



Chemical analysis reveals sex differences in the preen gland secretion of breeding Blue Tits

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Received: 29 March 2021 / Revised: 22 July 2021 / Accepted: 26 July 2021 / Published online: 11 August 2021
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Abstract

Acoustic and visual signals are well known to play important roles in social communication in birds. Growing evidence suggests that many bird species, including species of songbirds, additionally have a well-developed sense of smell. However, we are still at the beginning of understanding the potential importance of chemical communication in the social lives of birds, for example in mate choice. The secretion of the preen gland may be an important contributor to the chemical phenotype of birds. Here, we report on a first characterisation of the chemical composition of the preen gland secretion of the Blue Tit (*Cyanistes caeruleus*), a common songbird which is an often used model species in animal behaviour and ecology, in particular also in studies of sexual selection and (extra-pair) mate choice. We found sex differences in the composition of the preen gland secretion in breeding Blue Tits. Females further tended to have a larger number of putative compounds in their secretions compared to males. We briefly discuss the possible implications of these findings and speculate that the chemical composition of the preen gland secretion may be a sexually selected trait in Blue Tits. Our preliminary findings warrant follow-up research into the patterns of within- and among individual variation in the chemical composition of the preen gland secretion as well as the identification of the main chemical compounds involved.

Keywords Uropygial gland · VOCs · Avian olfaction · Sexual selection · *Cyanistes caeruleus*

Zusammenfassung

Chemische Analyse zeigt Geschlechtsunterschiede im Bürzeldrüsensekret brütender Blaumeisen

Singvögel sind vor allem für ihren Gesang und ihre Gefiedermerkmale, die bei der sozialen Kommunikation eine bedeutende Rolle spielen, bekannt. Immer mehr Hinweise deuten aber darauf hin, dass viele Vogelarten, darunter auch Singvogelarten, zusätzlich einen gut entwickelten Geruchssinn haben. Welche Bedeutung die geruchliche Kommunikation im sozialen Leben der Vögel, zum Beispiel bei der Partnerwahl, spielt ist allerdings weitgehend noch unbekannt. Das Bürzeldrüsensekret könnte in diesem Zusammenhang einen wichtigen Beitrag zum chemischen Phänotyp der Vögel leisten. In unserer Studie haben wir uns die chemische Zusammensetzung des Bürzeldrüsensekrets der Blaumeise (*Cyanistes caeruleus*) in der Brutzeit angeschaut. Blaumeisen sind eine viel genutzte Modellart in der Verhaltensforschung und Verhaltensökologie, insbesondere auch in Studien zur sexuellen Selektion und (außerpaarigen) Partnerwahl. Anhand von chemischen Analysen mittels Gas-Chromatographie fanden wir heraus, dass es bei brütenden Blaumeisen einen Geschlechtsunterschied in der Zusammensetzung des Bürzeldrüsensekrets gibt. Weibchen neigen außerdem dazu, eine größere Anzahl von vermeintlichen Substanzen in ihren Sekreten zu haben als Männchen. Wir diskutieren hier kurz die möglichen Implikationen dieser Ergebnisse und spekulieren, dass die chemische Zusammensetzung des Bürzeldrüsensekrets ein sexuell selektiertes Merkmal

Communicated by I. Moore.

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bei Blaumeisen sein könnte. Unsere vorläufigen Ergebnisse rechtfertigen Folgeuntersuchungen, in denen die Identifikation der wichtigsten chemischen Verbindungen des Bürzeldrüsensekrets und die Variation innerhalb und zwischen Individuen in der chemischen Zusammensetzung des Drüsensekrets im Fokus stehen.

Introduction

Birds are well known and much studied for their use of acoustic and visual signals in intraspecific communication (e.g. Catchpole and Slater 2003; Hill and McGraw 2006). The possibility that chemicals might also be involved in intraspecific communication has been widely ignored until recently (Roper 1999; Caro et al. 2015). Due to the misbelief that birds, and particularly songbirds, are anosmic (i.e. have no sense of smell)—in combination with the clear presence of acoustic and visual signals used in intraspecific communication—the existence and functionality of an avian olfactory phenotype has received little attention. This is surprising since chemical communication is omnipresent and can be found across the animal kingdom from one-cell organisms to humans (Müller et al. 2020).

Body odours are predestined to act as infochemicals transferring information about an organism (Müller et al. 2020) and evidence exists for several decades that chemicals are involved in intraspecific communication also in birds (Jacob et al. 1979; Balthazart and Schoffeniels 1979; Kolattukudy et al. 1987). In Mallards (*Anas platyrhynchos*), for example, females in contrast to males change the composition of the preen gland secretion between the reproductive and non-reproductive season (Jacob et al. 1979) and produce a specific substance during the breeding season only (Kolattukudy et al. 1987). Moreover, male mallards being deprived of their sense of smell showed reduced sexual behaviour compared to those with intact olfactory capabilities (Balthazart and Schoffeniels 1979). Although these early studies demonstrated sex differences in the chemical phenotype and the likely importance of chemicals in social communication in birds, only recently this phenomenon has received more extensive scientific investigation.

The secretion produced by the preen (or uropygial) gland, which is present in almost all bird species, is potentially a primary source of chemical cues in birds (Hagelin 2007). The preen gland—located on the lower back at the basis of the tail—is the only holocrine skin gland present in birds (Jacob and Ziswiler 1982). It releases a waxy, oily secretion that birds transfer onto their plumage during preening. The secretion has long been known to aid waterproofing and the physical integrity of the feathers (Elder 1954; Giraudeau et al. 2010; but see Salibian and Montalti 2009) and to protect them against feather-degrading bacteria (Jacob and Ziswiler 1982; Martín-Platero et al. 2006; Shawkey et al. 2003). Whilst few ornithologists would deny that the preen gland secretion plays an essential role in feather maintenance, this does not preclude other important functions. The

secretion consists of a large number of volatile compounds (Campagna et al. 2012), which could potentially be important in intraspecific chemical communication.

The chemical composition of the preen gland secretion has been found to hold information on sex in several species (Jacob et al. 1979; Reneerkens et al. 2007; Martín-Vivaldi et al. 2009; Whittaker et al. 2010; Zhang et al. 2010; Leclaire et al. 2011; Amo et al. 2012a; Mihailova et al. 2014; Tuttle et al. 2014; Grieves et al. 2019b), but not in some others (Reneerkens et al. 2007; Gabirot et al. 2018). In addition, it has been found that the secretion may hold information on age (Shaw et al. 2011), the major histocompatibility complex (Leclaire et al. 2014, 2017; Slade et al. 2016; Grieves et al. 2019b), genetic relatedness (Leclaire et al. 2012; Potier et al. 2018), individual identity (Whittaker et al. 2010) and health status (Grieves et al. 2018; Díez-Fernández et al. 2020). Moreover, behavioural studies have shown that olfactory cues are used in kin recognition (Bonadonna and Sanz-Aguilar 2012; Krause et al. 2012; Caspers et al. 2015, 2017) and influence mate choice (Balthazart and Taziaux, 2009; Caspers et al. 2015; Grieves et al. 2019a, b). Although the number of studies characterising chemical cues potentially involved in intraspecific communication in birds is increasing, we are still only at the beginning of unravelling the information that may be transferred via these chemical cues. This study is aimed at providing a first characterisation of the composition of the preen gland secretion in a well-studied songbird species, the Blue Tit *Cyanistes caeruleus*, which is an important model in studies of animal behaviour and ecology (reviewed by Stenning 2018).

There is evidence that Blue Tits have a sense of olfaction (Steiger et al. 2008), which they may use in different situations, such as nest building or social communication (Petit et al. 2002; Mennerat et al. 2009; Rossi et al. 2017). However, although the Blue Tit has long been used as a model particularly in studies of sexual selection and (extra-pair) mate choice (e.g. Kempenaers et al. 1992; Sheldon et al. 1999; Foerster et al. 2003; Korsten et al. 2006; Magrath et al. 2009; Schlicht et al. 2015), it remains unknown whether the chemical phenotype also plays a role in mate choice decisions in this species. To assess whether relevant information might be encoded in the preen gland secretion of Blue Tits, we investigated whether there is a sex difference in the preen gland secretion (i.e. whether the sexes differ in the composition of their preen gland secretion). To this aim, we collected preen gland secretion of male and female breeding Blue Tits and characterised its chemical composition, using gas chromatography.

Methods

Study population and field methods

We collected the preen gland secretion samples in a nest box population of Blue Tits in ‘De Vosbergen’ near Groningen, The Netherlands, which has been studied since 2001 (Korsten et al. 2006; Amininasab et al. 2016). During the breeding season of 2016, we sampled the preen gland secretion of 33 adult Blue Tits ($n = 13$ males; $n = 20$ females), which we captured inside their nest boxes during chick feeding (in May). We sexed the birds based on the presence (= female) or absence (= male) of an incubation patch. For collecting the samples, we used commercially available cotton wool buds (‘Jeden Tag Wattestäbchen’, Offenburg, Germany), with which we gently massaged the nipple of the birds’ preen glands (following e.g. Reneerkens et al. 2002). We stored the cotton wool buds with the preen gland secretion samples in Teflon-capped 20 ml glass vials (Labsolute®, Th. Geyer; Fig. 1). In the field, we also created blank samples without preen gland secretion as negative controls by taking the cotton wool buds briefly out of the sample vials (ca. 5 s) and placing them back in without sampling a bird ($n = 2$ blank samples). For further details on our standard field procedures, see Korsten et al. (2006). After collection in the field, samples were stored at -20 °C (on the day of their collection).

Sample processing and chemical analysis

At the start of the chemical processing, all samples were defrosted and 200 μl Dichloromethane (DCM, 99.9% purity) was added to each sample. Afterwards each sample was vortexed and 50 μl of DCM was extracted from the cotton, using a blunt-shaped glass syringe (Hamilton®, Bonaduz, Switzerland). The extracted samples were directly transferred into a 2 ml (Rotilabo®, Karlsruhe, Germany) glass vial containing a 100 μl glass inset. These extracted samples were analysed using gas chromatography with flame-ionisation detection (GC-FID, GC2010, Shimadzu) equipped with a VF-5 ms capillary column (30 m x 0.25 mm ID, DF 0.25, 10 m guard column, Varian Inc., Lake Forest, California, USA). For analysis, 1 μl of each sample was injected into a deactivated glass wool-packed liner at an inlet temperature of 250 $^{\circ}\text{C}$ and processed in a split 10 mode with 20 ml/min split flow. Hydrogen was used as carrier gas and its flow rate was held at 1 ml/min. The GC temperature started at 50 $^{\circ}\text{C}$ for an initial time of 3 min, followed by a 10 $^{\circ}\text{C}/\text{min}$ rate of increase to a final temperature of 280 $^{\circ}\text{C}$, which was kept for 20 min. A characteristic chromatogram is provided in Fig. 2. All of the chromatograms can be found in the supplementary material. For each chromatogram, the area underneath each



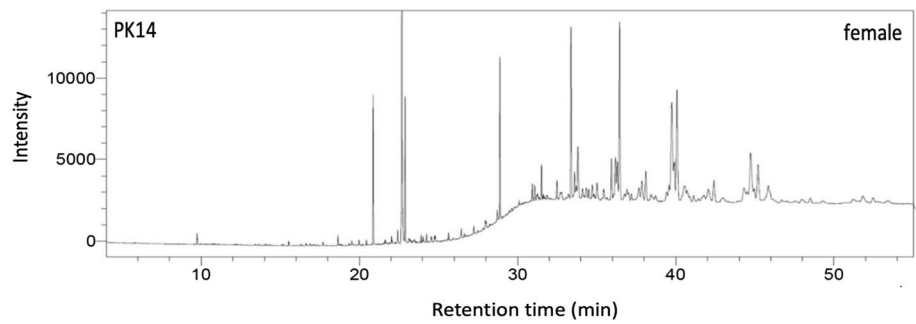
Fig. 1 Glass vial holding a cotton wool bud with a sample of preen gland secretion of a Blue Tit

peak was calculated and used for later analysis. The aim of this study was to investigate whether male and female breeding Blue Tits differ in the composition and diversity of their preen gland secretions. For this aim, we used GC-FID, which does not allow us to identify the single substances. Thus, we currently cannot identify the substances involved. However, this will be our aim in follow-up work.

Statistical analyses

Before further data analyses, we removed all recordings of putative substances also present in the cotton wool bud blank samples ($n = 2$) and removed all chemical singletons, i.e. substances that were only present in one of the samples (following Stoffel et al. 2015). In total, we found 475 different putative substances (based on retention time), which were recorded in at least 2 of our samples. Substances were aligned using the R package GCAIignR (Ottensmann et al. 2018) ran in R version 4.0.2 (R Core Team 2020). A large share of the substances (40%, $n = 193$) in this dataset were present in fewer than five individuals (Fig. 3a), with 3% of the substances ($n = 16$) being shared by all 33 individuals.

Fig. 2 Example chromatogram of the preen gland secretion of a Blue Tit female (chromatogram ID: PK14). All chromatograms can be found in the supplementary material



Next, for each sample, we calculated the relative contribution (%) of each substance to the total peak area of all substances (following Caspers et al. 2009; Leclaire et al. 2012; Stoffel et al. 2015). With this procedure, we ascertained that all chromatograms were comparable on a similar scale, since the total amount of preen gland secretion collected likely differed amongst individuals. Using these data, we compared the chemical diversity between the sexes, specifically the total number of substances, and the Shannon- and Simpson-Indices, by applying Mann–Whitney U tests.

For further analyses, we also created a reduced dataset by omitting all substances that contributed less than 0.1% to the samples' total peak area (Leclaire et al. 2012; Grieves et al. 2019b). A substance was only removed, if in none of the samples the contribution was larger than 0.1%. This reduced dataset consisted of 154 different putative substances. In the reduced dataset, 10% ($n = 16$) of substances were shared by all 33 individuals (Fig. 3b) and 12% of substances ($n = 19$) were shared by fewer than five individuals, giving more weight to those substances present in a larger proportion of the samples. Thereafter, peaks were again standardised by total peak area per individual (Stoffel et al. 2015). We compared the chemical composition between the sexes by computing a pairwise similarity matrix using the Bray–Curtis similarity index on the $\log(x + 1)$ transformed data. Then, we analysed potential differences between a priori defined groups (i.e. the sexes) with a non-parametric permutation-based analysis of similarities (ANOSIM). The ANOSIM is a permutation test that allows for determining whether samples within a priori defined groups are more similar on average

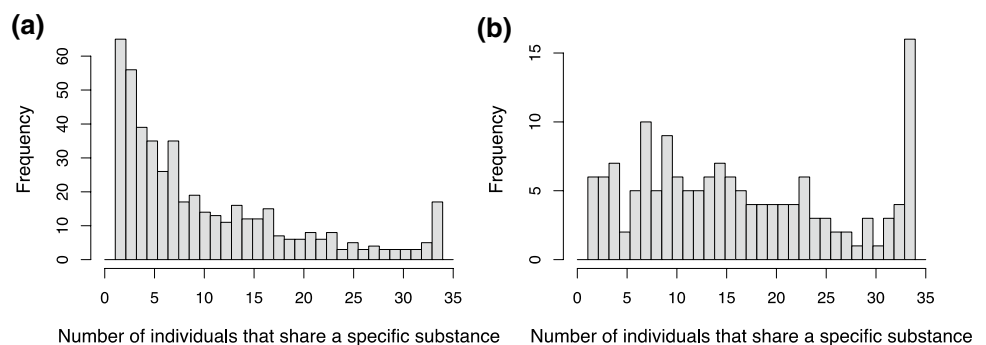
than samples between groups. The ANOSIM analyses were performed on the full dataset ($n = 475$ substances) and on the reduced dataset ($n = 154$ substances) using PRIMER 6.1.12 (Primer-E 2000 Ltd., Plymouth, UK). We visualised our data using a non-metric multidimensional scaling plot (nMDS). The nMDS plot gives a two-dimensional representation of the multidimensional matrix of pairwise similarities. The closer two symbols appear on such a plot the more similar the two samples are in their chemical composition. The axes are dimensionless. The significance level was set to 0.05 and we used two-tailed tests throughout.

Results

The chemical composition of the preen gland secretions differed markedly between the sexes, as shown by the ANOSIM analysis (ANOSIM: Global $R = 0.219$, $p < 0.001$; Fig. 4a), i.e. within-sex similarities were higher than between-sex similarities. The same pattern was found when analysing the full dataset of 475 substances (ANOSIM: Global $R = 0.274$, $p < 0.001$).

The preen gland secretion of females further tended to contain more putative substances (Mann–Whitney U test; $W = 181.5$, $p = 0.06$; Fig. 2b), with females having 163 putative substances (median) and males 110 (median). There was no difference in the diversity of the substances in the preen gland secretion between the two sexes as measured by

Fig. 3 Histograms showing how many individuals share a specific substance (a) based on the whole dataset ($n = 475$) and (b) based on the reduced subset, considering only substances contributing more than 0.1% of the total peak area of the samples ($n = 154$)



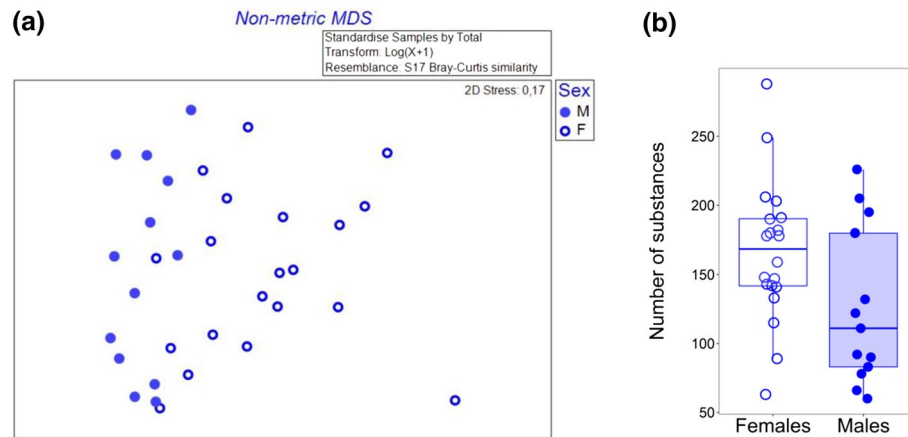


Fig. 4 Blue tits show significant sex differences in the composition (a) of the preen gland secretion with females tending to have a larger number of putative substances (b) in their preen gland secretion. **a** Non-metric multidimensional scaling (nMDS) plot of similarities between male and female Blue Tit preen gland secretions. Each

symbol represents an individual. nMDS plots are dimensionless, the closer two symbols appear on the plot, the more similar the chemical samples are in their composition. The similarity matrix was computed based on the Bray–Curtis Similarity Index. *F* female, *M* male. **b** Box plot of the number of putative substances in females and males

the Shannon and Simpson indices (Mann–Whitney *U* test; Shannon: $W = 148$, $p = 0.52$; Simpson: $W = 125$, $p = 0.87$).

Discussion

There is growing evidence that communication via chemicals is important in songbirds (e.g. Caro et al. 2015; Caspers et al. 2017; Grieves et al. 2019a; Whittaker et al. 2019). Blue tits likely have a well-developed sense of smell (Petit et al. 2002; Mennerat et al. 2009; Rossi et al. 2017), which raises the possibility that the chemical phenotype plays a role in intraspecific communication in this common songbird species. In this first explorative study we found significant differences in the chemical composition between males and females. Female secretions further tended to have a larger number of different putative substances compared to the secretions of males. The latter finding is in line with the pattern in the majority of birds studied so far in which females had larger diversities in case of sex differences in the chemical phenotypes (Whittaker and Hagelin 2020).

Functional explanations for a sex difference in preen gland secretion may be a potential role of chemicals from the female preen gland secretion in protecting the eggs from microbes, in suppressing the number of ectoparasites in the nest, or in providing crypsis to avoid olfactory hunting predators. Given that nest building and maintenance as well as incubation of the eggs are largely restricted to the female in Blue Tits, the production of certain molecules that play a role in nest hygiene, or egg and chick, or self-protection (Jacob et al. 1979) might be higher in females compared to males. Protection of eggs or chicks due to the presence of less detectable molecules has been described as the chemical crypsis

hypothesis (Reneerkens et al. 2005). In ground-breeding sandpipers, for example, it is known that seasonal changes from monoester-fatty-acids to diesters occur only in the incubating sex (Reneerkens et al. 2007). These diesters are less volatile and thus less detectable by olfactory hunting predators (Reneerkens et al. 2005). A larger diversity might not intuitively hint at a less detectable bouquet. However, it is noteworthy that in another songbird species, the dark-eyed junco (*Junco hyemalis*), female preen gland secretion contains more substances also found in plants, and thus an increased diversity might indeed help to provide chemical camouflage to the nest in its natural environment (Soini et al. 2007).

Another explanation for the sex difference in preen gland composition might be a potential role during intraspecific communication, which has been put forward as the sex semiochemical hypothesis (Grieves 2020). The sex semiochemical hypothesis posits that sex differences in preen gland secretion are associated with reproduction and the odour cues are involved in intraspecific chemical communication. Variation in the composition of the preen gland secretion might be correlated with sex specific characteristics and consequently influence mate choice (Amo et al. 2012a, b; Whittaker et al. 2013, 2018; Grieves et al. 2019b). In dark-eyed-juncos, for example, it was found that males with a relatively more ‘male-like’ odour profile sired more offspring in their own nest, which had a higher survival rate (Whittaker et al. 2013). Preen gland secretions may also provide information on MHC similarity or diversity (Leclaire et al. 2014, 2017; Slade et al. 2016; Grieves et al. 2019a, b) and relatedness (Krause et al. 2012; Leclaire et al. 2012; Potier et al. 2018), which both may also influence mate choice. This would require aspects of the chemical composition to be repeatable within individuals over time, thereby allowing

for reliable signalling of certain genotypes, something that would need to be further investigated.

In summary, we found sex differences in the chemical phenotype of breeding Blue Tits, in particular in the composition of the preen gland secretion. Furthermore females may have a larger number of different substances in the preen gland secretion (although this result was marginally non-significant). Although at this point we do not know whether the differences between the sexes have an adaptive value, either in protection or intraspecific communication, our finding clearly opens up the possibility that the chemical phenotype carries certain information which could play a role in mate choice. Further research is needed to investigate to what extent the chemical phenotype varies across seasons and within individuals, as well as to identify the main chemical compounds involved.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10336-021-01921-w>.

Acknowledgements We thank the Kraus-Groeneveld foundation for permission to work at the ‘De Vosbergen’ estate. We thank A. Hulshof, S. Kloek, P. van Kronenberg, A. Roelofs and S. Zondervan for help with the fieldwork. N. Lillie kindly helped with drawing the graphs. We are grateful to the two anonymous referees for helpful comments on an earlier version of the manuscript.

Funding Open Access funding enabled and organized by Projekt DEAL. This study was financially supported by a Freigeist-Fellowship of the Volkswagen Foundation to BAC.

Data accessibility All data are submitted as supplementary material.

Declarations

Conflict of interest The authors declare no conflicts of interest.

Ethical standards The study was conducted in full compliance with Dutch law and regulations.

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