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Research article

Chemical signatures in the preen oil of pied flycatchers: testing reproducibility and exploring ontogeny

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Preen oil – the secretion from the uropygial gland of birds – may have diverse functions in avian reproduction: protection against eggshell bacteria, olfactory crypsis against nest predators and olfactory mate choice. To investigate such functions, we should first characterise variation in preen oil composition, but also confirm that previously described patterns are robust. Replication studies are crucial to test the reproducibility of previous findings, but are rarely undertaken in chemical ecology. Here, we conducted an almost exact replication of a previous study on the chemical composition of preen oil in a wild passerine bird, the pied flycatcher *Ficedula hypoleuca*. We aimed to estimate the reproducibility of the previous results using larger sample sizes and following a pre-registered analysis. In addition, we explored the ontogeny of preen oil composition by comparing nestling and adult preen oil. In line with previous findings, preen oil composition was similar between breeding partners and not repeatable within individual females across breeding stages. Female preen oil changed across breeding stages more clearly than in the original study (higher richness, diversity and volatility during incubation than nestling-rearing), further refuting a role of preen oil in olfactory crypsis in this species. Unlike the original study, we found no difference in chemical profiles between sexes (nestling-rearing), casting doubt on the proposed role of preen oil as a sex semiochemical in this species. Nestling preen oil differed from adults, was more similar to adult males than to adult females, but was not more similar to parents than to non-parents. We found family chemical signatures, which, along with the breeding pair signature, suggests an influence of the nest environment on preen oil composition. Our study highlights the importance of replication and provides novel insights into the function and development of preen oil.

Keywords: Avian body odour, gas chromatography-mass spectrometry (GC-MS), intraspecific chemical communication, pre-registration, preen wax, replication study

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Introduction

The uropygial gland (or preen gland) is the main secretory gland of the skin of birds, producing preen oil, a waxy secretion which birds spread onto their plumage during preening (Jacob and Ziswiler 1982). Preen oil consists of a complex cocktail of chemicals that varies in composition within and between individuals (Grieves et al. 2022). The adaptive function of preen oil has been extensively studied but remains debated (reviewed by Moreno-Rueda 2017). Preen oil appears to be multifunctional, serving notably plumage maintenance and waterproofing (Giraudeau et al. 2010), chemical protection against ectoparasites (Alt et al. 2020), olfactory crypsis against nest predators (Reneerkens et al. 2005) and olfactory intraspecific communication (Grieves et al. 2022). The function(s) of preen oil likely depend on the species, but also on the season (Grieves et al. 2022). Indeed, in many species, at the onset of the breeding period, the chemical composition of preen oil changes and the amount of preen oil secreted increases (reviewed by Whittaker and Hagelin 2021 and Grieves et al. 2022). This suggests that, in addition to its year-round role in plumage maintenance, preen oil serves specific roles in reproduction.

Three main hypotheses for a reproductive function of preen oil have been proposed. First, preen oil may serve as chemical protection against ectoparasites during breeding. For example, the preen oil from incubating female Eurasian hoopoes *Upupa epops* has antimicrobial properties which may protect the eggs from pathogenic eggshell bacteria (Martín-Vivaldi et al. 2009, 2010, 2014). Second, preen oil may serve olfactory crypsis during breeding. For instance, in several ground-nesting shorebirds, the preen oil of incubating birds becomes less odorous during breeding, which may reduce the detectability of the clutch or incubating parent(s) by olfactorily-searching nest predators (Reneerkens et al. 2005, 2007a). Third, preen oil has been hypothesised to play a role in intraspecific olfactory communication, such as sex signalling during mate choice (Whittaker and Hagelin 2021, Grieves et al. 2022). For example, in two passerine species (dark-eyed juncos *Junco hyemalis* and song sparrows *Melospiza melodia*), the preen oil of females and males undergoes different chemical changes prior to breeding. This leads to sex differences in preen oil composition during the breeding season, which allow birds to discriminate between sexes by smell (Whittaker et al. 2010, 2011a, Grieves et al. 2019a,b). The two latter hypotheses are based on the odoriferous nature of preen oil. Indeed, preen oil is a major source of avian body odour (Alves Soares et al. 2024).

Before experimentally testing hypothetical functions of preen oil, it is important to describe the natural variation in its chemical composition (i.e. 'chemical fingerprint' or 'chemical profile') within and among individuals. For example, the existence of sex differences in chemical fingerprints should be established before testing for olfactory sex discrimination (Grieves et al. 2019a,b). In addition, the detected chemical patterns should ideally be corroborated by replication studies to verify their reproducibility. This may avoid inauspicious research investment based on spurious results, which thrive under a research paradigm that prioritises novelty over robustness (Forstmeier et al. 2017). While dedicated replication studies are extremely valuable, they are still very rare in the fields of ecology and evolution (Nakagawa and Parker 2015, Kelly 2019), especially exact or close replication studies (i.e. with a high degree of fidelity to the original study protocol). Studies on chemical fingerprints are particularly difficult to replicate, given the complexity of the methodology of chemical extraction and analysis, as well as processing and multivariate statistical analysis of the chromatographic data (Tebbe et al. 2020, Alves Soares et al. 2024). Even when using the same methodology, studies conducted across several years often report strong among-year differences, which may be due to fluctuations in environmental conditions (e.g. weather, food availability) or subtle differences in protocols like the preservation of samples (Mardon et al. 2010). In fact, in the few replicated studies in chemical ecology, results are poorly reproducible (Wyatt 2015). Indeed, studies on chemical fingerprints often search for subtle effects based on relatively small sample sizes, increasing the risk of false positives or exaggerated effect sizes (Wyatt 2015).

In recent years, studies on preen oil composition have accumulated and revealed differences between sexes, seasons, life stages and individuals (reviewed by Grieves et al. 2022 and Alves Soares et al. 2023), but also effects of diet (Thomas et al. 2010), food stress (Grieves et al. 2020), hormones (Whittaker et al. 2018), parasitic infection (Talbott et al. 2022), microbiota (Whittaker et al. 2019) or major histocompatibility complex (MHC) genotype (Leclaire et al. 2014). Yet, only a handful of these studies have been replicated and successfully reproduced the original findings. Among these, we can cite studies on the preen oil of song sparrows, where sex differences were found repeatedly across populations and in both wild and captive individuals (Grieves et al. 2018, 2019a,b, 2020). Furthermore, in this species, the covariation of preen oil composition with MHC genotype was also reproducible (Slade et al. 2016, Grieves et al. 2019c, Grieves et al. 2021). Sex and seasonal differences in preen oil profiles were also reproducible in six shorebird species (Reneerkens et al. 2002, 2007a,b), dark-eyed juncos (Soini et al. 2007, Whittaker et al. 2010, 2011b, 2013) and white-throated sparrows *Zonotrichia albicollis* (Tuttle et al. 2014, Forrette 2018). However, in the two latter species, chemical patterns were only partly reproducible. For example, the sex differences in the preen oil of dark-eyed juncos reported in Whittaker et al. (2010, 2013) were not reproduced in Whittaker et al. (2016). Similarly, the sex differences in the preen oil of white-throated sparrows in captivity (Tuttle et al. 2014) were mostly not reproducible in the wild (Forrette 2018).

In a previous study on the chemical composition of the preen oil of wild European pied flycatchers *Ficedula hypoleuca*, we detected interesting patterns of natural variation (Gilles et al. 2024a). Analysing samples from eleven breeding pairs during the period of nestling-rearing, we found a high similarity between pair mates and subtle differences between

sexes, notably a higher volatility and a slightly higher chemical diversity in females than in males (Gilles et al. 2024a). In the preen oil from eight females that were sampled repeatedly during both incubation and nestling-rearing, we found no repeatability within individuals but systematic changes across breeding stages, such as an increased volatility (lower proportion of low-volatility compounds) during incubation (Gilles et al. 2024a). Based on these patterns, we speculated on the possible function of preen oil in this species. Observing sex differences during breeding, we hypothesised that pied flycatchers may use preen oil for olfactory sex signalling during mate choice ('sex semiochemical hypothesis', Grieves et al. 2022). Further, the increased volatility observed during incubation in females suggested that preen oil does not play a role in olfactory crypsis at the nest in this species (Grieves et al. 2022). However, these results were based on relatively small sample sizes, and we chose to refrain from designing experiments on the possible functions underlying these patterns. Instead, we aimed to establish that the patterns are reproducible.

In this study, we therefore conducted a close replication of Gilles et al. (2024a). We returned to the study site one year later and collected preen oil samples from the same population of pied flycatchers. We used the same methodology for the sampling, storage and extraction of the preen oil, as well as for the processing and statistical analysis of the chromatographic data, which we pre-registered (Jeanjean et al. 2023, https://osf.io/tbcug). Pre-registered analyses increase the trustworthiness of results by limiting practices such as cherry-picking, p-hacking and HARKing (Fraser et al. 2018). The only methodological difference compared to the original study was the use of gas chromatography-mass spectrometry (GC-MS) instead of gas chromatography-mass spectrometry with flame ionisation detector (GC-FID) for the chemical analysis. For this reason, our study is not an exact but a close replication of Gilles et al. (2024a) (Nagakawa and Parker 2015). Sample sizes were much larger than in the original study, with samples from 46 breeding pairs during nestling-rearing (compared to 11 in the original study) and from 29 females repeatedly sampled across breeding stages (compared to 8 in the original study). Like in the original study, we measured the alpha diversity (richness and Shannon diversity), volatility and beta diversity (Bray–Curtis dissimilarity) in preen oil composition. We tested for sex differences and similarity between pair mates during nestling-rearing, and for changes across breeding stages and individual signatures in females sampled both during incubation and nestling-rearing. We expected to reproduce the results from the original study, i.e. sex differences (with higher volatility and diversity in females) and similarity between pair mates during nestling-rearing, and changes across breeding stages within females (with higher volatility during incubation) but no individual signature among females.

In addition to our replication effort, we explored proximate causes of variation in preen oil composition. First, we tested whether the similarity observed between pair mates is caused by their spatial proximity (same territory including potentially a similar diet) as suggested in the original study. Second, we investigated the ontogeny of preen oil composition in pied flycatchers, by sampling preen oil from nestlings and adults from 16 families during the nestling-rearing period. We expected to find differences between life stages, as reported in other species (reviewed by Alves Soares et al. 2023), which could reflect differences in physiology (e.g. related to reproduction). We also predicted that nestlings would secrete a preen oil more similar to their mother than to their (social) father, as females spend more time brooding, potentially transferring more preen oil substances, or microbiota affecting preen oil substances, to the nestlings (Whittaker et al. 2016). Finally, we predicted to find family signatures (i.e. higher similarity between family members than with other individuals), as found in another passerine species (dark-eyed junco, Whittaker et al. 2016), which would indicate an effect of the rearing environment and/or genetics on the development of preen oil composition.

Material and methods

Sampling

The sampling methods were the same, and were performed by the same person, as in Gilles et al. (2024a), except that we sampled preen oil from nestlings in addition to adults. Fieldwork took place between 1 May and 8 June 2020, during the breeding season of pied flycatchers, in a nestbox population based in a lowland mixed coniferous forest near Elbergen in northwest Germany (52°27′N, 7°15′E; for details on the study site see Altenkirch and Winkel 1991). The GPS position of the nestboxes was recorded as northing and easting coordinates. Adult females and males were sampled during both the incubation (6.1 days ± SD 3.6 before hatching) and the nestling-rearing period (10.6 days ± SD 2.6 after hatching). Nestlings from 16 broods (5.7 nestlings ± SD 1.0 per brood) were sampled on average at 12.2 days (± SD 1.4) after hatching. In total, 249 preen oil samples were taken (161 from adults during both incubation and nestling-rearing, and 88 from nestlings). Birds were caught directly in their nestbox, using custom-made wire swing traps. To capture males during incubation, we used mist-nets placed around the nestbox, as they do not enter the nestbox during this period. We sampled preen oil by gently rubbing a cotton swab on the preen gland of the bird for 5 s, and immediately placed the cotton swab in a 20 mL screw neck glass vial. In addition, 17 field blanks were taken by agitating a cotton swab in the air, to control for environment contamination of the preen oil samples. Samples were stored at −20°C in the evenings of sampling days, and at −40°C at the end of the field season pending chemical analysis.

Chemical analysis

The preparation of the samples prior to chemical analysis was the same, and were performed by the same person, as in

Gilles et al. (2024a). Samples were defrosted before sample preparation. We injected 100 µl dichloromethane into the cotton swab, which was then squeezed out using a 100 µl blunt point glass syringe to extract the preen oil (and dichloromethane). Extracts were then concentrated by evaporation (for 10–30 min) to a volume of approximately 5 µl at ambient temperature, under a fume hood, in 2 ml glass vials equipped with a 100 µl glass inlet. For the chemical analysis, we deviated from the methodology of the original study, as we used GC-MS instead of GC-FID, and helium instead of hydrogen as a carrier gas. This is because our laboratory transitioned from GC-FID to GC-MS analyses. The main difference is that the mass spectrometry (MS) of GC-MS allows for the identification of compounds in addition to the retention time, whereas GC-FID only provides retention times. However, in our case the identification of substances (using the National Institute of Standards and Technology library) was not certain enough to be used in the study, but we used it to improve our confidence during manual adjustments of the data. We used a GC-MS model GC2030-QP2020NX (Shimadzu) with a VF-5ms capillary column (30 m × 0.25 mm ID, DF 0.25, 10 m guard column, Varian Inc., Lake Forest, USA) and helium (at a 1 ml/min flow rate) as a carrier gas. The GC temperature was first set at 60°C for 3 min, and then increased at 10°C/min to reach the final temperature of 280°C, kept for 20 min. In addition to the preen oil samples and the field blanks, 33 GC blanks (containing dichloromethane only) were added to the analysis to control for instrument contamination of the preen oil samples.

Processing of chemical data

We processed the chemical data following Gilles et al. (2024a).

From the chromatograms, we extracted the retention time of each peak (i.e. substance) and its abundance (area under the peak) using GC Solutions v2.41. For simplicity, we refer to the number of peaks as 'the number of substances' in a sample, but note that a peak could also represent a mixture of substances with very similar retention times (i.e. coelution). When the chromatogram appeared to carry either too much noise or no preen oil substances, samples were excluded from the analysis (N = 23 samples). We also discarded two samples for which information on breeding stage was missing, and six samples from individuals that were sampled twice during nestling-rearing (in that case we kept the second sample, the first one being too close to the hatching date). Using the 218 remaining samples, we aligned the retention times with the 'GCalignR' package (Ottensmann et al. 2018) in the R software ver. 4.2.0 (www.r-project.org) to associate each substance with a single retention time across all samples (Supporting information). To make sure that major environmental and instrument substances were not analysed as preen oil substances, the substances present in field blanks and/or GC blanks were excluded during the alignment. We also performed manual adjustments to improve the *GCalignR* alignment, using shade plots in the PRIMER software v7.0.21 (Clarke and Gorley 2015) for visualisation (Supporting information). Blank substances with retention times closely resembling those of preen oil substances were manually removed, after verification of their distinctiveness using the MS identification.

Because cotton swabs were used for sampling preen oil, the exact quantity of preen oil collected in each sample was unknown. We thus used relative abundances (area under the peak divided by the total area under the chromatogram) instead of absolute abundances of each substance in our analyses. This means that we were not able to study the variation in the absolute abundance of preen oil substances, although we acknowledge that variation in absolute abundances could be important (Whittaker and Hagelin 2021). To attenuate the disproportionate influence of high-abundance substances compared to low-abundance substances, the chemical data were log-transformed (log(X + 1)) prior to analysis (following Clarke et al. 2014). In samples showing a relatively small total area under the chromatogram (likely due to a low concentration of preen oil in the sample), low-abundance substances may not be detected ('concentration bias' as identified in Gilles et al. 2024a). To make sure that the concentration of the samples would not affect our analyses, we plotted the Shannon diversity in relation to a proxy of the mean concentration of preen oil (the total area under the chromatogram divided by the number of substances recorded in the sample) in each sample (Supporting information). We found a positive relationship between concentration and chemical diversity, but there was no obvious concentration threshold below which Shannon diversity dropped (i.e. no concentration threshold), in contrast to Gilles et al. (2024a). Thus, we decided to keep all of the samples in the analysis.

To further analyse the variation in preen oil composition, we took measures of alpha diversity for each sample, namely chemical richness (number of substances) and diversity (Shannon index). We also measured the volatility of each sample, i.e. the proportion of highly volatile compounds, as the total area under the chromatogram before peak C at retention time 10.12 min (Supporting information). This retention time threshold was used in Gilles et al. (2024a) to select approximately the 10% most volatile molecules (the molecules included within the first 10% of the total abundance). In this study, we used the same threshold, but it selected only 2.5% of the compounds, which might be due to among-year differences in the chemical analysis or in environmental conditions. Note that this is only a proxy of volatility, as, to our knowledge, there is no method to measure the overall volatility (in vapour pressure) of preen oil or other biological samples. Unlike the original study however, we decided not to use the proportion of low-volatility substances as a proxy of volatility, because we are uncertain whether the presence of low-volatility substances imply a lower volatility. Indeed, low-volatility substances may break into more volatile compounds and take part in body odour once applied on the plumage (Mardon et al. 2011, Maraci et al. 2018).

Statistical analysis

The statistical analyses were conducted in accordance with the pre-registration (Jeanjean et al. 2023, https://osf.io/tbcug).

Replication

Following Gilles et al. (2024a), we tested the effect of sex and pair identity using samples from complete breeding pairs (i.e. where both the male and female were successfully sampled) during nestling-rearing (N = 92 samples from 46 pairs), and the effect of breeding stage and individual identity using samples from females with repeated samples (i.e. sampled successfully during both incubation and nestling-rearing) (N = 58 samples from 29 females). Because permutational analysis of variance (PERMANOVA) can be sensitive to differences in dispersion under unbalanced designs (Anderson et al. 2008), we made sure that our designs were perfectly balanced. Note that the sample sizes for the latter analysis are slightly lower than in the pre-registration (repeated samples for 29 females instead of 33). We discarded four samples from incubating females for which the alignment did not accurately reflect the chromatogram. Including the four mis-aligned samples in the analysis does not change any of our conclusions (Supporting information).

We first investigated the effect of sex (fixed effect) and pair identity (random effect), and of breeding stage (fixed effect) and individual identity (random effect), on the beta diversity (overall composition) of preen oil composition using PERMANOVA (Anderson 2008, 2014) on Bray–Curtis dissimilarities (Borcard et al. 2011) in PRIMER ver. 7.0.21 (Clarke and Gorley 2015). In addition, we used non-metric multidimensional scaling (NMDS) on Bray–Curtis dissimilarities for visualisation (Borcard et al. 2011). p values for the PERMANOVAs were obtained using 9999 permutations under a reduced model with type III (partial) sums of square (SS), and considered significant when below $\alpha = 0.05$. In addition to the statistical analyses outlined in the pre-registration, we performed dispersion tests (PERMDISP) using PRIMER. Given that a significant result in PERMANOVA may indicate differences in both location and dispersion between groups, conducting a PERMDISP enables us to account for variations in dispersion across sexes and breeding stages.

We then studied the same effects (one model with sex (fixed) and pair identity (random) and one model with breeding stage (fixed) and individual identity (random)) on the chemical richness, Shannon diversity and volatility of preen oil using linear mixed models (LMM) with Gaussian distributions, using the 'lme4' package (Bates et al. 2014) in R ver. 4.2.0 (www.r-project.org). We assessed the significance of fixed effects by checking whether the 95% confidence interval of the beta estimates contained 0 using the 'broom.mixed' package (Bolker et al. 2022), and also checked p values using the 'lmerTest' package (Kuznetsova et al. 2017). The significance of random effects was evaluated by checking whether the 95% confidence intervals of the repeatability estimates contained 0, and by checking the p value based on permutations, using the 'rptR' package (Stoffel et al. 2017). In addition, we measured the variance explained (marginal R^2) by each fixed effect using the 'partR2' package (Stoffel et al. 2021). We verified the assumptions for LMMs using the 'performance' package (Lüdecke et al. 2021).

To compare the results from the replication study and the original study, we calculated effect sizes for the LMM analyses (chemical richness, Shannon diversity and volatility). For fixed effects, we calculated a corrected version of the standardised effect size Cohen's d (Cohen 1988) for small sample sizes, the Hedges' g (Hedges and Olkin, 1985) and its 95% confidence interval, using the 'effsize' package (Torchiano 2020) in R. We used the repeatabilities with their confidence interval as effect sizes for random effects (Stoffel et al. 2017). To guide our inference on whether the results of the original study were reproduced (replication success), for each effect studied, we answered two questions (Valentine et al. 2011): 1) is the effect significant or non-significant (i.e. p value under or above the significance threshold of 0.05) in both studies? 2) Does the effect size estimate of the replication study fall into the confidence interval of the effect size estimate of the original study? For the PERMANOVAs, we could not calculate confidence intervals of effect sizes, and thus could not answer question 2).

Exploratory analyses

In addition to the replication study, we conducted exploratory analyses, as mentioned in the pre-registration (Jeanjean et al. 2023).

Gilles et al. (2024a) found a high similarity in preen oil composition between breeding partners and proposed that this may be due to their spatial proximity, as they share the same territory and the same food availability (i.e. spatial autocorrelation). To test for the effect of spatial proximity on preen oil composition, we ran Mantel tests of the spatial versus chemical distance (Bray–Curtis dissimilarity), along with Mantel correlograms (Borcard et al. 2011) and scatterplots for visualisation, using the 'vegan' package (Oksanen et al. 2022) in R (www.r-project.org). This method tests whether chemical similarity covaries with spatial proximity by comparing pairwise chemical distances with pairwise spatial distances. We used all the samples from adult males and females during nestling-rearing for which we had the GPS position of the nestbox (regardless of whether they were part of a complete breeding pair). We tested females (N = 44) and males (N = 42) separately to control for the effect of breeding partner proximity. A higher similarity between breeding partners could also be the result of a temporal autocorrelation, as breeding partners were sampled at around the same date and time. To control for this, we extracted the pairwise Bray–Curtis similarities of nestling-rearing females (N = 50) and males (N = 48) separately, and tested for the effect of the time difference (fixed effect) between each pair of samples on the Bray–Curtis similarity with generalised linear mixed models (GLMM) with beta distribution using the 'glmmTMB' package (Brooks et al. 2017) in R (www.r-project.org).

To explore the ontogeny of preen oil composition, we tested whether preen oil composition differs between nestlings

and adults, and whether it contains (social) family signatures (i.e. high similarity between members of the same nest). We used samples from 16 broods (100 samples, 31 from adults and 69 from nestlings) collected during nestling-rearing, and employed the same analytical method as for the replication analysis. We tested the effect of life stage (fixed effect) and family identity (random effect) on beta diversity (Bray–Curtis dissimilarities) using PERMANOVA, PERMDISP and NMDS, and on chemical richness, Shannon diversity and volatility using LMMs. In addition, we tested whether the preen oil of nestlings is more similar to that of their mother or father (social father as we did not control for extra-pair paternity, but hereafter simply referred to as 'father'), to that of adult females or males (other than their mother and father), and to that of their parents or other nestling-rearing adults. First, we extracted the pairwise Bray–Curtis similarity for each nestling–adult pair from the Bray–Curtis matrix, and separated them in pairs of nestling–mother, nestling–father, nestling–adult female and nestling–adult male (Raulo et al. 2021). We could then study the effect of adult and parent sex, as well as the effect of being the mother/father (fixed effect) on the similarity between samples, while controlling for the effect of family identity (random effect) and nestling identity (random effect nested within family identity). As Bray–Curtis similarity data range between 0 and 1, we used GLMM with beta distribution using the 'glmmTMB' package (Brooks et al. 2017) in R (www.r-project.org). Because chemical data from the same individuals were used in different subsets of nestling-adult pairs, and because each subset was tested twice (e.g. nestling–mother pairs tested both against nestling–father and nestling–other adult female pairs), we adjusted the p values according to the Holm procedure for multiple testing correction (Holm 1979).

As indicated in the pre-registration, we initially intended to test for the interaction between sex and breeding stage, and to test whether pair similarity changes across breeding stages. However, with only seven samples from males during incubation, we were not able to obtain meaningful results and did not run this analysis.

Results

In the 218 samples retained for alignment, a total of 88 substances were detected, with an average of 24 substances per sample (SD = 7).

Sex differences

We found no sex difference in overall preen oil composition (Bray–Curtis dissimilarities) during the nestling-rearing period (PERMANOVA; $p = 0.17$, component of variation = 0.01, Table 1, Fig. 1a), despite the fact that this effect had been significant in the original study. The dispersion test however indicates a slightly greater dissimilarity in preen oil composition among males than among females (PERMDISP; difference in mean distance to centroid = 2.99, $p = 0.01$). We also found no effect of sex on chemical richness (LMM; males: $\beta = 1.09$ [95% CI: −1.60, 3.38]), $p = 0.36$, marginal $R^2 = 0.01$), diversity (LMM; males: $\beta = 0.04$ [−0.06, 0.14]), $p = 0.45$, marginal $R^2 = 0.01$) and volatility (LMM; males: $\beta = -0.36$ [−0.77, −0.004], $p = 0.07$, marginal $R^2 = 0.04$) (Table 2, and Supporting information). In the original study, the effect of sex was either non-significant or marginally significant on richness and diversity, but significant on volatility (Table 2, Fig. 2). Note, however, that the effect size estimates of the replication results were all encompassed by the 95% confidence intervals of the effect size estimates of the original study (Fig. 2).

Similarity between breeding partners

Pied flycatchers had a preen oil slightly more similar to that of their breeding partner than to that of other nestling-rearing individuals (PERMANOVA; $p = 0.05$, component of variation = 0.05; Table 1, Fig. 1b), corroborating the result from

Table 1. Results from PERMANOVA on the overall preen oil chemical composition (Bray–Curtis dissimilarities) of pied flycatchers from both the original and the replication study. 1) Effect of sex (fixed effect) within breeding pairs (random effect) sampled during nestling-rearing (Noriginal = 22 samples from 11 pairs; Nreplication = 92 samples from 46 pairs). 2) Effect of breeding stage (fixed effect) within individual females (random effect) sampled during both incubation and nestling-rearing (Noriginal = 16 samples from 8 females; Nreplication = 58 samples from 29 females).

	Original study					Replication				
	df	SS	Ps-F	P(perm)	sq. component of variation	df	SS	Ps-F	P(perm)	sq. component of variation
1) Sex and pair similarity										
Sex	1	156.7	3.23	**0.035**	3.14	1	182.5	1.67	0.173	1.26
Pair	10	1172.0	2.42	**0.006**	5.86	45	6831.6	1.39	**0.050**	4.63
Residuals	10	484.6	–	–	6.96	45	4904.1	–	–	10.44
2) Breeding stage and individual similarity										
Breeding stage	1	458.0	5.74	**0.014**	6.88	1	1124.5	9.44	**< 0.001**	5.89
Individual	7	691.6	1.24	0.293	3.08	28	3951.5	1.18	0.215	3.32
Residuals	7	558.5	–	–	8.93	28	3335.8	–	–	10.90

Analysis based on Bray–Curtis dissimilarities of log-transformed relative abundances. 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).

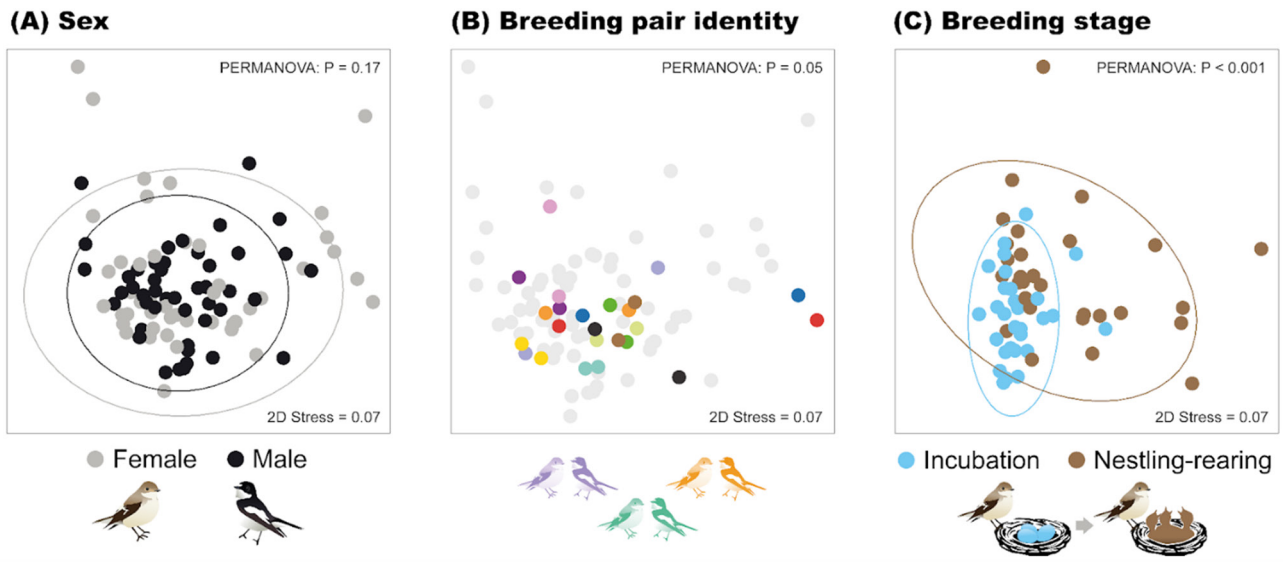

Figure 1. Differences in overall preen oil composition between sexes, among breeding pairs and between breeding stages in pied flycatchers. Non-metric multidimensional scaling (NMDS) plots represent Bray–Curtis dissimilarities. Each circle represents a preen oil sample and ellipses the 95% confidence intervals for each group assuming a multivariate t-distribution. p values were calculated from PERMANOVAs. 2D Stress is a measure of the fit between the distance among samples in the plot (i.e. in two-dimensional space) and the actual distance among samples in multivariate space, with values below 0.1 indicating a very good fit. (A) Difference between sexes (N = 92 samples from 46 females and 46 males during nestling-rearing). (B) Similarity between pair mates (N = 92 samples from 46 pairs during nestling-rearing). Circles of the same colour represent samples from pair mates (only a random selection of 12 pairs is coloured for illustration purposes, the remaining 34 pairs are greyed out to improve readability). (C) Difference between breeding stages (N = 58 repeated samples from 29 individual females).

the original study. However, breeding partners did not show a more similar preen oil in terms of chemical richness (LMM; repeatability = 0.06 [95% CI: 0, 0.33]), p = 0.34), diversity (LMM; repeatability = 0 [0, 0.29], p = 1) or volatility (LMM; repeatability = 0.01 [0, 0.32], p = 0.51) (Table 2). In the original study, the effect of pair identity was significant on chemical richness and diversity, but not on volatility (Table 2, Fig. 2). The effect size from the replication study was only included in the confidence interval of the effect size estimate of the original study for the effect on volatility (Fig. 2). We found no evidence of spatial autocorrelation, as the chemical distance (Bray–Curtis dissimilarity) between samples did not correlate with the spatial distance between nestboxes, neither in females (Mantel test; r = −0.01, p = 0.5) nor in males (r = 0.03, p = 0.30) (see also scatterplot and Mantel correlograms, Supporting information). We found limited evidence of temporal autocorrelation, with a weak correlation between chemical distance (Bray–Curtis dissimilarity) and difference in sampling time (hours) in females (GLMM; β = 0.0006; p < 0.001), and no correlation in males (GLMM; β = 0.0002; p = 0.15) (Supporting information).

Changes across breeding stages

Females differed in their overall preen oil composition during incubation and nestling-rearing (PERMANOVA; p < 0.001, component of variation = 0.06; Table 1, Fig. 1c). Furthermore, incubating and nestling-rearing females differed in dispersion (greater dissimilarity among nestling-rearing females than among incubating females: PERMDISP; difference in mean distance to centroid = 4.48; p = 0.002). The effect of breeding stage on the overall preen oil composition was significant in both the original and replication studies (along with the difference in dispersion). We also detected a higher chemical richness (LMM; nestling-rearing: β = −4.90 [−8.25, −1.70], p = 0.004, marginal R^2 = 0.14), diversity (LMM; nestling-rearing: β = −0.15 [−0.27, −0.05], p = 0.016, marginal R^2 = 0.1) and volatility (LMM; nestling-rearing: β = −1.34 [−2.01, −0.68], p < 0.001, marginal R^2 = 0.20) during incubation than nestling-rearing (Table 2, Fig. 3). In contrast, the effects of breeding stage on chemical richness, diversity and volatility were not significant in the original study (Table 2, Fig. 2). The effect sizes from the replication results were included in the 95% confidence interval of the effect sizes of the original study for the effect of breeding stage on both chemical richness and diversity, but not for the effect on volatility (Fig. 2).

Individual signatures

We found no evidence for individual chemical signatures in overall preen oil composition in females across breeding stages (PERMANOVA; p = 0.22, component of variation = 0.03; Table 1), as in the original study. We also found no repeatability in chemical richness (LMM; repeatability = 0 [0, 0.38], p = 0. 38), diversity (LMM; repeatability = 0 [0, 0.37], p = 1) or volatility (LMM; repeatability = 0.06 [0, 0.42], p = 0.41) within individual females across breeding stages, as in the original study (Table 2, Fig. 2). All of the effect size estimates from the replication study were included in the confidence interval of the original study (Fig. 2).

Table 2. Results of GLMMs on the chemical richness (number of substances), diversity (Shannon index) and volatility (proportion of high-volatility substances) from both the original and the replication study. 1) Sex differences within breeding pairs (Noriginal = 22 samples from 11 pairs; Nreplication = 92 samples from 46 pairs). 2) Breeding stage differences within individual females (Noriginal = 16 samples from 8 females; Nreplication = 58 samples from 29 females) in chemical richness (number of substances), diversity (Shannon index) and volatility (proportion of high-volatility substances) in preen oil composition.

	Original study				Replication			
1) Sex and pair similarity								
Richness								
Fixed effect	df	β [95% CI]	P	R^2 [95% CI]	df	β [95% CI]	P	R^2 [95% CI]
Sex	10	−2.73 [−11.30,5.8]	0.53	0.01 [0,0.10]	45	1.09 [−1.60,3.38]	0.36	0.01 [0,0.08]
Random effect		Variance (SD)	P	Rep. [95% CI]		Variance (SD)	P	Rep. [95% CI]
Breeding pair		241.36 (15.54)	**0.01**	0.71 [0.30,0.92]		2.12 (1.46)	0.34	0.06 [0,0.33]
Diversity								
Fixed effect	df	β [95% CI]	P	R^2 [95% CI]	df	β [95% CI]	P	R^2 [95% CI]
Sex	10	**−0.08 [−0.15,−0.01]**	**0.05**	0.07 [0,0.29]	90	0.04 [−0.06,0.14]	0.45	0.01 [0,0.08]
Random effect		Variance (SD)	P	Rep. [95% CI]		Variance (SD)	P	Rep. [95% CI]
Breeding pair		0.01 (0.12)	**0.01**	0.67 [0.21,0.91]		0 (0)	1	0 [0,0.29]
Volatility								
Fixed effect	df	β [95% CI]	P	R^2 [95% CI]	df	β [95% CI]	P	R^2 [95% CI]
Sex	20	**−1.35 [−2.46,−2.20]**	**0.03**	0.21 [0.01,0.53]	45	−0.36 [−0.77,−0.004]	0.07	0.04 [0,0.14]
Random effect		Variance (SD)	P	Rep. [95% CI]		Variance (SD)	P	Rep. [95% CI]
Breeding pair		0 (0)	1	0 [0,0.60]		0.01 (0.08)	0.51	0.01 [0,0.32]
2) Breeding stage and individual similarity								
Richness								
Fixed effect	df	β [95% CI]	P	R^2 [95% CI]	df	β [95% CI]	P	R^2 [95% CI]
Breeding stage	14	1.12 [−18.00,21.40]	0.91	0 [0,0.30]	56	**−4.90 [−8.25,−1.70]**	**0.004**	0.14 [0.02,0.31]
Random effect		Variance (SD)	P	Rep. [95% CI]		Variance (SD)	P	Rep. [95% CI]
Individual		0 (0)	1	0 [0,0.68]		0 (0)	0.38	0 [0,0.38]
Diversity								
Fixed effect	df	β [95% CI]	P	R^2 [95% CI]	df	β [95% CI]	P	R^2 [95% CI]
Breeding stage	14	0.05 [−0.13,0.24]	0.56	0.02 [0,0.36]	56	**−0.15 [−0.27,−0.05]**	**0.016**	0.1 [0.01,0.26]
Random effect		Variance (SD)	P	Rep. [95% CI]		Variance (SD)	P	Rep. [95% CI]
Individual		0 (0)	1	0 [0,0.73]		0 (0)	1	0 [0,0.37]
Volatility								
Fixed effect	df	β [95% CI]	P	R^2 [95% CI]	df	β [95% CI]	P	R^2 [95% CI]
Breeding stage	7	−1.12 [−2.72,0.34]	0.20	0.06 [0,0.35]	28	**−1.34 [−2.01, −0.68]**	**< 0.001**	0.20 [0.06,0.39]
Random effect		Variance (SD)	P	Rep. [95% CI]		Variance (SD)	P	Rep. [95% CI]
Individual		2.42 (1.55)	0.13	0.49 [0,0.89]		0.11 (0.34)	0.41	0.06 [0,0.42]

df: degrees of freedom. β [95% CI]: beta estimate and 95% confidence interval. R^2: marginal R^2. SD: standard deviation. Rep.: adjusted repeatability. p values of random effects are based on permutations. Intercepts are 'females' for the sex and pair similarity analysis, and 'incubation' for the breeding stage and individual similarity analysis.

Life stage differences and family signatures

Nestlings and adults had a different overall preen oil composition (PERMANOVA; $p < 0.001$, component of variation = 0.06; Fig. 4a, Supporting information), with no difference in dispersion (PERMDISP; difference in mean distance to centroid = 2.37; $p = 0.07$). There were no differences in terms of chemical richness (LMM; nestling: $\beta = -1.15$ [−3.33, 1.07], $p = 0.34$, marginal $R^2 = 0.01$) or diversity (LMM; nestling: $\beta = -0.05$ [−0.13, 0.04], $p = 0.27$, marginal $R^2 = 0.01$), but the preen oil from adults was on average more volatile (LMM; nestling: $\beta = -0.69$ [−1.04, −0.40], $p < 0.001$, marginal $R^2 = 0.12$) than that of nestlings (Supporting information). We also found that family members (i.e. adults and nestlings sharing the same nestbox) had a more similar preen oil composition to each other than to members of other families (PERMANOVA; $p < 0.001$, component of variation = 0.05; Fig. 4b, Supporting information). Furthermore, preen oil composition was repeatable within families in terms of chemical richness (LMM; repeatability = 0.17 [0, 0.36], $p = 0.01$) and volatility (LMM; repeatability = 0.25 [0.04, 0.44], $p = 0.001$) but not diversity (LMM; repeatability = 0.08 [0, 0.25], $p = 0.11$) (Supporting information). In addition, the preen oil composition of nestlings was more similar to that of nestling-rearing males than to nestling-rearing females (non-parent) in the population (GLMM; nestling-rearing males: $\beta = 0.14$ [0.11, 0.17], $p < 0.001$, adjusted $p < 0.001$). They also showed marginally greater similarity to their fathers than to their mothers, although this result was non-significant after adjusting for multiple testing (GLMM; mother: $\beta = -0.13$ [−0.24, −0.02], $p = 0.02$, adjusted $p = 0.06$). However, they did not exhibit greater similarity to their mother than to other nestling-rearing females (GLMM; nestling-rearing females: $\beta = -0.01$ [−0.59, 0.56], $p = 0.97$, adjusted $p = 1$), or to their father than to other nestling-rearing males (GLMM; nestling-rearing males: $\beta = -0.01$ [−0.63, 0.62], $p = 0.98$, adjusted $p = 1$) (Fig. 4c and Supporting information).

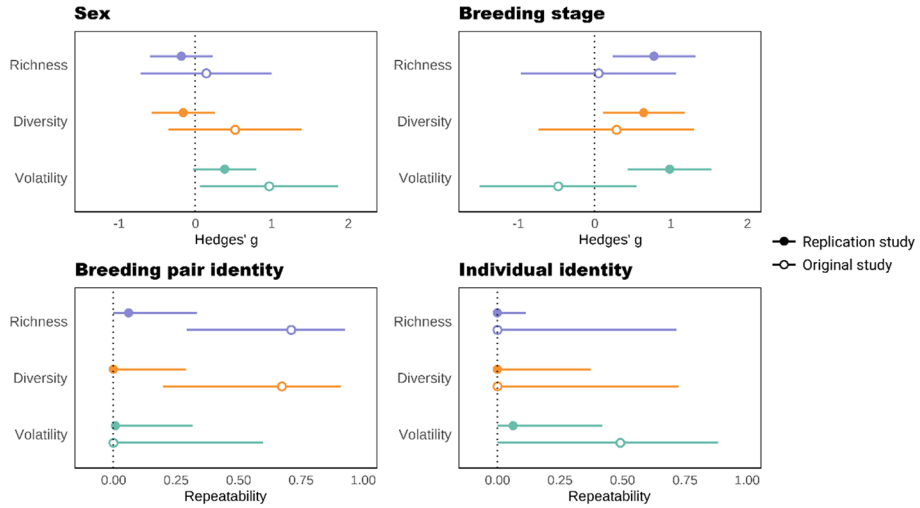

Figure 2. Reproducibility of sex and breeding stage effects as well as pair and individual signatures in preen oil composition (chemical richness, chemical diversity and volatility). Circles represent effect sizes (Hedges' g for fixed effects of sex and breeding stage, repeatability for random effects of breeding pair and individual identity) obtained from linear mixed effects models in the replication (filled circles) and the original study (empty circles). Whiskers represent 95% confidence intervals of the effect sizes.

Discussion

Replication studies are essential to assess the validity of prior findings, but are rarely ever done in the fields of ecology and evolution (Nakagawa and Parker 2015), especially close or exact replications (Parker 2013, Kelly 2019, Fraser et al. 2020). This is concerning, as many effects are assumed to be true once logged into the peer-reviewed literature although they are actually not robust across studies, and likely heavily influenced by type I error (false positives) and publication bias (Seguin and Forstmeier 2012, Parker 2013, Sánchez-Tójar et al. 2018). We performed a close replication study of Gilles et al. (2024a) investigating variation in preen oil composition in pied flycatchers during the breeding season. As in the original study, we found that preen oil composition was similar between pair mates, differed between breeding stages within females, but contained no detectable individual chemical signature in females. It should be noted that the evidence for a similarity between pair mates was more subtle than in the original study, while evidence for a breeding stage difference in females was stronger. Importantly, however, we did not find any evidence that preen oil composition differs between sexes, although

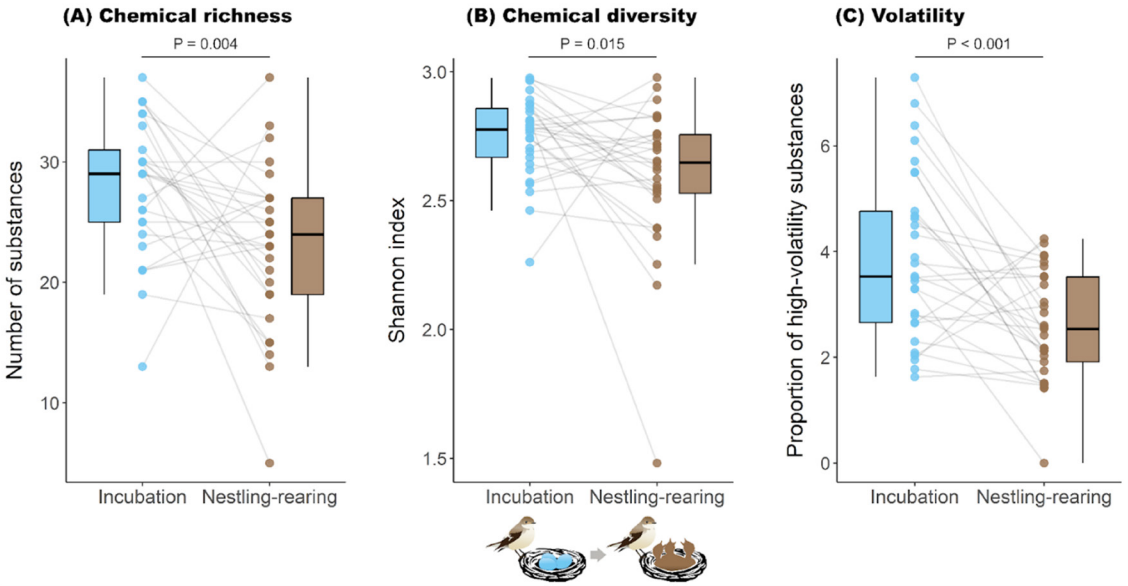

Figure 3. Boxplots showing the changes in preen oil composition between breeding stages within individual female pied flycatchers in (A) chemical richness (number of substances), (B) chemical diversity (Shannon index) and (C) volatility (proportion of high-volatility substances). N = 58 repeated samples from 29 individual females. p values were calculated from linear mixed effects models. Each point represents a preen oil sample. Lines connect the repeated samples of an individual.

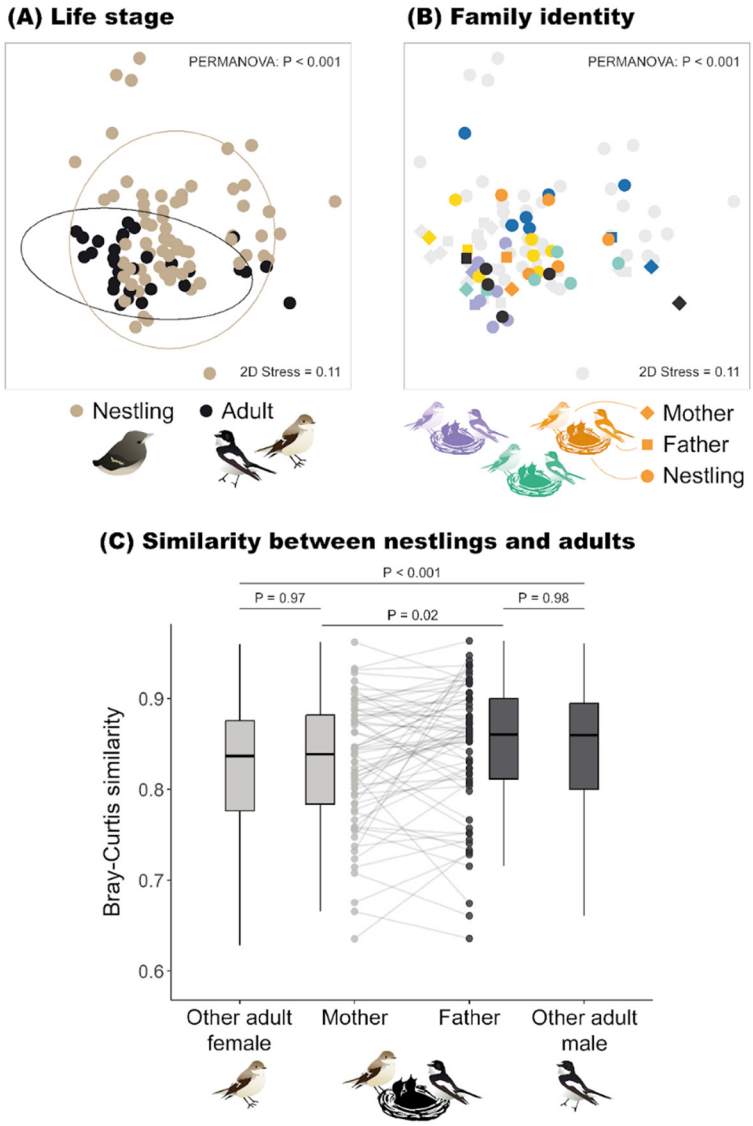

Figure 4. Life stage differences and family signatures in preen oil composition in pied flycatchers. (A, B) Non-metric multidimensional scaling (NMDS) plots represent Bray–Curtis dissimilarities. Each circle represents a preen oil sample and ellipses the 95% confidence intervals for each group assuming a multivariate t-distribution. p values were calculated from PERMANOVAs. 2D Stress is a measure of the fit between the distance among samples in the plot (i.e. in two-dimensional space) and the actual distance among samples in multivariate space, with values below 0.15 indicating a good fit. (A) Differences between life stages (N = 100 samples, 31 from adults and 69 from nestlings). (B) Similarity between family members (N = 100 samples from 16 families). Points of the same colour represent samples from the same family (only a random selection of six families are coloured for illustration purposes, the remaining ten families are greyed out to improve readability). (C) Boxplot of the similarity in overall preen oil composition (Bray–Curtis similarity) between nestlings and adults (parents and other nestling-rearing adults). Each point represents the similarity between a nestling and a parent. Lines connect the similarity of a nestling with its mother and the similarity with its father. p values were calculated from linear mixed effects models testing the effect of social relationship (mother, father, other adult nestling-rearing female and other adult nestling-rearing male) as a fixed effect while controlling for the random effects of nestling identity and family identity.

subtle sex differences were found in the original study. In addition to our close replication, we explored further causes of variation in preen oil composition. First, we found no correlation between similarity in preen oil chemical composition and the spatial proximity of nestboxes. Second, we found that preen oil composition was similar among family members but differed between life stages (nestling versus adult). Third, we found that the preen oil of nestlings was slightly more similar to that of their father than to that of their mother, and overall more 'male-like' than 'female-like'.

Sex differences

Unlike Gilles et al. (2024a), we did not find any evidence for a sex difference in adult preen oil composition during nestling-rearing. In the original study, marginally significant

sex differences were detected in overall preen oil composition, but this result was not reproduced in our study. Our replication study had larger sample sizes, and therefore more statistical power to reject a false null hypothesis (Asendorpf et al. 2013), making the replication findings more reliable. Our failure to reproduce the original result thus suggests that it was a false positive rather than the result of true among-year differences in the degree of sexual dimorphism. Furthermore, the original study found a more volatile preen oil composition in females than males, which could also not be reproduced here. But as the effect size from the replication is still close to the original and not far from statistical significance (Valentine et al. 2011), we so far cannot completely dismiss the possibility of an effect of sex on preen oil volatility in this species. Finally, the original study also found that females had a slightly greater chemical diversity than males, and this marginal effect was, yet again, not reproducible. Overall, these findings reduce our confidence in the presence of a sex difference in preen oil composition in pied flycatchers during the nestling-rearing period.

This changes the inference made by Gilles et al. (2024a) on the potential function of preen oil as an olfactory cue used to discriminate between sexes during mate choice (Grieves et al. 2022). However, as argued in the original study, sex differences should be investigated during the peak of the mate choice period, rather than during nestling-rearing, before dismissing this hypothesis. In song sparrows for instance, sex differences were found during nest building and early egg laying (i.e. when mate choice still occurs) but not after fledging (Grieves et al. 2019a). Similarly, in dark-eyed juncos, sex differences were found repeatedly before egg laying started (Whittaker et al. 2010, 2013) but not at the time of fledging (Whittaker et al. 2016). As we sampled preen oil in the middle of the nestling-rearing period (on average ca 11 days after hatching), this may have been too long after mate choice for sex differences to be detected. Note, however, that even if preen oil does not convey information about the sex of individuals, it may still be used as an olfactory signal during mate choice by encoding information on relatedness or individual quality (Whittaker et al. 2013, Potier et al. 2018, Gilles et al. 2024b). Our new results also diminish the credibility of preen oil playing a role in chemically protecting eggs and/or nestlings. Indeed, we would expect such a function to cause sex differences in preen oil, as females incubate the eggs and brood young nestlings, whereas males only take part in food provisioning.

Similarity between breeding partners

As in Gilles et al. (2024a), our study showed that breeding partners secrete a preen oil of similar composition. However, the evidence for a breeding pair chemical signature is much weaker than in the original study. Indeed, the effect of pair identity was only successfully reproduced on the overall preen oil composition. For this effect, the only method to assess replication success is to compare p values, and with a p value just under the significance threshold in the replication ($p = 0.0495$), whether the effect is considered significant or not strongly comes to chance (Piper et al. 2019). In addition, we could not reproduce the pair similarity in terms of chemical richness and diversity that were found in the original study. Overall, these results hint toward a very subtle (or even potentially absent) breeding pair similarity in preen oil composition.

As discussed in Gilles et al. (2024a), several hypotheses could explain similarity between breeding partners. First, pair similarity could be attributable to spatial autocorrelation, in case of phenotype-environment correlations outside of the nest (e.g. pairs share the same territory and thus possibly the same diet). For example, zebra finches *Taeniopygia guttata* that nest closer together have more similar skin microbiota (although this may also result from direct exchange of bacteria in addition to environmental effects) (Engel et al. 2020). But as we found no correlation between the spatial proximity between nests and the similarity in preen oil composition, this hypothesis seems very unlikely. Second, pair similarity could be explained by a temporal autocorrelation, as breeding partners were often sampled on the same day and at approximately the same time of day. Although we found a correlation between the proximity in the time of sampling and the similarity in preen oil composition in females, the effect was negligible, and absent in males, so this hypothesis seems rather unlikely as well. Third, preen oil composition could be impacted by an exchange of substances through physical contact or allopreening. However, since we collected preen oil directly from the uropygial gland, such exchanges are unlikely. A fourth hypothesis is that the similarity in preen oil between pair mates is the result of assortative mating based on preen oil odours. But as explained in Gilles et al. (2024a), this seems unlikely in a species like the pied flycatcher, in which we expect low levels of inbreeding (Kruuk et al. 2002). Finally, the fifth – and most likely – hypothesis is that preen oil composition is influenced by the social and/or microbial environment in the nestbox. This explanation seems particularly plausible as we also found an effect of family identity on preen oil composition. Nest and social environment seem to be key factors in shaping preen oil composition (Whittaker et al. 2016) as well as microbial communities (Kulkarni and Heeb 2007, Kreisinger et al. 2015, Leclaire et al. 2023), which was also found in our study species (plumage microbiota, Goodenough et al. 2017). Although the relationship between microbiota and preen gland secretions remains unclear at present (Whittaker et al. 2016, Grieves et al. 2021), there is some evidence that uropygial microbial communities influence preen oil composition (Martin-Vivaldi et al. 2010, Jacob et al. 2014, Whittaker and Theis 2016, Whittaker et al. 2019).

Changes across breeding stages

Both the original study and our replication study found that preen oil composition changes between the incubation and nestling-rearing periods within individual females. The breeding stage effects were clearer than in the original study. Indeed, we not only found changes in overall preen

oil composition as in the original study, but also in chemical richness, diversity and volatility. Note that an effect on volatility had also been detected in the original study, but on a different measure of volatility (i.e. proportion of low-volatility substances) which was not used in the replication (Material and methods). The effect of breeding stage on chemical diversity from the replication was readily included in the 95% confidence interval of the original study, suggesting that it may have been non-significant originally due to a lack of statistical power (Valentine et al. 2011).

Overall, our replication results reinforce the suggestions from Gilles et al. (2024a) regarding the potential functions associated with the changes in preen oil across breeding stages in females. This shift may reflect a use of preen oil as an olfactory signal during mate choice (e.g. signalling individual quality or reproductive state; Grieves et al. 2022). However, we note that preen oil composition during incubation may not necessarily reflect preen oil composition during mate choice (e.g. drop in volatile compounds in female dark-eyed juncos after egg-laying; Whittaker et al. 2011b). Our results also align with previous evidence in favour of a role of preen oil in parental care, as in many species, seasonal changes in preen oil composition or preen gland size are found primarily in the incubating sex (Grieves et al. 2022) and often occur during (or shortly before) specific breeding stages, such as the incubation (Reneerkens et al. 2002) or nestling-rearing period (Pap et al. 2010, Amo et al. 2012). Our results could therefore reflect a role of preen oil as an olfactory signal for parent–offspring communication (Caspers et al. 2017) or as a chemical defence against harmful eggshell bacteria (Martin-Vivaldi et al. 2010). These hypotheses are not mutually exclusive and should be tested experimentally. Besides, our results could also simply result from nonadaptive mechanisms, such as changes in diet (Thomas et al. 2010) or hormones during the course of the breeding season (Whittaker et al. 2011b). Finally, our results show that preen oil composition can change rapidly (i.e. over a period of approximately two weeks), as found in other species (Reneerkens et al. 2007b, Whittaker et al. 2011, Amo et al. 2012, Grieves et al. 2018).

Replication outcome

While it is tempting to put more confidence in the more powerful replication study than in the original study, it is important to note that a replication study on its own can never confirm or disconfirm the results of an original study (Earp and Trafimow 2015). This is because replication studies are never exact replications, especially when studying wild populations, where many factors cannot be controlled for (Fidler et al. 2017), and when conducting chemical analyses, which are sensitive to slight alterations in methodology (Tebbe et al. 2020, Alves Soares et al. 2024). For instance, although the chromatograms in the replication had a very similar appearance to the chromatograms from the original study, we found substantially fewer substances overall (88 instead of 119 in the original study), and the nine most abundant substances were not exactly the same as in the original study (compare the Supporting information between both studies). With only two studies, it is impossible to know whether these differences in chemical data are due to biological (i.e. among-year differences), methodological differences (Tebbe et al. 2020), or both. Instead, replication studies increase or decrease our confidence in a given hypothesis, while contributing to the general estimation of the effect studied (Heirene 2021). All the results from both studies should therefore be taken into account to inform inference, especially when considering that our methods to assess replication outcome are not flawless. As already explained, using a dichotomous approach such as statistical significance to assess replication success is limited as it only tells us whether the effect was different from zero in both studies, but not whether the effect sizes were different (Heirene 2021). This method is especially unreliable when comparing studies with different sample sizes as we did. Indeed, simply by changing the sample sizes, one could find very different p values associated with the same effect size (Piper et al. 2019, Heirene 2021). The comparison of effect sizes and their confidence intervals present an amelioration by giving information about the magnitude and direction of the effect on a continuous scale, making use of most of the statistical information available (Asendorpf et al. 2013, Heirene 2021). However, this method is still sensitive to under-powered analyses, like those from the original study, which provide only relatively uncertain estimates (i.e. with wide confidence intervals) (Verhagen and Wagenmakers 2014). Consequently, all the confidence intervals from the replication were overlapping with that of the original study, incorrectly implying that all of the effects were reproducible. This is why it may be informative to consider multiple measures of reproducibility before drawing conclusions (Valentine et al. 2011, Asendorpf et al. 2013). Improving the quality of replication studies could be facilitated by the development of straightforward methods to compare results across studies, including Bayesian approaches like the Bayes factor (Verhagen and Wagenmakers 2014).

Ontogeny of preen oil composition

As found in numerous bird species (reviewed by Alves Soares et al. 2023), we found a significant difference in overall preen oil composition between nestlings and adults. This difference could reflect non-adaptive processes such as differences in diet or physiology. It may also reflect a role of preen oil olfactory signalling for reproduction, with adults, and not nestlings, secreting reproduction-related semiochemicals. Preen oil odours may even be used to advertise sexual readiness or maturity, although this remains to be tested. In line with this, our study showed that adults had a more volatile preen oil than nestlings, as in grey catbirds *Dumetella carolinensis* (Shaw et al. 2011). Juvenile birds, which are still developing their preening behaviour, could benefit from a less volatile preen oil, as it may adhere longer to the plumage, therefore requiring less preening (Shaw et al. 2011), but this remains speculative.

In addition, our study revealed that preen oil composition was similar within families, suggesting an influence of a shared environment (i.e. the nestbox) and/or genetic relatedness.

Although we cannot disentangle the relative influences of these two factors in the absence of a cross-fostering experiment, a study on dark-eyed juncos showed that the social (nest) environment was a stronger predictor of preen oil composition than genes (Whittaker et al. 2016). A strong effect of the nest environment has also been suggested in cooperatively breeding smooth-billed anis *Crotophaga ani*, in which members of the same breeding group (i.e. sharing the same nest) have a similar preen oil composition and preen gland microbiota although they are not genetically related (Grieves et al. 2024). In line with this, we found that breeding partners, which are in theory relatively unrelated (Kruuk et al. 2002), produce a similar preen oil, further suggesting that the nest environment has an effect on preen oil composition in pied flycatchers ('Similarity between breeding partners' above). Several features of the nest environment could affect preen oil composition, notably the nest microbiome and the nest occupants themselves (i.e. social environment) (Jacob et al. 2014, Whittaker et al. 2016, Goodenough et al. 2017).

Our study also indicated that, although not significant after multiple testing correction, the preen oil composition of nestlings resembled marginally more that of their father than that of their mother. As mothers spend more time with nestlings than fathers, we would expect to find the opposite pattern. We also found that nestlings secrete a preen oil that resembled marginally more that of adult males (other than their father) than females (other than their mother), which was surprising since we found no difference between sexes in adults. Furthermore, the preen oil of nestlings was not more similar to the preen oil of their mother than to that of other adult females, nor was it more similar to the preen oil of their father than to that of other adult males. We expected the opposite, since we found pair and family signatures, suggesting an influence of the nest environment on preen oil composition. However, these unexpected results align almost exactly with the results of a study on dark-eyed juncos (Whittaker et al. 2016). Indeed, although dark-eyed junco chicks are more in physical contact with their mother, their preen oil is more similar to that of their father than to that of their mother. Besides, although no sex differences were found in adults at the time of fledgling, dark-eyed junco fledglings secrete a preen oil that is more similar to that of males than to that of females. Finally, even though Whittaker et al. (2016) found strong effects of the social environment in the nest on preen oil, the preen oil of dark-eyed junco chicks is not more similar to that of their father than to that of other adult males. In dark-eyed juncos, the male-like/father-like preen oil of nestlings has been suggested to be driven by the fact that nestlings have a lower abundance of compounds that are typically abundant in adult females (female-like) (Whittaker et al. 2016). In many bird species, females tend to exhibit greater richness and diversity of substances in their preen oil than males (Whittaker and Hagelin 2021). Such a pattern may explain why young birds secrete a more male-like preen oil, as they may lack substances secreted by adult females or contain them in lower proportions. The male-like preen oil of pied flycatcher nestlings could also be due to their reduced volatility, as we also found that volatility was marginally lower in males than females. But this remains speculative, especially since we found no sex difference in adult pied flycatchers. Note also that since we did not determine the sex of the nestlings, it is possible that the male-like preen oil we observed in our results is due to a higher proportion of male nestlings in our data. To our knowledge, family signatures on preen oil composition had only been studied in dark-eyed juncos (Whittaker et al. 2016). But similarities within families regarding microbiota have been more extensively investigated. Many of these studies highlight the importance of nest and social environments on avian microbiome (Ruiz-Rodriguez et al. 2014, Engel et al. 2020, Maraci et al. 2022, Grieves et al. 2024). Taking inspiration from these studies, future research should conduct cross-fostering experiments to disentangle environmental and genetic effects on preen oil composition. The heritability of preen oil composition could be further investigated by studying pedigreed populations (e.g. using 'animal models', which is now possible with compositional data; Wilson et al. 2010, Sweeny et al. 2023).

Conclusion

In a dedicated replication study, we showed the robustness of the effect of breeding stage on preen oil composition within individual female pied flycatchers, while revealing the fragility of the effects of sex and pair identity. The non-reproducibility of a substantial portion of the original results emphasises the critical need for more replication studies in the field of avian chemical ecology and beyond. To more comprehensively describe chemical signatures in preen oil, future research should also investigate sexual differences during the mate choice period, and determine whether individual plasticity across breeding stages is exclusive to the incubating sex. Our study also provides novel insights into the ontogeny of preen oil composition in this species, but further investigations are needed to validate whether family signatures and male-like preen oil in nestlings are consistent patterns in this and in other species. Finally, future research should conduct experiments (e.g. bioassays on antimicrobial activity, behavioural trials on olfactory preferences) to examine the role of preen oil in reproduction.

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Author contributions

Laurence Jeanjean: Data curation (lead); Formal analysis (lead); Funding acquisition (supporting); Methodology (supporting); Visualization (equal); Writing - original draft (equal); Writing - review and editing (equal). **Barbara A. Caspers**: Conceptualization (equal); Funding acquisition (lead); Methodology (equal); Project administration (equal); Resources (lead); Supervision (equal); Writing - review and editing (supporting). **Tim Schmoll**: Conceptualization (equal); Funding acquisition (equal); Investigation (lead); Methodology (equal); Project administration (equal); Supervision (equal); Writing - review and editing (equal). **Marc Gilles**: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Funding acquisition (equal); Methodology (equal); Project administration (equal); Supervision (lead); Visualization (equal); Writing - original draft (equal); Writing - review and editing (equal)

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Data availability statement

Data are available from the Publications at Bielefeld University public repository: https://doi.org/10.4119/unibi/2993792 (Jeanjean et al. 2024).

Supporting information

The Supporting information associated with this article is available with the online version.

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