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Preen oil composition of Pied Flycatchers is similar between partners but differs between sexes and breeding stages

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Preen oil, the secretion of the uropygial gland, may be an important source of body odour in birds. By characterizing the chemical composition of preen oil, we can describe the olfactory phenotypes of birds and investigate whether odours could have a function in sexual signalling or other chemical communication. Here we analysed the preen oil of a wild passerine, the European Pied Flycatcher *Ficedula hypoleuca*, to find out whether it holds socially relevant information. We sampled both the female and male of breeding pairs during nestling rearing to test for sex differences and within-pair similarity. We additionally sampled the females during incubation to test for changes across breeding stages and for individual repeatability of chemical profiles. Pair mates had similar chemical profiles in comparison with other breeding adults. Furthermore, we found evidence for sex differences and for changes across breeding stages. Notably, the preen oil of females was more diverse and more volatile than that of males, and the preen oil secreted by females during incubation was more volatile than that secreted during nestling rearing. However, we found no evidence for individual repeatability of chemical profiles across breeding stages in females. Our results point towards a function of preen oil in sexual signalling, although other functions should not be excluded. Our study is a first step towards understanding the role of odours in the social life of an important avian model species used in the study of mate choice and sexual selection.

Keywords: bird olfaction, chemical profile, *Ficedula hypoleuca*, olfactory communication, passerine, scent, seasonal change, sex semiochemical, uropygial gland secretion.

Birds were long believed to have no or little sense of smell, notably because of their relatively small olfactory bulbs, their lack of a vomeronasal system and the absence of sniffing behaviour (Roper 1999, Hagelin & Jones 2007, Caro *et al.* 2015). In addition, their often elaborate songs and colourful plumages misled researchers into thinking that birds essentially rely on acoustic and visual cues for communication, overlooking the potential importance of olfaction (Bonadonna & Mardon 2013). Over the last two decades, however, evidence has accumulated demonstrating that birds have a well-developed sense of smell (Clark & Smeraski 2022), which they use in a variety of contexts, including during foraging (Nevitt 2008, Wikelski *et al.* 2021), navigation (Wallraff 2004,

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Gagliardo 2013) and nest-building (Petit *et al.* 2002, Gwinner & Berger 2008). Evidence further suggests that birds also use olfaction to communicate with conspecifics, in particular during reproduction (reviewed in Hagelin & Jones 2007, Balthazart & Taziaux 2009, Caro *et al.* 2015, Whittaker & Hagelin 2021). However, how general and important chemical communication is across bird taxa remains to be established.

The occurrence of chemical communication is conceivable in birds because their olfactory phenotype often holds socially relevant information. Preen oil is a waxy substance secreted by the preen (or uropygial) gland, which birds smear on their plumage during preening (Jacob & Ziswiler 1982). Preen oil, or chemical substances derived from preen oil (e.g. via physical or bacterial degradation), may be a major source of infochemicals in birds (Hagelin & Jones 2007, Caro *et al.* 2015), although not all avian infochemicals come from the preen oil (Hagelin & Jones 2007, Golüke *et al.* 2021). In fact, preen oil is often used as a proxy for avian body odour, for example in olfactory preference trials (e.g. Whittaker *et al.* 2011a, Grieves *et al.* 2019a). In addition to its potential role in chemical communication, preen oil serves diverse other functions such as plumage maintenance, waterproofing, and protection against bacteria and ectoparasites (reviewed in Moreno-Rueda 2017).

Preen oil is usually composed of non-volatile compounds, including wax esters, and volatile compounds, including alcohols, aldehydes, carboxylic acids, methyl ketones, benzoates, terpenes, lactones and phenols (Haahti *et al.* 1964, Jacob & Ziswiler 1982, Soini *et al.* 2013, reviewed in Campagna *et al.* 2012). The chemical composition of preen oil can be affected by diet (Thomas *et al.* 2010, Kanakri *et al.* 2018), season (Reneerkens *et al.* 2002, reviewed in Grieves *et al.* 2022), preen gland microbiota (Martín-Vivaldi *et al.* 2010, Whittaker *et al.* 2019b) and hormones (Bohnet *et al.* 1991, Whittaker *et al.* 2011b). Many studies have analysed the chemical composition of preen oil to decipher whether it contains socially relevant information. Preen oil can hold information about species identity (Mardon *et al.* 2010, Van Huynh & Rice 2019), population identity (Whittaker *et al.* 2010, Grieves *et al.* 2019b), sex (Caspers *et al.* 2022, reviewed in Grieves *et al.* 2022), age (Sandilands *et al.* 2004, Díez-Fernández *et al.* 2021), breeding status (Reneerkens *et al.* 2007, Martín-Vivaldi *et al.* 2009), individual identity (Mardon *et al.* 2010, Jennings & Ebeler 2020), genetic heterozygosity (Whittaker *et al.* 2019a), major histocompatibility (MHC) genotype (Leclaire *et al.* 2014, Slade *et al.* 2016) and genetic relatedness (Potier *et al.* 2018). Importantly, experimental studies have shown that birds can perceive such socially relevant variation in preen oil composition using their sense of smell (Whittaker *et al.* 2011a, Leclaire *et al.* 2017, Grieves *et al.* 2019a).

Sex differences and seasonal changes in preen oil composition are major targets to study the role of odour in intraspecific communication in birds. For example, a recent review found that in most species the preen oil of females contains a higher number and diversity of volatile substances than that of males (Whittaker & Hagelin 2021). This suggests that preen oil could play a role in olfactory signalling between the sexes, although sex differences in preen oil composition could also point towards other sex-specific functions. For example, the sex and seasonal differences described in the preen oil of ground-breeding shorebirds (order Charadriiformes) may serve as olfactory crypsis to avoid predation (Reneerkens *et al.* 2002). Indeed, the preen oil of individuals of the incubating sex becomes less volatile during incubation, which may hinder the olfactory detection of the nest by predators (Reneerkens *et al.* 2005). A recent quantitative review analysed the occurrence of sex and seasonal differences in preen oil composition in 59 species and found chemical patterns to be consistent with a role of preen oil in sexual signalling during mate choice, as well as olfactory crypsis during incubation (Grieves *et al.* 2022). Investigating how the volatility of preen oil differs between the sexes and breeding stages would help assess the relative importance of these two hypotheses. A role in olfactory crypsis would be supported by a lower volatility in the incubating sex and during incubation, whereas a role in sexual signalling would be supported by any difference in volatility. Indeed, either high- or low-volatility compounds can transmit chemical information and act as honest, sexually selected signals – not just high-volatility compounds, as proposed in Grieves *et al.* (2022). Low-volatility compounds could act as sexually selected signals, for example, if they signal greater protection for the offspring (against predators via olfactory crypsis, or against pathogens via antimicrobial activity, Moreno-Rueda 2017). High concentrations of large, less-volatile compounds might also indicate mate quality if the bearer must use more energy to produce them. In addition, it

would be insightful to evaluate the (dis)similarity in preen oil composition between breeding partners. Birds may use odours to assess relatedness (Bonadonna & Sanz-Aguilar 2012, Krause *et al.* 2012, Caspers *et al.* 2015) and evidence from several species suggests that similarity in preen oil composition covaries with genetic relatedness and MHC genotype (Leclaire *et al.* 2014, Slade *et al.* 2016, Potier *et al.* 2018, Grieves *et al.* 2021). Individuals should generally pair up with unrelated mates to avoid inbreeding (e.g. Kruuk *et al.* 2002, but see de Boer *et al.* 2021), or with mates dissimilar at the MHC to maximize the disease resistance of their offspring (e.g. Consuegra & Garcia de Leaniz 2008), and therefore partners can be expected to have a rather dissimilar preen oil composition (Grieves *et al.* 2019c). Finally, individual chemical signatures (i.e. repeatable preen oil composition within individuals over time) are also of interest. Individual signatures are considered a fixed aspect of an individual, and their presence may suggest that preen oil composition has a genetic component. Individual chemical signatures are essential for birds individually to recognize conspecifics and assess their relatedness and other characteristics via olfaction, notably during mate choice (Mardon *et al.* 2010).

In this study, we investigated the chemical composition of the preen oil of the European Pied Flycatcher *Ficedula hypoleuca* (hereafter Pied Flycatcher), a common passerine bird often used in studies on behaviour, ecology and evolution (e.g. Both *et al.* 2006, Ellegren *et al.* 2012, Nicolaus *et al.* 2022). Whereas visual and acoustic traits and their role in sexual selection have received great attention in this species (e.g. Lampe & Espmark 2003, Sirkiä & Laaksonen 2009), the potential role of olfactory phenotypes has been completely unexplored. We sampled the preen oil of Pied Flycatchers to analyse its chemical composition using gas chromatography and investigated sex differences and partner (dis)similarity during the period of nestling rearing, as well as changes across breeding stages (incubation vs. nestling rearing) and individual signatures in females.

METHODS

Study species and population

We studied Pied Flycatchers from an established nestbox population in a lowland mixed coniferous forest near Elbergen in NW Germany (52°27′N, 7°15′E; for details on the study site see Altenkirch & Winkel 1991). The Pied Flycatcher is a common medium-sized (13 cm; 9–22 g) passerine bird with a wide distribution in the Palaearctic, and is a trans-Saharan migrant that arrives between late March and early May on European breeding grounds. During the breeding season, Pied Flycatchers form social pair bonds (Glutz von Blotzheim & Bauer 1993). Although social monogamy prevails in the study population, typically a small proportion of males are socially polygynous each year (Lubjuhn *et al.* 2000, Huk & Winkel 2006). Furthermore, some extra-pair paternity occurs (Brün *et al.* 1996, Lubjuhn *et al.* 2000). Pied Flycatchers provide biparental care but only females build the nest and incubate, and both sexes contribute to nestling provisioning (Glutz von Blotzheim & Bauer 1993).

Field methods

During routine nestbox checks as part of a long-term monitoring programme, Pied Flycatchers were caught by hand or with nest traps during the breeding season in May and June 2019, and were ringed with uniquely numbered metal rings (issued by 'Vogelwarte Helgoland', Wilhemshaven, Germany). To collect preen oil, we gently swabbed the preen gland with a fresh cotton bud several times from both sides over a period of approximately 5 s, and immediately placed the cotton bud in a 20-mL screw neck glass vial (following Caspers *et al.* 2022). Upon return from the field site in the evenings of sampling days, vials were stored at −20 °C until further analysis. Females were sampled during incubation and both pair partners were sampled during nestling rearing (often on the same day). In all, we collected 103 preen oil samples over a period of 22 days. In addition, we took blank (control) samples in the field (four in total) for which an identical handling protocol was applied, although without sampling a bird.

Laboratory methods

Vials were defrosted for at least 60 min prior to sample preparation. To extract preen oil, we injected 100 µL of dichloromethane directly into the cotton bud, and squeezed out the content of the bud (consisting of dichloromethane and preen oil) using a 100-µL blunt point glass syringe. We

transferred the extracts into 2-mL glass vials equipped with a 100-µL glass inlet. The extracts were concentrated by evaporation – by leaving the glass vials open at ambient temperature under a fume hood for 10–30 min – to a volume of approximately 5 µL before analysis. Samples were analysed by gas chromatography (GC) with a flame ionization detector (GC-FID, GC 2010 plus, Shimadzu, Duisburg, Germany) equipped with a VF-5 ms capillary column (30 × 0.25 mm ID, DF 0.25, 10-m guard column, Varian Inc., Lake Forest, CA, USA). One microlitre (1 µL) of each sample was injected into a deactivated glasswool-packed liner at an inlet temperature of 220 °C and processed in a split 10 mode with 20 mL/min split flow. Hydrogen was used as carrier gas and its flow rate was maintained at 1 mL/min. The GC temperature started at 60 °C for 3 min, followed by a 10 °C/min increase rate to a final temperature of 280 °C, which was maintained for 20 min. Three GC blank samples (containing dichloromethane only) were analysed among the preen oil and field blank samples.

Chromatographic data processing

For a given sample, GC-FID produces a chromatogram in which each substance is represented by a peak, the area of which is proportional to the abundance of that substance in the sample. Substances are distinguished by their specific retention times. Peak areas and retention times were extracted using GC Solutions v2.41 (see Supporting Information Table S1 for details). The retention times of homologous substances may vary subtly among samples due to unavoidable stochastic variation in ambient temperature, flow rate of the carrier gas or column ageing. However, homologous peaks should be considered to represent a single substance and therefore need to be aligned on a unique retention time. Chromatograms were aligned using the *GCalignR* package (Ottensmann *et al.* 2018) in R v3.6.1 (R Core Team 2022). In total, 110 samples were used for the alignment procedure, consisting of 103 preen oil samples, three GC blank samples and four field blank samples. Any substances detected in the GC blank or field blank samples were removed to control for possible contamination (e.g. from the cotton swabs, the observer or the environment) during laboratory work or fieldwork, respectively. We excluded samples that contained no further substances after the removal of the substances detected in the blank samples from further analysis. Substances detected in only a single sample were ignored in further analysis. After alignment and filtering, 98 preen oil samples were retained. Details about the alignment procedure are available in the Supporting Information (Appendix S1, Table S1). From the 98 retained samples, we discarded 21 samples with chromatograms similar to the blank samples, where no preen oil had apparently been collected, resulting in 77 successful samples. We calculated the relative abundance of each substance by dividing its peak area by the total chromatogram area because the total amount of preen oil collected was not standardized and varied across samples. Relative abundances are pertinent to assess differences in potential information content, although we may miss differences in absolute abundances, which can also be important (Mardon *et al.* 2011, Whittaker & Hagelin 2021). Relative abundances were log-transformed log $(x + 1)$ to prevent high-abundance substances from having a disproportionate influence during the analysis (following Clarke *et al.* 2014).

We verified that our chromatographic data were properly aligned and transformed by inspecting shade plots (Supporting Information Fig. S1) in PRIMER v7.0.20 (Clarke & Gorley 2015). We calculated the chemical richness (number of substances), diversity (Shannon index) and volatility (proportions of high-volatility and low-volatility substances) of each sample. The proportions of high-volatility and low-volatility substances were measured as the proportion of abundance (i.e. chromatogram area) *before peak* C and *after peak F*, respectively (Fig. 1, Supporting Information Fig. S2). We chose these specific thresholds to exclude the central part of the chromatograms (which contains most of the abundance and shows little variation among samples) while conserving sufficient and equivalent abundances in the early part (high-volatility; mean ± sd proportion = 9.9 ± 2.1%; mean ± sd number of substances = 14.1 ± 8.6) and the late part (low-volatility; mean ± sd proportion = 9.0 ± 3.1%; mean ± sd number of substances = 13.6 ± 8.3) of the chromatograms. Alternatively, we could have chosen another relevant set of thresholds, that is, *before peak B* and *after peak H*, to focus only on the most volatile and the least volatile substances, respectively. We repeated the analyses on volatility using this alternative set of thresholds and found

similar results, suggesting that our method is robust (see Supporting Information, SOM). These two measures of volatility inform us on two different mechanisms by which preen oil substances can affect the detectability of a bird (or its nest/clutch): high-volatility substances would directly increase its detectability, whereas low-volatility substances may reduce its detectability. The proportions of high- and low-volatility substances were not correlated (Spearman test: $rho = -0.11$, $P = 0.35$), which confirms that these measures represent two distinct traits.

Concentration bias

Upon further examination of the chromatographic data, it appeared that some samples had a remarkably limited chemical richness and diversity. As we had no measurement of the generally minute quantities of preen gland secretion we collected, it is likely that only a very small amount of secretion was collected in these samples, and that this low concentration made low-abundance substances hard to detect, resulting in low richness and diversity (Supporting Information Fig. S3). We examined the relationship between diversity and concentration, using the abundance of the most abundant substance across samples (substance F in Fig. 1) as a proxy for the total concentration of a given sample. Diversity drops abruptly below a certain concentration threshold (abundance of substance F = 70 000), revealing a likely concentration bias (Supporting Information Fig. S4). Scarcer substances are less well detected in low-concentration samples, resulting in an underestimated chemical richness and diversity. Because low-concentration samples may introduce noise in our data, we discarded samples below the concentration threshold ($n = 16$ samples) and conducted our analyses on the remaining 61 samples (Fig. S4). We ran additional analyses where we included the low-concentration samples ($n = 77$ samples) to verify the robustness of our results (see 'complete dataset' in the SOM).

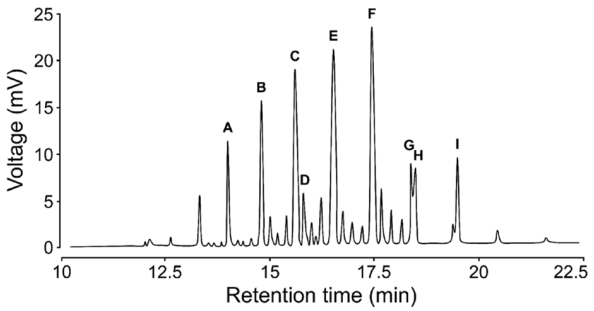

Figure 1. Representative GC-FID chromatogram of the preen oil of a female Pied Flycatcher sampled during nestling rearing. Analyses were performed on the complete chromatograms (all substances) and on the nine most abundant substances (indicated with letters). The abundance of the most abundant substance across all samples (substance F) was used as a proxy for the concentration of preen oil in each sample.

Statistical analyses

To investigate differences between the sexes and among breeding pairs, we used the samples from breeding pairs where both the female and the male were successfully sampled. Our data included 22 samples from 11 pairs. To investigate differences between breeding stages and among individuals, we used the samples from females sampled on two occasions, namely, during incubation and during nestling rearing (mean ± sd number of days between the two samples = 16.8 ± 2.8). Our data included 14 samples from seven females. We made sure that our designs were perfectly balanced (i.e. equal sample sizes across groups), in particular because permutational multivariate analyses of variance (PERMANOVA) can be sensitive to differences in dispersion under unbalanced designs (Anderson *et al.* 2008).

We tested for differences in the overall composition of preen oil using PERMANOVA with the PERMANOVA+ v1 add-on (Anderson *et al.* 2008) in PRIMER v7.0.20 (Clarke & Gorley 2015). We first constructed a resemblance matrix based on pairwise Bray–Curtis dissimilarities between samples. Bray–Curtis dissimilarity, which is commonly used in chemical ecology studies (Brückner & Heethoff 2017), is a well suited measure for the analysis of abundance data because it ignores joint absences (Clarke *et al.* 2014). PERMANOVA models were run with 9999 permutations and type III (partial) sums of squares. Although the type of sums of squares should not matter with our balanced designs, we chose type III because it is the most conservative (Anderson *et al.* 2008). To test for sex and pair differences, *sex* was included as a fixed effect and *pair ID* as a random effect. To test for breeding stage and individual differences, *breeding stage* was included as a fixed effect and *individual ID* as a random effect. We verified the homogeneity in dispersion with PERMDISP tests,

even though PERMANOVA is robust to heterogeneity in dispersion under balanced designs (Anderson *et al.* 2008). We repeated all PERMANOVA models considering only the nine most abundant substances (i.e. only substances that were consistently the most abundant substances in all samples; Fig. 1 and Fig. S1) to assess whether differences observed in preen oil composition are solely driven by differences in the most abundant substances, or scarce substances also play a role. In combination with PERMANOVA, we visualized our data with metric multidimensional scaling (mMDS) plots in PRIMER.

In addition, we investigated differences among preen oil samples in chemical richness, chemical diversity and volatility (proportion of high- and low-volatility substances). To do so, we ran generalized linear mixed models (GLMMs) with Gaussian distribution and identity link using the *lmer* function of the *lme4* package (Bates *et al.* 2009) in R. In the models testing for sex and pair differences, *sex* was included as a fixed effect and *pair ID* as a random effect. In the models testing for breeding stage and individual differences, *breeding stage* was included as a fixed effect and *individual ID* as a random effect. The significance of fixed effects was assessed (at $\alpha = 0.05$) by checking whether their 95% confidence interval (95% CI) contained zero. We additionally indicated *P*-values for both fixed effects and random effects, which were obtained with the *lmerTest* (Kuznetsova *et al.* 2017) and the *rptR* (Stoffel *et al.* 2017) packages, respectively. We calculated the marginal R^2 explained by fixed effects using the *partR2* package (Stoffel *et al.* 2021) and the adjusted repeatability of traits based on random effects with the *rptR* package. Assumptions of normality and homoscedasticity of the residuals were verified by visual inspection of plots with the *performance* package (Lüdecke *et al.* 2021). Boxplots were produced with the *ggplot2* package (Wickham 2011). Details on the analyses in R are available in Appendix S1.

Discarding the low-concentration samples may not be sufficient to control for the concentration bias. Therefore, we repeated all models where significant effects were detected, adding *concentration* (area of the most abundant peak across samples) as a covariate, and checked whether the effects were robust. We also verified that (1) there was no difference in concentration between the groups (females vs. males, incubation vs. nestling rearing period) with Wilcoxon rank-sum tests, and (2) there was no correlation in concentration between paired samples (both sexes of a pair, both breeding stages of an individual) with Spearman correlation tests.

RESULTS

The 61 preen oil samples that were retained for analysis contained a total of 119 different substances with an average of 46 substances (sd = 16) per sample.

Sex differences during nestling rearing period

We found a difference between females and males in the overall composition of preen oil (PERMANOVA; $P = 0.035$, *component of variation* = 3.1%; Table 1, Fig. 2). However, no sex difference was detected when including the low-concentration samples (Supporting Information Table S2) or when considering only the nine most abundant substances (Supporting Information Table S3). Females had on average a slightly more diverse preen oil than males (GLMM; $\beta = 0.08$ (95% CI:

Table 1. Results from PERMANOVA on the preen oil chemical composition of Pied Flycatchers. (a) Effect of sex (fixed effect) within breeding pairs (random effect) sampled during nestling rearing ($n = 22$ samples from 11 pairs). (b) Effect of breeding stage (fixed effect) within individual females (random effect) sampled during both incubation and nestling rearing ($n = 16$ samples from eight females).

	df	SS	*F* (pseudo)	*P* (perm)	Component of variation
(a) Sex and pair					
Sex	1	156.7	3.23	**0.035**	3.14
Pair	10	1172.0	2.42	**0.006**	5.86
Residuals	10	484.6	—	—	6.96
(b) Breeding stage and individual					
Breeding stage	1	458.0	5.74	**0.014**	6.88
Individual	7	691.6	1.24	0.293	3.08
Residuals	7	558.5	—	—	8.93

Analysis based on Bray–Curtis dissimilarities of log-transformed values. *P*-values were obtained using 9999 permutations under a reduced model with type III (partial) sums of square (*SS*), and are indicated in bold if the effect is significant at $\alpha = 5\%$. Components of variation are 'pseudo' multivariate analogues of univariate variance components and were square-root-transformed to represent relative effect sizes in Bray–Curtis units (i.e. % of Bray–Curtis dissimilarity).

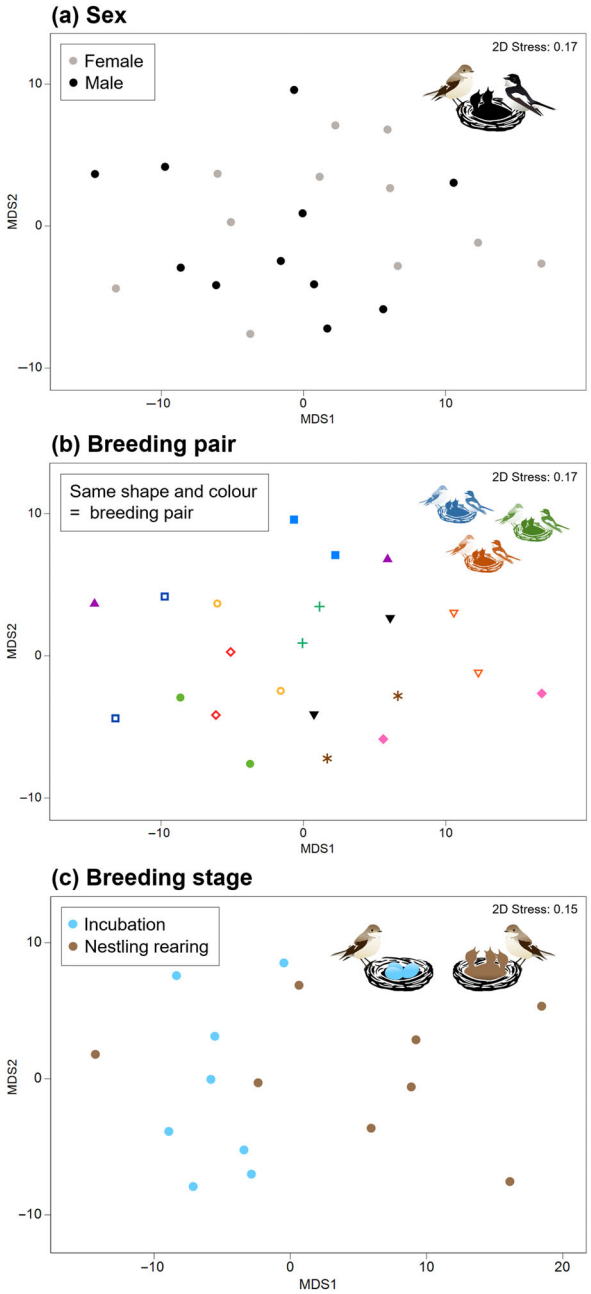

Figure 2. Two-dimensional metric multidimensional scaling (mMDS) plots representing Bray–Curtis dissimilarity in the preen oil composition of Pied Flycatchers. (a) Sex differences within pairs during nestling rearing. (b) Differences among breeding pairs during nestling rearing. (c) Differences between breeding stages within individual females. 2D Stress is a measure (between 0 and 1) of the fit between the distance among samples in two-dimensional space and the actual distance among samples in multivariate space (values near 0 indicating a better fit).

0.01–0.15), *marginal* R^2 = 0.07; Supporting Information Table S4). The preen oil of females was also more volatile than that of males, as it contained more high-volatility substances (β = 1.35 (95% CI: 0.29–2.46), *marginal* R^2 = 0.21; Fig. 3, Table S4). However, we found no evidence that the sexes differed in chemical richness (males: β = −2.73 (95% CI: −11.30 to 5.80), *marginal* R^2 = 0.01) or in the proportion of low-volatility substances (males: β = 0.03 (95% CI: −0.83 to 0.95), *marginal* R^2 = 0; Table S4).

Partner similarity during nestling rearing period

We detected similarity between pair members (i.e. partners) in the overall composition of preen oil (PERMANOVA; P = 0.006, *component of variation* = 5.9%; Table 1, Fig. 2). This pair effect was also evident when including the low-concentration samples (Table S2) and when considering only the nine most abundant substances (Table S3). The similarity between partners may be partly explained by the fact that they were sampled close in time, at a similar temperature and by the same observer. Possibly as a result of this, the concentration in preen oil in samples obtained from pair members was correlated (*rho* = 0.74, P = 0.01). However, when controlling for concentration, and for date and time of sampling, the pair effect remained (Supporting Information Tables S5 and S6). Partners also had similar preen oil in terms of chemical richness (GLMM; *repeatability* = 0.71), diversity (*repeatability* = 0.67) and proportion of low-volatility substances (*repeatability* = 0.58; Table S4). The proportion of high-volatility substances, however, was not repeatable within pairs (*repeatability* = 0; Table S4).

Change across breeding stages within females

The overall composition of preen oil of females changed significantly from incubation to nestling rearing (PERMANOVA; P = 0.014, *component of variation* = 6.9%; Table 1, Fig. 2). The effect of breeding stage was also detected when including low-concentration samples (Table S2) and when considering only the nine most abundant substances (Table S3). The preen oil secreted during incubation

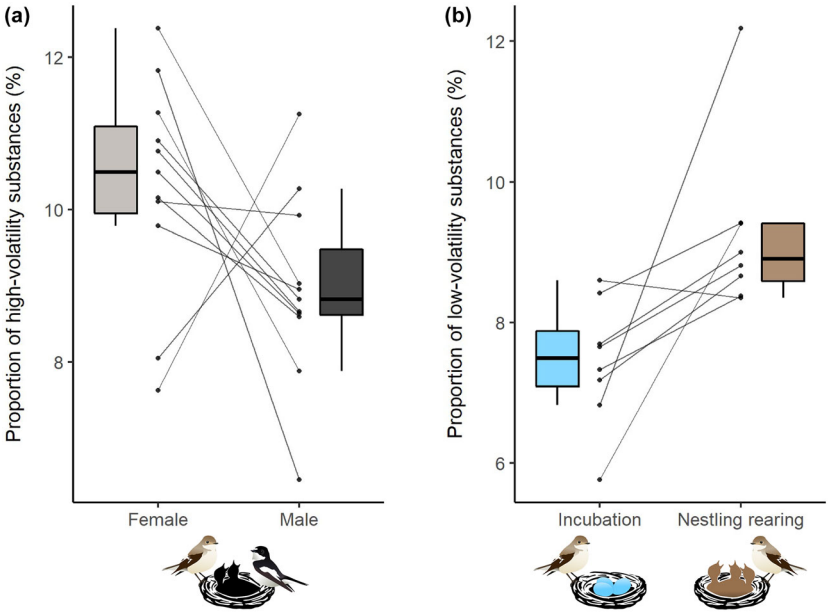

Figure 3. Sex and breeding stage differences in the volatility of Pied Flycatcher preen oil. (a) Sex differences in the proportion of *high-volatility* substances within pairs during nestling rearing. Lines connect breeding pair mates. (b) Breeding stage differences in the proportion of *low-volatility* substances within individual females. Lines connect observations of individual females.

contained a lower proportion of low-volatility substances (GLMM; β = 1.84 (95% CI; 0.89–2.94), *marginal* R^2 = 0.43; Fig. 3, Table S7). However, no breeding stage differences were detected in chemical richness (β = 1.12 (95% CI: –18.00 to 21.40), *marginal* R^2 = 0), diversity (β = 0.05 (95% CI: –0.13 to 0.24), *marginal* R^2 = 0.02) or the proportion of high-volatility substances (β = −1.12 (95% CI: –2.72 to 0.34), *marginal* R^2 = 0.06; Supporting Information Table S7).

Individual repeatability within females

We found no evidence for individual-specific chemical signatures in the females sampled twice, either for the overall composition (PERMANOVA; P = 0.29, *component of variation* = 3.1%; Table 1) or for the nine most abundant substances (Table S3). In addition, neither richness, diversity nor volatility were repeatable within individual females (Table S7).

DISCUSSION

We investigated natural variation in preen oil chemical composition in a wild population of Pied Flycatchers. We found that preen oil composition is similar between pair mates and differs between the sexes during the period of nestling rearing, and differs between breeding stages in females. However, we found no evidence for any repeatable, individual chemical signature in females across breeding stages. Based on these findings, we discuss potential functions of preen oil and olfactory phenotypes in this species. We focus in particular on odour-related functions, due to the potential importance of preen oil for olfactory communication (Whittaker & Hagelin 2021, Grieves *et al.* 2022), but also touch upon other, non-olfactory functions of preen oil (reviewed in Moreno-Rueda 2017).

Sex differences

Our study revealed sex differences in preen oil composition during the period of nestling rearing. Sex differences were detected in the overall composition of preen oil only when excluding low-concentration samples, where scarce substances could not be reliably detected. This suggests that the sex difference is mainly driven by scarce substances. Hence, it is no surprise that, when only looking at the nine most abundant substances, we found no sex difference. In addition, sexes differed

in chemical diversity and volatility, with a higher diversity and higher proportion of high-volatility substances in females. This is in line with the findings of Whittaker and Hagelin (2021), who demonstrated that avian chemical signals are often more pronounced in females. Indeed, females often have larger preen glands (e.g. Golüke & Caspers 2017), therefore secreting greater amounts of preen oil (Martín-Vivaldi *et al.* 2009), and produce a preen oil with a higher diversity and a higher number of volatile substances than males (e.g. Jacob *et al.* 2014, Caspers *et al.* 2022, reviewed in Whittaker & Hagelin 2021).

Different functional hypotheses have been proposed to explain why females and males secrete a different preen oil during the breeding season. First, the sex semiochemical hypothesis posits that preen oil provides chemosignals that function in mate choice, which can be associated with a sex difference in the proportions of some substances (Whittaker *et al.* 2010, Grieves *et al.* 2022). Secondly, the olfactory crypsis hypothesis posits that the preen oil secreted by the incubating sex is used to minimize olfactory cues from eggs, nestlings or the incubating parent, thereby reducing nest predation from olfactorily searching predators, and leading to sex differences in species with uniparental incubation (Reneerkens *et al.* 2002, Grieves *et al.* 2022). Thirdly, the incubating and/or chick-rearing sex may produce preen oil substances that limit infection of the eggs and/or chicks by parasitic bacteria, fungi or arthropods (Moreno-Rueda 2017), which could also lead to sex differences in preen oil composition in species with uniparental incubation and/or care (e.g. Martín-Vivaldi *et al.* 2009). Although this third hypothesis does not make any assumption based on the odorous nature of preen oil, it is possible that preen oil odours are used as a signal indicating an individual's ability to battle against pathogens, but this remains to be investigated.

The sex difference observed in Pied Flycatchers more likely reflects a function in sexual signalling than in olfactory crypsis, because the preen oil of females (incubating sex) was more volatile than that of males. It is nonetheless possible that the more volatile preen oil of females serves olfactory crypsis via chemical background matching, if the volatile substances blend with the odour of the environment surrounding the nest. This hypothesis has previously been proposed based on the observation that the preen oil of Dark-eyed Juncos *Junco hyemalis* contains volatile linear alcohols similar to that produced by plants surrounding their nest (Soini *et al.* 2007). Dark-eyed Juncos are a ground-nesting species and olfactory crypsis may be more important for such species with less protection from predators than for cavity-nesting species, such as the Pied Flycatcher (Grieves *et al.* 2022).

Olfactory acuity may be highest during courtship (Groof *et al.* 2010) and the preen gland is often largest at the time of hatching (Martín-Vivaldi *et al.* 2009, Golüke & Caspers 2017), suggesting that the perception and production of chemical substances may be magnified during early breeding. It would be important to analyse sex differences in preen oil even before egg-laying, when sexual selection is most in effect. In our study, sex differences were assessed during nestling rearing (mean ± sd number of days after hatching = 9.2 ± 2.2), that is, after mate choice and incubation. For stronger inference on the role of preen oil in either sexual signalling or chemical protection, sex differences should ideally be evaluated during mate choice or incubation, respectively. We can speculate that the sex difference would be more pronounced if we had considered absolute abundances, instead of relative abundances, notably as females may have a larger preen gland size and produce more preen oil than males (Whittaker & Hagelin 2021). To allow such analyses, future studies should measure the amount of preen oil collected. We should not overlook the possibility that the observed sex differences may also be the result of non-adaptive mechanisms, such as sex differences in diet or preen gland microbiota (Grieves *et al.* 2022). For example, sex differences in diet have been documented in the Pied Flycatcher, with females foraging more often in trees searching for caterpillars and males foraging more often on aerial lepidopterans (Alatalo & Alatalo 1979).

Similarity between breeding partners

Despite the sex differences in preen oil composition, breeding partners secreted preen oil with relatively similar compositions (in terms of overall composition, chemical richness and diversity, proportion of low-volatility substances) in comparison with other synchronously breeding pairs, suggesting that partners may have similar odours. In another passerine species, the Dark-eyed Junco, it has also been found that breeding partners had

similar preen oil composition (Whittaker *et al.* 2016). We cannot exclude that this similarity may partly be due to sampling bias, as both members of a pair were sampled on the same day and around the same time of day. Pair members were therefore sampled under similar environmental conditions (e.g. temperature, humidity), which may have affected the viscosity and thereby the amount of the preen oil collected, and which could explain the correlation in preen oil concentration between the samples of pair mates. However, the pair effect was robust and remained similar in magnitude when statistically controlling for both concentration and date and time of sampling. We thus propose that the similarity in preen oil composition observed between mates may be due to: (1) preferences for mates with similar odours; (2) a transfer of preen oil substances between mates; or (3) phenotype-environment correlations, as mates share the same environment (e.g. nest microbiota, available food).

The first possibility is that Pied Flycatchers mate preferentially with individuals that have an odour similar to their own, resulting in assortative mating. One of the possible functions of a preference for breeding partners with a similar odour is the avoidance of outbreeding, i.e. mating with individuals that are genetically very different (Luo *et al.* 2015). Indeed, like inbreeding, outbreeding can have deleterious consequences (outbreeding depression, Marshall & Spalton 2000, Szulkin *et al.* 2013). For this hypothesis to be relevant, it should be verified that similarity in preen oil composition covaries with genetic similarity in Pied Flycatchers, as has been shown in other bird species (Leclaire *et al.* 2014, Slade *et al.* 2016, Potier *et al.* 2018). However, we think it is unlikely that the similarity in preen oil observed within breeding pairs is the result of an increased relatedness between pair members, as highly dispersive passerines with large population sizes often have very low rates of inbreeding (e.g. < 1% in the closely related Collared Flycatcher *Ficedula albicollis*; Kruuk *et al.* 2002).

The second possibility is that breeding partners transfer preen oil substances or preen gland microbes to one another. Such transfers could be direct during social interactions (copulation, allopreening) or indirect when sharing the same breeding environment (e.g. via nestbox surfaces, nest material, nestlings; Hagelin 2007, Hagelin & Jones 2007). Once smeared on the plumage, preen oil compounds could be exchanged directly between partners, just like feather microbes are transmitted during social interactions (Kulkarni & Heeb 2007). However, we are unsure how likely it is that such an exchange of preen oil would lead to a different composition of the freshly secreted preen oil that we collected. Mates may also secrete a similar preen oil because they have similar preen gland microbiota. Indeed, breeding partners often have similar microbiota (preen gland, Whittaker *et al.* 2016; skin around the preen gland, Engel *et al.* 2020; cloaca, Kreisinger *et al.* 2015, Whittaker *et al.* 2016), possibly due to their spatial proximity or similarity in diet. Preen gland microbiota may shape preen oil composition (Whittaker *et al.* 2019b, but see Whittaker *et al.* 2016, Grieves *et al.* 2021) by modifying chemical substances in the preen oil (Martín-Vivaldi *et al.* 2010, Whittaker *et al.* 2019b). However, it is currently not known whether Pied Flycatchers harbour microbes in their preen gland that produce specific substances in the preen oil.

A third possibility is that partners adjust their preen oil phenotypes to their shared environment in a similar way (i.e. phenotype-environment correlations; Snowberg & Bolnick 2012, Fokkema *et al.* 2021, Trappes *et al.* 2021). Mated pairs may for instance exhibit a parallel chemical adjustment to the communities of bacteria and ectoparasites that are specific to their nests (i.e. nest microbiota). Indeed, it has been shown that in Great Tits *Parus major* the preen oil of both females and males changes in response to experimental modifications of nest microbiota (Jacob *et al.* 2014). However, the induced changes were greater in females than in males, probably because females spend more time in the nest. In Pied Flycatchers, females are also in contact considerably longer with the nest environment than are males, as they build the nest and incubate the eggs alone, whereas males only briefly enter the nest to feed the female during incubation and the chicks during brood care. The effect of nest microbiota is therefore expected to have a greater impact on females than on males. Accordingly, it was found that nest microbiota affected the plumage microbiota of female but not male Pied Flycatchers (Goodenough *et al.* 2017). Furthermore, even though the sexes can differ in their overall diet (Alatalo & Alatalo 1979), breeding partners may still consume a relatively similar diet, which is known to affect preen oil composition (Thomas *et al.* 2010). Pied Flycatcher pairs exploit the same territory and

therefore have the same food resources available (Grundel 1990, Moreno *et al.* 1995). Breeding partners may thus have a similar preen oil composition because they feed on similar food resources in their territory. This could be investigated by testing whether pairs with neighbouring or partly overlapping territories have similar preen oil, but such a test would require a larger sample size.

Change across breeding stages in females

Almost all species studied to date exhibit seasonal changes in preen oil composition (Whittaker & Hagelin 2021, Grieves *et al.* 2022). The preen oil of female Pied Flycatchers, in our study, changed from the incubation to the nestling rearing phase. Thereby, our study provides further evidence that systematic seasonal changes in preen oil can occur over relatively short periods of time, with a change detected over only 17 days (average time period between the two samples). Other rapid changes were documented in Red Knots *Calidris canutus* (Reneerkens *et al.* 2007), Dark-eyed Juncos (Whittaker *et al.* 2011b) and Song Sparrows *Melospiza melodia* (Grieves *et al.* 2018), where preen oil composition changed in less than 2 weeks.

We found that the preen oil secreted during incubation was on average more volatile, as it contained a lower proportion of low-volatility substances, than that secreted later in the breeding season. Despite the lack of change in chemical richness and diversity, this result is consistent with the sex semiochemical hypothesis (Grieves *et al.* 2022), as a change in volatility could be used for chemical communication. Similarly, a number of studies on other bird species found that the preen oil produced during breeding is more volatile than that produced during non-breeding, and have hypothesized that the preen oil produced during breeding serves as a chemosignal for reproduction (e.g. White-throated Sparrows *Zonotrichia albicollis*, Tuttle *et al.* 2014; Grey Catbirds *Dumetella carolinensis*, Shaw *et al.* 2011; Dark-eyed Juncos, Soini *et al.* 2007). For example, in White-throated Sparrows, individuals held in breeding conditions produced four volatile compounds that are not secreted under non-breeding conditions (Tuttle *et al.* 2014), and the preen oil of females contained higher abundances of volatiles before laying (i.e. during the mate choice period) than during incubation (Forrette 2018). However, our finding of a higher volatility of preen oil during incubation than nestling rearing seems inconsistent with a role of preen oil in olfactory crypsis (Grieves *et al.* 2022). In precocial species, such as sandpipers, mobile chicks leave the nest shortly after hatching, and it is therefore important to avoid olfactory detection of the nest by predators during the egg phase (incubation), but not necessarily during the chick phase (Reneerkens *et al.* 2002). Sandpipers chemically camouflage their nest by secreting a less volatile preen oil specifically during the period of incubation (Reneerkens *et al.* 2002, 2005). In contrast, in altricial species like the Pied Flycatcher, chicks are raised in the nest, and olfactory detection of the nest by predators should be avoided both during the egg and chick phases. Presumably, the vulnerability of Pied Flycatchers to olfactorily searching nest predators does not vary across breeding stages, and thus preen oil volatility should not change across breeding stages. It is also possible that the changes across breeding stages observed in the preen oil of females are related to a role in olfactory parent–offspring communication (Caspers *et al.* 2017). An alternative explanation for our finding that female preen oil is more volatile during incubation is that it may contain specific volatile compounds that inhibit the growth of eggshell bacteria, as was shown in Eurasian Hoopoes *Upupa epops* (Martín-Vivaldi *et al.* 2010).

CONCLUSION AND FUTURE RESEARCH

This study provides the first characterization of the chemical composition of the preen oil of Pied Flycatchers. Our results warrant further investigation in the chemical ecology of an important model species for studying sexual selection and mate choice in birds. In future work, sampling females during mate choice (e.g. before egg-laying) and sampling males during both mate choice and incubation would be informative. More pronounced sex differences in preen oil during mate choice, possibly in combination with either increased or reduced volatility, can be an indication that it plays a role in sexual olfactory signalling; but note that a function in sexual signalling should not be ruled out in case there are no sex differences in preen oil composition. If the similarity within pairs is caused by the transmission of

chemicals between mates, it is predicted to increase across the breeding stages, as mates spend more time in proximity. The possible presence of individual signatures in preen oil chemical profiles should be further investigated in females (with additional repeated samples during peak periods of sexual selection, and within and across breeding stages), as well as in males. Finally, behavioural trials should be conducted to test whether Pied Flycatchers can readily detect differences in chemical profiles, particularly in the context of mate choice and reproduction.

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AUTHOR CONTRIBUTIONS

Marc Gilles: Data curation; formal analysis; methodology; validation; visualization; writing – original draft; writing – review and editing. **Rienk W. Fokkema:** Conceptualization; data curation; formal analysis; methodology; validation; writing – original draft; writing – review and editing. **Peter Korsten:** Conceptualization; methodology; writing – review and editing. **Barbara A. Caspers:** Conceptualization; funding acquisition; methodology; resources; writing – original draft; writing – review and editing. **Tim Schmoll:** Conceptualization; data curation; investigation; writing – original draft; writing – review and editing.

CONFLICT OF INTEREST

None.

ETHICS AND PERMITTING

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Data Availability Statement

Data and code are available at the repository PUB – Publications at Bielefeld University (https://pub.uni-bielefeld.de/record/2965523, doi: https://doi.org/10.4119/unibi/2965523).

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SUPPORTING INFORMATION

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

Appendix S1. R code.

Figure S1. Chromatographic data from the GC-FID analysis of 77 preen oil samples of Pied Flycatchers.

Figure S2. Calculation of the proportion of high-volatility and low-volatility substances.

Figure S3. Concentration bias, illustrated by representative GC-FID chromatograms of three samples with varying overall concentrations of preen oil.

Figure S4. Chemical diversity (Shannon diversity index) in relation to the overall concentration in preen oil (abundance of substance F), revealing a concentration bias.

Table S1. Settings used for the integration of chromatographic data using the software GC Solutions (version 2.41) and for the alignment of chromatographic data using the *align_chromatograms* function of the *GCalignR* package in R.

Table S2. Results from permutational multivariate analysis of variance (PERMANOVA) on the preen oil chemical composition of Pied Flycatchers including the low-concentration samples (complete dataset).

Table S3. Results from permutational multivariate analysis of variance (PERMANOVA) on the preen oil chemical composition of Pied Flycatchers considering only the nine most abundant substances.

Table S4. Results of generalized linear mixed models (GLMMs) investigating sex differences within breeding pairs in several chemical aspects of the preen oil of Pied Flycatchers: richness

(number of substances), diversity (Shannon index), proportion of high-volatility substances and proportion of low-volatility substances. $n = 24$ samples from 12 pairs (12 females and 12 males).

Table S5. Results from permutational multivariate analysis of variance (PERMANOVA) examining the effects of factors on the preen oil chemical composition of Pied Flycatchers while controlling for the concentration in preen oil (abundance of the most abundant substance).

Table S6. Results from permutational multivariate analysis of variance (PERMANOVA) examining sex differences (fixed effect) within breeding pairs (random effect) on the preen oil chemical composition of Pied Flycatchers sampled during nestling rearing, while controlling for the temporal effects of sampling date (fixed effect) and time of day (fixed effect). $n = 34$ samples from 17 pairs using the complete dataset; $n = 22$ samples from 11 pairs using the reduced dataset. The complete dataset includes all samples, whereas the reduced dataset includes only high-concentration samples

Table S7. Results of generalized linear mixed models (GLMMs) investigating the effect of breeding stage within individuals in several chemical aspects of the preen oil of Pied Flycatchers: richness (number of substances), diversity (Shannon index), proportion of high-volatility substances and proportion of low-volatility substances. $n = 16$ samples from 8 individual females (8 during incubation, 8 during nestling rearing).

Table S8. Results of the tests for homogeneity of multivariate dispersions (PERMDISP) to test for differences between sexes and breeding stages in dispersion (i.e. in deviations from centroid) in preen oil chemical composition.

Table S9. Results of generalized linear mixed models (GLMMs) on volatility using the alternative thresholds to measure the proportion of high-volatility and low-volatility substances (see Fig. S2).