*Danio rerio*), MHC peptides may function as chemical signals for kin recognition[27,28]. Although in several species, non-peptide compounds, including carboxylic acids, were found to correlate with MHC profiles[29,30], it is not known whether a non-peptide mechanism can drive MHC-mediated behavior[31]. At the molecular level, the link between MHC and non-peptide odours may stem from excreted odorants becoming conjugated with amino acids and therefore being

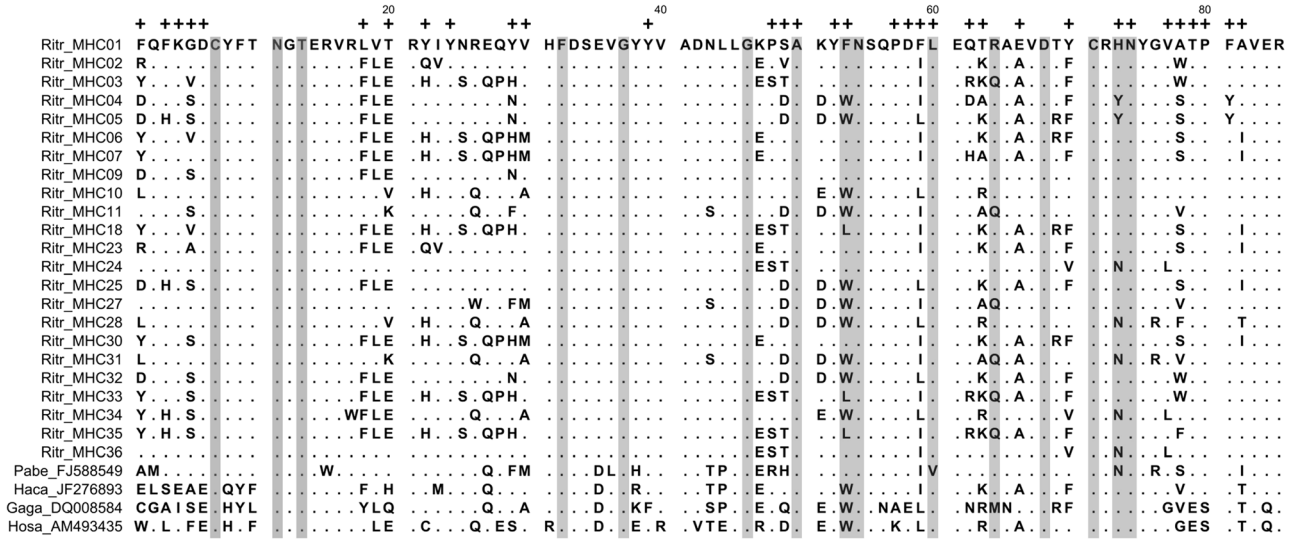


**Figure 1 | Amino acid alignment of MHC Class II DRB alleles isolated from black-legged kittiwakes.** Dots indicate matching amino acids relative to Ritr_MHC01 and shaded areas indicate conserved residues as outlined in[50]. Plus signs above alignment correspond to putative antigen binding sites as inferred from[46]. Additional species and their associated GenBank accession numbers included in the alignment are *Pachyptila belcheri* (FJ588549), *Halobaena caerulea* (JF276893), *Gallus gallus* (DQ008584) and *Homo sapiens* (AM493435).

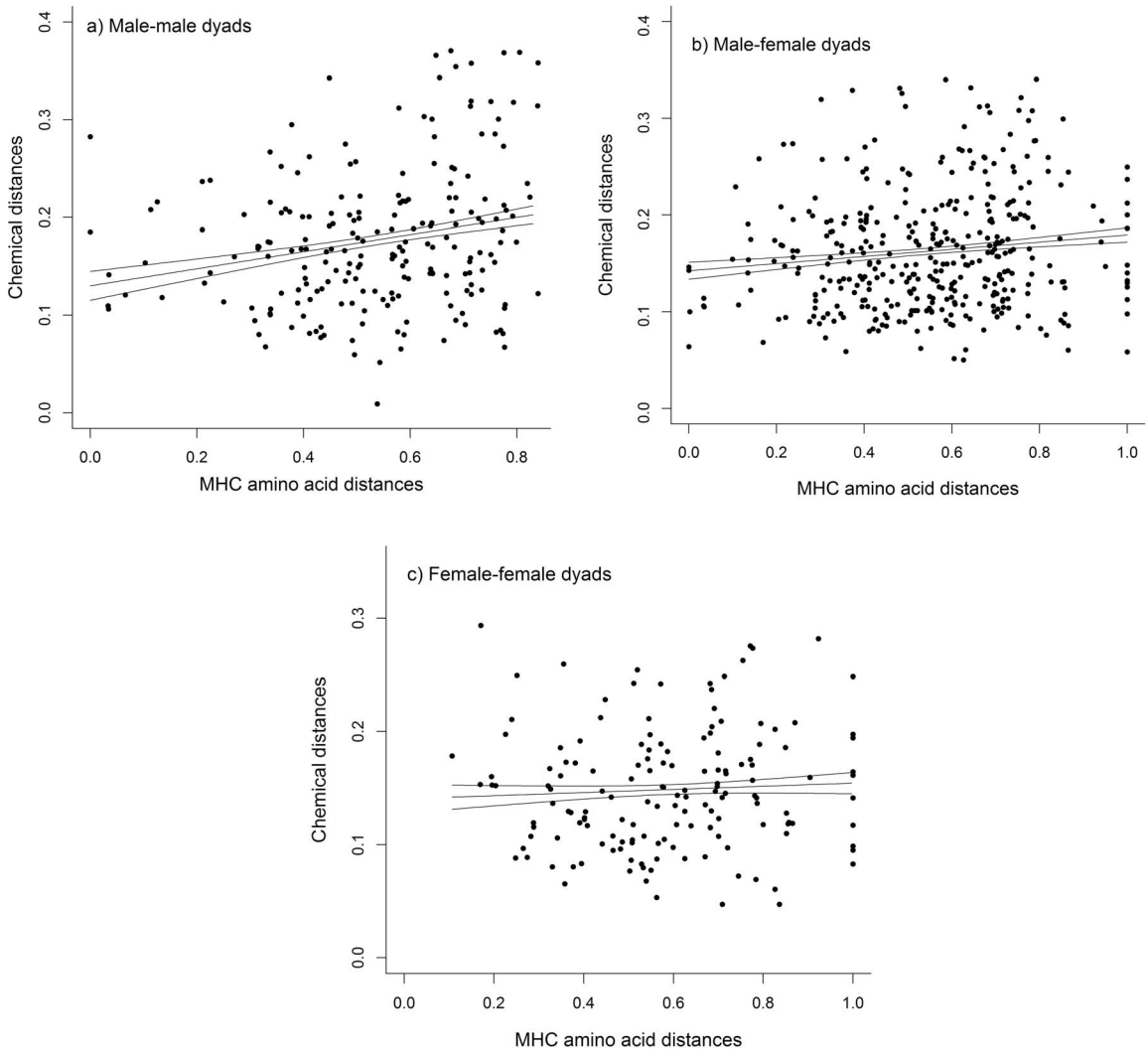


**Figure 2 | Relationship between MHC amino-acid distances and chemical distances (as described by Euclidean distances) in (a) male-male dyads, (b) cross-sex dyads, and (c) female-female dyads. Solid lines are linear model predicted values and SE.**

bound by MHC proteins[29], or from odorants being secondary metabolites of biochemical pathways moderated by MHC or MHC-linked genes. Our study is correlative. Therefore, our results do not allow us to determine the degree to which preen secretion chemicals are influenced by MHC compared to other polymorphic genes whose variations usually covary with those in the MHC, such as the major urinary protein (MUP) genes in natural populations of mice[32]. Experiments with MHC-congenic birds, for instance, are needed to disentangle these two hypotheses.

In our study, we analyzed the composition in preen secretion wax esters. Wax esters are long-chained compounds with low volatility at normal temperatures and pressure. Birds lack a vomeronasal organ[33], and whether they can detect nonvolatile olfactory cues by the main olfactory system, as shown in mice[34], is unknown. Non-exclusively, wax esters may be metabolized by odor-producing bacteria present on feathers or in the preen gland, and lead to the release of volatiles encoding MHC information[35].

Although preen secretions are spread onto the plumage during preening, they may represent only one component of the body odour emitted by birds. However, we do know that in kittiwakes, chemical profiles of preen oil are highly similar to the chemical profiles of down feathers surrounding the preen gland[36] and to the chemical profiles of neck feathers (our unpublished data). Another sampling protocol (e.g., as described in[37]) would be necessary to characterise the entire body odour of kittiwakes and to determine to what extent preen secretions contribute to the odour emitted by the birds. In addition, variance in chemical distances was high for any particular MHC distance. Some compounds are likely to be influenced by non-MHC factors, which may have led to the small effect sizes detected in this study. Although our approach has led to new insights into chemical signalling in birds, an important next step is to determine which specific chemical compounds are central in odour-based kin recognition (as suggested in[38]).

In conclusion, our results provide the first evidence that preen secretion can encode information on MHC relatedness in birds. How MHC-related odours influence kittiwake behaviour needs now to be studied. Our findings open the door for further studies that may comprehensively link mate choice and immunity in birds.

## Methods

**Study site.** Samples were collected in the pre-laying period, between 15 April and 20 May 2011 (first laying in the population: 27 May 2011), in a population of black-legged kittiwakes nesting on an abandoned US Air Force radar tower on Middleton Island (59°26′N,146°20′W), Gulf of Alaska. Preen secretion and blood samples were collected from 18 females and 21 males. Experiments were approved by the US Fish and Wildlife Service and State of Alaska.

**Preen secretion collection and analyses.** Preen secretion collection, extraction and GC analyses were adapted from our protocol described earlier[36]. Preen secretion samples were stored at −20°C until chemical analyses in March 2012. Samples were immersed in 0.5 ml dichloromethane/ nonadecane (internal standard, 20 μg/ml), agitated for 2 h at ambient temperature and then kept frozen until analysis. They were analyzed on a DANI GC-1000 gas chromatograph (DANI Instruments SpA), equipped with a flame-ionization detector and a Restek Rtx®-5MS (30 m × 0.25 mm, 0.25 μm film thickness) capillary column. Helium was used as a carrier gas. The flame-ionization detector was operated at 300°C and the injector was used at 280°C. Samples were injected in splitless mode. The oven was programmed as follows: 7°C/min from 50°C to 200°C and then 3°C/min to 290°C and a 10 min hold-on at 290°C. Blanks were regularly interspersed throughout the sample analyses. We retained peaks that comprised at least 0.1 per cent of the total area of the chromatogram (n = 120 peaks) and analyzed all samples in a short period of time to minimize inter-assay variability.

In kittiwakes, all individuals of both sexes have the same compounds making gas chromatograph – mass spectrometer (GCMS) analysis not necessary for profile alignment. Two samples were, however, run on a GCMS to further identify the chemical compounds. The analyses were performed on a Finnigan Trace 2000 chromatograph (Thermo Scientific) directly coupled to a mass spectrometer quadrupole detector (electron impact at 70 eV). The temperature source was set at 200°C, the interface between GC and MS modules at 250°C and the splitless injector at 280°C. Helium was the carrier gas and the flow rate was 1.2 ml/min. 1 μl of sample was injected in an apolar capillary column (Restek Rtx®-5MS; 30 m × 0.25 mm, 0.25 μm film thickness, 5% diphenyl and 95% dimethylpolysiloxane). The oven temperature program was as set for the GC analyses. The mass spectra were scanned from 60 to 500 m/z. All identified peaks were wax esters (i.e., esters of long aliphatic carboxylic acids and fatty alcohols) as found in our earlier study[16].

Because we could not control for the amount of secretion collected, each chromatogram peak was quantified as the relative proportion of the peak size to the overall area of the chromatogram. Chromatograms were analyzed with the Peak Simple integration software (Version 3.77, Buck Scientific Inc.). To measure similarity in preen secretion composition between each dyad of individuals, we calculated the Euclidean distance after chord-transformation (Chord distance)[39]. This distance is mainly influenced by compounds with large absolute differences between individuals[40] which are, as a rule, compounds with high abundance[41] (Pearson correlation between mean and standard deviation of each compound: $r = 0.99$, $P < 0.0001$, showing that larger differences are also found in more abundant compounds in our dataset). Therefore, we also calculated pairwise Euclidean distances using prior normalization of the relative abundances, so that all compounds were considered equally. Prior normalization was realized using the "range" method in decostand() function (VEGAN package in the R software)[42].

**Genetic analyses.** Upon capture, blood was taken from the alar vein. DNA extraction was performed as described in[17]. We amplified a 258 bp fragment of exon 2 of the MHC Class II DRB locus using the primers KWMHC2_ex2_F (5′-GCACGAGCAGGGTATTTCCA-3′) and KWMHC2_ex2_R (5′-GTTCTGCCACACACTCACCT-3′), which we designed based on previously-published black-legged kittiwake sequences containing exon 2 and its flanking regions (K.D. McCoy, unpublished data: GenBank Accession numbers: HQ822398-HQ822472). PCR was performed in 25 μl reaction volumes containing the forward and reverse primers (0.2 mM each), 1.25 units of GoTaq Flexi DNA polymerase (Promega), 1× reaction buffer (Promega), 1.5 mM $MgCl_2$ (Promega), 0.2 mM dNTPs and approximately 50 ng of genomic DNA. The PCR conditions consisted of an initial denaturation step (94°C, 3 min), followed by 34 cycles of 30 s at 94°C, 30 s at 57°C, 45 s at 72°C, and a final extension step for 10 min at 72°C. The success of our amplifications was confirmed on a 1.5% agarose gel stained with ethidium bromide. Primers and excess dNTPs were removed from the amplified products by digesting exonuclease-shrimp alkaline phosphatase (Fermentas Life Sciences).

As we were expecting multiple MHC alleles per individual, we cloned each PCR product. Cloning was conducted using a TOPO TA Cloning kit (Invitrogen) as per the manufacturer's protocol. We then picked between 24 and 48 transformed colonies for each individual (depending on the success of the cloning) and amplified and sequenced the inserts as described in[43]. Sequence editing and alignment was conducted using CLC DNA Workbench 5.7 (CLC bio).

MHC alleles were classified as unique when they differed by 3 or more bp from all other known alleles and were found more than once within or between samples[44]. All potential alleles found only once in one individual were verified by reamplifying the locus for that individual in a new PCR and cloning the product. When we could not verify a particular allele, it was assumed to be a PCR artefact. However, MHC screening was very repeatable across multiple PCRs. We conducted multiple PCRs for 34 individuals and in all cases we identified the same alleles from both PCRs (van Dongen, unpublished data). Ideally, studies should survey a larger region of the MHC than MHC-DRB, but this requires a level of knowledge of MHC structure that is lacking for non-model organisms. However, the MHC region is characterized by strong linkage disequilibrium[45], meaning that relatively small segments of the MHC provide valuable information about the larger complex.

To confirm that we were genotyping a putatively functional MHC locus and not a pseudogene, we took a number of steps. First, we searched for 1) the presence of frame shift mutations or stop codons in the translated alleles and 2) characteristic features of functional Class II molecules, such as conserved residues and putative peptide binding regions (inferred from[46]). We tested for selection by comparing the ratio of non-synonymous ($d_N$) over synonymous ($d_S$) substitutions using MEGA 5[47] both within and outside of the peptide binding regions. We used the Z-test for selection implementing the modified Nei-Gojobori method with Jukes-Cantor correction and calculated standard errors with 1000 bootstraps. Last, to confirm that both loci are transcribed in blood cells, we amplified MHC alleles from the cDNA of two individuals. A RiboPure - Blood kit (Ambion) was used to extract RNA from 250-500 μl of blood that had been stored in RNAlater. The RNA was subsequently treated with DNase that was provided with the RNA extraction kit and reverse transcribed using a QuantiTect Reverse Transcription Kit (Qiagen). The cDNA was subsequently used as a PCR template and cloned, as described above, using the primers KWMHC2_F (5′ CCA ATG GTA CYG AGC GGG TGA 3′) and KWMHC2_R (5′ ACG GCG CAA ACC AGC CTG T 3′), which are located within exon 2 and exon 3, respectively.

Genetic MHC similarity was calculated based on the amino acid sequences of each allele. To estimate pairwise distances in MHC genotypes between all individuals, we used UniFrac[48], a phylogenetic comparison tool originally developed for measuring phylogenetic distances between microbial communities. Using this approach, two individuals were classified as similar at the MHC if they shared the same alleles or shared alleles that were phylogenetically clustered. A maximum-likelihood phylogenetic tree of all alleles for the UniFrac analysis was inferred using MEGA 5. The maximum likelihood tree was based on a WAG model[49], which was selected as the most appropriate evolutionary model to explain the variability among protein sequences. Evolutionary rate differences among sites were modelled using a discrete Gamma distribution (number of categories = 5).

**Statistical analyses.** Correlations between preen secretion similarity and MHC relatedness were tested using Mantel tests (VEGAN package[42]) and 5000 data randomizations. The correlation between preen secretion similarity and MHC relatedness may stem from one sex only. As Mantel tests cannot handle interactions, we performed Mantel tests in all dyads, in male-male dyads and in female-female dyads separately. The male – female matrix was not square and thus we could not use the Mantel test. Instead, we used a Spearman's correlation permutation test to test for the correlation between chemical distances and MHC distances. All statistical tests were performed with the R statistical software (R Development Core Team 2014).


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## Author contributions

S.L., W.F.D.vD. and R.H.W. conceived and designed the study. T.M. collected the samples. S.L., S.V. and C.D. performed the chemical analyses. W.vD. performed the genetic analyses. S.L., W.F.D.vD., S.A.H., P.B., E.D. and R.H.W. wrote the manuscript.

## Additional information

**Competing financial interests:** The authors declare no competing financial interests.

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