Chemical Communication in Songbirds

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Biology
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Abstract

Avian chemical communication has been understudied due to the misconception that olfaction is unimportant or even lacking in birds. Early work focused on the olfactory foraging capabilities of seabirds because of their ecology (open ocean foraging) and large olfactory bulbs. In contrast, olfaction in passerine birds, comprising over half of all extant avian taxa, was long overlooked due to their relatively small olfactory bulbs. It is now well established that passerines can smell, and their olfactory acuity is comparable to that of macrosmatic mammals such as rats. Much of our theory on communication and mate choice has involved studying visual and acoustic signals in birds, especially passerines. However, there is mounting evidence that chemical cues are a previously overlooked but important element of avian communication and mate choice. I used gas chromatography to explore sources of variation in song sparrow (Melospiza melodia) preen oil. I then performed behavioural experiments to test whether song sparrows are capable of discriminating among preen oil odour cues. Finally, I explored the hypothesis that major histocompatibility complex (MHC) genotype underlies variation in preen gland microbiota and that this contributes to variation in preen oil chemical composition, providing a potential mechanism for MHC-based mate assessment. Preen oil differed between birds experimentally infected with haemosporidian malaria parasites (Plasmodium sp.) and sham-inoculated controls; between populations, ages, sexes, and breeding versus postbreeding seasons; and with MHC genotype. Song sparrows used preen oil odour to discriminate between the sexes, and to discriminate the MHC similarity and diversity of potential mates. Preen gland microbes differed between populations and sexes, and covaried with MHC genotype but not with preen oil composition. Collectively, my thesis establishes that preen oil is information-rich and that birds use preen oil odour cues in ecologically relevant contexts. I provide some of the first evidence that pathogen exposure alters chemical cues in birds, that birds use odour cues to discriminate the MHC genotype of potential mates, and that MHC genotype is positively correlated with both preen gland microbes and preen oil chemical composition.
Keywords

Animal communication, bacteria, chemical communication, malaria, mate choice, MHC, olfaction, preen oil, songbird, uropygial gland
Summary for Lay Audience

Most birds have a specialized preen gland that secretes preen oil, a waxy substance involved in both feather maintenance and chemical communication. I measured chemical differences in preen oil from different groups of birds and tested whether song sparrows use smell to detect such differences.

Avian malaria is a disease that affects over 70% of the world’s bird species, impacting their reproduction and survival. I compared preen oil from malaria-infected and uninfected birds, showing that preen oil changed with exposure to malaria parasites. I then tested whether birds avoid the preen oil of infected individuals, but found no evidence for this. Next, I showed that preen oil differs between species, populations, ages, sexes, and seasons. I tested song sparrows’ responses to preen oil from same versus opposite sexes and from brood parasites, species that rely on other species to raise their young. Both sexes spent more time with opposite-sex than same-sex preen oil, while males spent more time and females spent less time with brood parasite oil.

An essential part of immune defense in vertebrate animals is a set of genes called the major histocompatibility complex (MHC). High MHC allelic diversity can increase disease resistance, so animals should prefer mates with MHC genes different from their own. Offspring from MHC-dissimilar mates should have greater MHC diversity and disease resistance. Because this is so important, natural selection likely provides animals with ways to assess MHC. Fish and mammals use smell, but we do not know how birds assess MHC. Preen oil can reflect MHC genotype, so birds may use preen oil odour to choose MHC-dissimilar mates, thereby protecting their offspring from disease. Using behavioural trials, I showed that song sparrows spent more time with preen oil from MHC-dissimilar and MHC-diverse potential mates. Finally, I used genetic sequencing to identify the bacteria living in song sparrows’ preen gland, showing that bacteria differ between sexes and populations, and with MHC genotype. Birds with more similar MHC genotypes had more similar preen gland bacteria and oil. Overall, my thesis showed that scent-based communication in birds is more common and more complex than previously believed.
Co-Authorship Statement

All data chapters are co-authored with Dr. Elizabeth MacDougall-Shackleton as the senior author. She provided funding for all projects, assisted with field data collection, contributed to study design, and provided statistical advice and editorial comments.

A version of Chapter 2 was published in *The Auk*. Myself (LAG), TRK, and EAM-S designed the study. LAG and TRK conducted the fieldwork, and collected the data. I collected and analyzed all preen oil data while TRK conducted infection trials and assessed infection status. EAM-S and MAB supervised the research. LAG and EAMS wrote the paper with input from TRK and MAB. Because I was not the sole author who collected data, I use the pronoun “we” to describe all procedures in the methods section of this chapter.

**Citation:** Grieves LA, Kelly TR, Bernards MA, MacDougall-Shackleton EA. 2018. Malarial infection alters wax ester composition of preen oil in songbirds: results of an experimental study. Auk. 135(3):767–776.

A version of Chapter 3 is being prepared for submission to the *Journal of Parasitology*. LAG and EAM-S designed the study. EAM-S supervised the research. LAG collected and analyzed the data, with input from EAM-S. LAG wrote the paper with input from EAM-S.

**Citation:** Grieves LA, MacDougall-Shackleton, EA. No evidence that songbirds use odor cues to avoid malaria-infected conspecifics. J Parasitol. In prep.

A version of Chapter 4 was published in the *Journal of Chemical Ecology*. LAG and EAM-S designed the study. LAG collected and analyzed the data with input from MAB and EAM-S. LAG wrote the paper with input from MAB and EAM-S. MAB and EAM-S supervised the research.

**Citation:** Grieves LA., Bernards MA, MacDougall-Shackleton EA. 2019. Wax ester composition of songbird preen oil varies seasonally and differs between sexes, ages, and populations. J Chem Ecol. 45(1):37–45.
A version of Chapter 5 was published in *Animal Behaviour*. LAG and EAM-S designed the study. LAG collected and analyzed the data with input from MAB and EAM-S. LAG wrote the paper with input from MAB and EAM-S. EAM-S supervised the research. **Citation:** Grieves LA, Bernards MA, MacDougall-Shackleton EA, 2019. Behavioural responses of songbirds to preen oil odour cues of sex and species. Anim Behav. 156:57–65.

A version of Chapter 6 was published in *Animal Behaviour*. LAG and EAM-S designed the study. LAG collected and analyzed the data with input from GBG, MAB, and EAM-S. LAG wrote the paper with input from GBG, MAB, and EAM-S. EAM-S supervised the research. **Citation:** Grieves LA, Gloor GB, Bernards MA, MacDougall-Shackleton EA. 2019. Songbirds show odour-based discrimination of similarity and diversity at the major histocompatibility complex. Anim Behav. 158:131–138.

A version of Chapter 7 is being prepared for submission to *Proceedings of the Royal Society B* with Dr. Gregory B. Gloor and Dr. Elizabeth A. MacDougall-Shackleton. LAG and EAM-S designed the study with input from GBG. GBG processed the raw sequencing data through custom pipelines prior to analysis. LAG, TRK, and EAM-S collected the data, and LAG completed all analyses with input from GBG and EAM-S. LAG wrote the chapter with input from GBG and EAM-S. GBG and EAM-S supervised the research.
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<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>16S</td>
<td>bacterial 16S gene, a component of the 30S small subunit of a prokaryotic ribosome that binds to the Shine-Dalgarno sequence (a ribosomal binding site)</td>
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<tr>
<td>2D</td>
<td>two dimensional</td>
</tr>
<tr>
<td>AFAR</td>
<td>Advanced Facility for Avian Research</td>
</tr>
<tr>
<td>AIC</td>
<td>Akaike information criteron</td>
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<tr>
<td>ANOSIM</td>
<td>analysis of similarities</td>
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<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
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<tr>
<td>bp</td>
<td>base pairs</td>
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<td>C</td>
<td>carbon</td>
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<tr>
<td>CHCl₃</td>
<td>chloroform</td>
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<tr>
<td>Class II</td>
<td>major histocompatibility complex class II</td>
</tr>
<tr>
<td>D</td>
<td>dark (hours of)</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>dNTPs</td>
<td>deoxynucleoside triphosphates</td>
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<td>GC</td>
<td>gas chromatography</td>
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<td>light (hours of)</td>
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<td>principal components analysis</td>
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<td>PC</td>
<td>principal component</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>potential hydrogen</td>
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<tr>
<td>PTFE</td>
<td>polytetrafluoroethylene</td>
</tr>
<tr>
<td>P-SOSP</td>
<td>lineage of <em>Plasmodium</em> spp. (avian malaria) found in song sparrows</td>
</tr>
<tr>
<td>R</td>
<td>coefficient of determination, proportion of variance in dependent variable predictable from independent variable</td>
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<td>the square of the correlation, R²</td>
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<td>ribosomal ribonucleic acid</td>
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<td>SE</td>
<td>standard error</td>
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Sosp-DAB* putative functional MHC class II exon 2 allele in song sparrows
SV sequence variant (synonym for OTU, operational taxonomic unit)
t test statistic following a Student’s t-distribution under null hypothesis
Taq * Thermus aquaticus
TMA trimethylamine
Tris-HCl trisaminomethane-hydrochloric acid
TURDUS lineage of Plasmodium spp. (avian malaria) found in thrushes (Turdus sp.)
UniFrac unique fraction metric, a measure of phylogenetic distance between taxa or between alleles
UV ultraviolet
V volts
V4 hypervariable region 4 of the bacterial 16S gene 30S small ribosomal subunit
WAG Whelan and Goldman (model)
\( \chi^2 \) test statistic following a chi-squared distribution under null hypothesis
Appendix A Table A1 Studies and species included in meta-analysis testing for an effect of time of year (breeding versus nonbreeding stage) and incubation type (uniparental versus biparental) on the probability of detecting sex differences in preen oil chemical composition. Effect size calculations are based on reported sample sizes and test statistics (e.g. F, t, U, and Z), where possible. NA indicates that effect size calculations were not possible due to missing or unclear data.

Appendix B Table B1 Chemical composition of song sparrow (Melospiza melodia) preen oil wax esters as determined by gas chromatography-mass spectrometry (GC-MS; N = 21; all sexes, seasons, age classes, and populations combined). Carbon numbers refer to the total number of carbons in the monoester. Percent of ester is an average for all birds measured. Percent of ester at each carbon number represents the total amount of isomeric monoesters by combining the contributions from the individual isomers (i.e., peaks A and B combined). Monoester peak A and B refers to two peaks resolved by GC-MS that had the same carbon number but different retention times (see Chapter 4 text for details).

Appendix B Figure B1 Complete wax ester composition of breeding stage adult song sparrow preen oil at Newboro (N females = 3, N males = 3, mean ± SD). Peaks that were at least 0.1% of the total chromatogram area were retained for analysis, while peaks that were < 0.1% were counted as zero (see Chapter 4 text for details).

Appendix B Figure B2 Selected wax ester composition of breeding stage adult song sparrow preen oil at Newboro (N females = 3, N males = 3, mean ± SD). For complete wax ester composition see Appendix B, Fig. B1.

Appendix B Figure B3 Wax ester composition of song sparrow preen oil from post-breeding stage adults and juveniles (sexes pooled for juveniles, sampled at Newboro). Peaks that were at least 0.1% of the total chromatogram area were retained for analysis, while peaks that were < 0.1% were counted as zero (see Chapter 4 text for details).
Appendix B Figure B4 Complete wax ester composition of breeding-stage song sparrow preen oil at Newboro and Cambridge (N_{Newboro males} = 3, N_{Newboro females} = 3, N_{Cambridge males} = 3, N_{Cambridge females} = 3, mean ± SD). Peaks that were at least 0.1% of the total chromatogram area were retained for analysis, while peaks that were < 0.1% were counted as zero (see Chapter 4 text for details). .................................................................253

Appendix B Figure B5 Selected wax ester composition of breeding-stage song sparrow preen oil at Newboro and Cambridge (N_{Newboro males} = 3, N_{Newboro females} = 3, N_{Cambridge males} = 3, N_{Cambridge females} = 3, mean ± SD). For complete wax ester composition, see Appendix B, Fig. B4. .................................................................................................................254

Appendix B Figure B6 Complete wax ester composition of adult song sparrow preen oil sampled during breeding and post-breeding stages at Newboro (N_{early-season females} = 3, N_{late-season females} = 2, N_{early-season males} = 3, N_{late-season males} = 3, mean ± SD). Peaks that were at least 0.1% of the total chromatogram area were retained for analysis, while peaks that were < 0.1% were counted as zero (see Chapter 4 text for details). .................................................................................................................255

Appendix B Figure B7 Selected wax ester composition of adult song sparrow preen oil sampled during breeding and post-breeding stages at Newboro (N_{early-season females} = 3, N_{late-season females} = 2, N_{early-season males} = 3, N_{late-season males} = 3, mean ± SD). For complete wax ester composition see Appendix B, Fig. B5. .................................................................................................................256

Appendix C Table C1 MHC class II exon 2 alleles and associated GenBank accession numbers (Acc. No.). ..................................................................................................................................................257

Appendix D Detailed bacterial DNA extraction protocol. .........................................................................................260

Appendix D Table D1 Bayesian Ribosomal Database Project taxonomic assignment of sequence variants (SVs) collected from the uropygial gland of adult song sparrows that were removed from further analysis as putative contaminants (see text for details). .....262

Appendix D Table D2 Bayesian Ribosomal Database Project taxonomic assignment of sequence variants (SVs) collected from the uropygial gland of adult song sparrows. Superscripts indicate taxa previously reported from the uropygial gland, feathers.
surrounding the gland, feathers on rump (near the gland), or from body and wing feathers of other bird species.

Appendix D Figure D1 The mean number of A) MHC class II alleles and B) preen oil peaks per individual differs between free-living song sparrows sampled from London and Cambridge breeding populations separated by approximately 100 kms. Open circles show individual values, lines show mean ± SE. Sample sizes are reported in parentheses.

Appendix E Ethics statement.
Chapter 1

1 General introduction

1.1 Animal communication

All animals use communication to navigate their social environment. Communication is the process by which information is transferred between individuals. In turn, this information affects the behaviour of the individual receiving the information. Thus, communication requires both a sender (signaler) and a receiver, as well as a signal (Searcy and Nowicki 2005). Signals are defined as structures or actions that alter the behaviour of a receiver, that evolved because of that effect on receiver behaviour, and that are effective (i.e., maintained by selection) because the receiver response has also evolved (Maynard-Smith and Harper 2003). In contrast, cues are features, structures, or actions that can be used to guide an individual’s actions and behaviour, but that did not evolve for that purpose (i.e., cues did not evolve to have an effect on receivers) (Maynard-Smith and Harper 2003). For example, in many frog species, male song is a signal that serves to attract sexually receptive females. On the other hand, frog song can also be used as a cue by hunting bats to locate frog prey. While a structure or action may act as both signal and cue, depending on the context (e.g., frog song), this need not be the case. For example, carbon dioxide emitted by breathing mammals is used as a cue by mosquitoes seeking blood meals.

Animals communicate using a variety of sensory modalities, including chemical, vibrational, acoustic, and visual signals. Chemical communication, which includes olfactory and gustatory communication, is one of the oldest forms of communication (Bradbury and Vehrencamp 1998). Chemical communication is taxonomically widespread; all cellular life from bacteria to animals are sensitive to chemical information (Wyatt 2014). However, the majority of research on chemical communication in animals comes from studies of insects and mammals, with other taxa having been largely overlooked (Johansson and Jones 2007).
1.2 Avian chemical communication

Avian chemical communication has been understudied because, historically, birds were believed to possess little to no sense of smell (Audubon 1826; Stager 1967; Bang and Cobb 1968). The main reason early researchers came to this conclusion appears to be due in large part to the poor design of many early experiments on avian olfaction (discussed in Stager 1967). Yet the misinformation that birds are microsmic or even anosmic has persisted to the present day, even alongside the publication of groundbreaking research demonstrating the olfactory capabilities of birds (Averett 2014). One potential explanation for the persistence of this myth is our anthropomorphic view that the rigid nostrils and bill of birds seem incapable of performing behaviours we associate with smelling (Balthazart and Taziaux 2009).

Nevertheless, our understanding of avian chemical communication is growing rapidly. It is probable that all bird species have a fully functional olfactory system (Wenzel 1971; Clark 1993; Steiger et al. 2008; Steiger et al. 2009; Zelenitsky et al. 2011). Indeed, birds use smell in a variety of contexts, including food location (Healy and Guilford 1990; Nevitt et al. 2008; Potier et al. 2019), predator avoidance (Hagelin et al. 2003; Amo et al. 2008; Amo et al. 2017; Mahr and Hoi 2018; but see Amo et al. 2018; Blackwell et al. 2018; Stanback et al. 2019), and in nest building, putatively to protect nests against parasites through the selection of repellent aromatic herbs (Clark 1991; Lambrechts and Dos Santos 2000; Lambrechts and Hossaert-McKey 2006).

Smell is also used by birds in many social contexts, including the recognition of mates (Bonadonna and Nevitt 2004) and kin (Coffin et al. 2011; Bonadonna and Sanz-Aguilar 2012; Caspers et al. 2013; Caspers et al. 2017), and in the discrimination of species (Zhang et al. 2013; Krause et al. 2014; Van Huynh and Rice 2019), individuals (Bonadonna et al. 2007; Bonadonna et al. 2009; Fracasso et al. 2018), and the sexes (Hirao et al. 2009; Whittaker et al. 2011a; Amo et al. 2012). The use of smell in avian social communication necessitates that there must be some source or sources of avian body odour that contain information which can then be transferred among individuals. That is, signals or cues that alter receiver behaviour must exist.


1.3 Preen oil as a chemical cue

Avian odours may be derived from a number of sources, including feces, blood, stomach oils, powder down, plumage, and from secretions of the anal gland, salt gland, salivary gland, ear glands, sebokeratocytes, and the uropygial or preen gland (reviewed in Hagelin and Jones 2007). In birds, the entire skin is lipogenic and acts as a sebaceous secretory organ, with the uropygial gland acting as a specialized part (Salibian and Montalti 2009). The uropygial or preen gland is a large holocrine integumentary gland located near the base of the tail in most bird species (Jacob and Ziswiler 1982; Salibian and Montalti 2009). The uropygial gland is present in the embryonic stages of all bird species that have been studied, but it is absent in the adults of some species in the orders Struthioniformes, Piciformes, Psittaciformes, and in some varieties of rock pigeon (*Columba livia*; order Passeriformes) (Moreno-Rueda 2017).

Preen oil secreted from the uropygial gland is widely considered to be the main source of avian body odour (Jacob 1978; Caro et al. 2015), and likely also contributes to plumage odour (Soini et al. 2007). Preen oil secretions are typically monoester waxes comprised of a fatty acid esterified to an alcohol moiety. These secretions usually consist of a mixture of fatty acids and alcohols with varying chain lengths and branching patterns, resulting in a complex mixture of potentially hundreds of individual wax esters of variable molecular weight (Dekker et al. 2000; Campagna et al. 2012). Higher molecular weight diester waxes have also been identified in the preen oil secretions of some Charadriiform shorebirds (Piersma et al. 1999).

Preen oil secretions serve multiple non-mutually exclusive functions in birds, including waterproofing, feather maintenance, protection against ectoparasites, pollutant depuration (reviewed in Moreno-Rueda 2017), olfactory crypsis (Reneerkens et al. 2002; Reneerkens et al. 2005), and social communication via cosmetic colouration (Amat et al. 2011) and chemical cues (reviewed in Caro et al. 2015; Moreno-Rueda 2017). Importantly, preen oil secretions should be regarded as chemical cues rather than chemical signals because, while these secretions can have an effect on an individual’s (i.e., a receiver’s) actions and behaviour, preen oil did not likely evolve for this purpose.
The chemical composition of preen oil is dynamic and can be affected by diverse factors, such as diet (Thomas et al. 2010; Leclaire et al. 2019a), food stress (Reneerkens et al. 2007a; Grieves et al. 2020), time of year (Bhattacharyya and Chowdhury 1995; Soini et al. 2007; Martín-Vivaldi et al. 2009; Fischer et al. 2017), age (Shaw et al. 2011), sex (Jacob et al. 1979; Mardon et al. 2010; Whittaker et al. 2010; Tuttle et al. 2014), circulating androgen levels (Whittaker et al. 2011b), major histocompatibility complex (MHC) genotype (Leclaire et al. 2014; Slade et al. 2016a), and skin and preen gland microbiota (Jacob et al. 2014; Whittaker et al. 2019).

Avian preen oil thus has the potential to act as a chemical cue that may convey a wealth of information to receivers. As outlined in section 1.2, there is growing evidence that birds are capable of using preen oil cues in social contexts. However, more work is needed to understand how widespread the use of preen oil chemical cues are among avian taxa, and there are many research areas that remain relatively unexplored. One of these is the role of odour cues in identifying individuals that may be harbouring infectious disease.

1.4 Odour cues and disease

A major cost of interacting with conspecifics is the increased risk of exposure to pathogens. As a result, diverse behavioural adaptations have evolved that enable animals to detect and avoid diseased conspecifics (Hedrick 2017). Olfactory avoidance mechanisms have evolved at least in part because infection can alter host body odour (Kavaliers et al. 2005; Shirasu and Touhara 2011; Olsson et al. 2014). In mammals, experimental work has shown that mice and rats are capable of using odour cues to discriminate and avoid infected conspecifics (Kavaliers and Colwell 1995; Penn and Potts 1998; Kavaliers et al. 2004). In birds, avian influenza alters fecal odour in mallards (*Anas platyrhynchos*) and mice can detect these odour cues (Kimball et al. 2013), but whether avian conspecifics are capable of detecting such cues is unknown.
Chemical cues of infection status clearly have the potential to benefit hosts, but they can also be adaptive to the pathogen. Malaria parasites (*Plasmodium* spp.) produce volatile compounds that, when emitted by infected mammalian hosts, attract insect vectors (Lacroix et al. 2005; De Moraes et al. 2014; Kelly et al. 2015; de Boer et al. 2017; Correa et al. 2017). Malaria infection increases the attractiveness of birds to uninfected mosquito vectors of *Plasmodium* (Cornet et al. 2013) and these mosquitoes are also attracted to avian preen oil (Russell and Hunter 2005), but whether this is related to malarial infection status is unknown. Given the paucity of data on this subject, I test for preen oil odour cues of malarial infection in Chapter 2 of this thesis, and I test whether hosts can use odour cues to discriminate between *Plasmodium*-infected and uninfected conspecifics in Chapter 3.

### 1.5 Factors affecting sex differences in preen oil

Understanding the factors affecting sex differences in preen oil is also of interest because chemical cues in preen oil are increasingly thought to play a role in avian mate choice and reproduction (Balthazart and Taziaux 2009; Caro et al. 2015). However, the evidence for sex differences in the chemical composition of preen oil is mixed. For example, sex differences in preen oil chemical composition have been found in breeding mallards (Jacob et al. 1979), herring gulls (*Larus argentatus*; Fischer et al. 2017), and dark-eyed juncos (*Junco hyemalis*; Whittaker et al. 2010), but not in red knots (*Calidris canutus*; Reneerkens et al. 2007a), Cory’s shearwaters (*Calonectris borealis*; Gabirot et al. 2016), or New Zealand silveryes (*Zosterops lateralis*; Azzani et al. 2016).

Seasonal changes in preen oil are related to breeding *versus* nonbreeding seasons (e.g., Bhattacharyyya and Chowdhury 1995; Soini et al. 2007) and are influenced by sex hormones such as estradiol (Bohnet et al. 1991) and testosterone (Whittaker et al. 2011b). Furthermore, the chemical characteristics of preen oil have the potential to influence mate choice (Jacob et al. 1979; Hirao et al. 2009; Leclaire et al. 2017). Thus, I propose the ‘sex semiochemical hypothesis’, which posits that sex differences in preen oil are associated with reproduction and that preen oil odour cues are involved in mate recognition.
(identifying the appropriate sex to mate with) and mate choice (identifying a suitable mate, e.g., a genetically compatible mate). The sex semiochemical hypothesis predicts that there should be an effect of breeding stage (breeding versus nonbreeding season) on preen oil, such that sex differences in the chemical composition of preen oil should be found only during the breeding stage. This hypothesis also predicts that birds should use preen oil odour cues to discriminate between the sexes and among individuals.

The ‘olfactory crypsis hypothesis’ posits that incubating birds switch from lower molecular weight (more odorous) monoester secretions to higher molecular weight (less odorous) diester secretions during incubation as a means of reducing odour cues at the nest, thereby protecting eggs and young from olfactory-searching predators (Reneerkens et al. 2002, 2007b). This hypothesis predicts an effect of both breeding stage and incubation type. Preen oil changes should only occur in breeding stage birds during incubation, leading to sex differences in uniparentally incubating, but not biparentally incubating, species. This hypothesis also predicts that mammalian predators should be better at detecting low molecular weight than high molecular weight preen oil secretions (Reneerkens et al. 2005).

The sex semiochemical and olfactory crypsis hypotheses are not mutually exclusive. I hypothesized that the probability of detecting sex differences in preen oil depends on both time of year and incubation type. Specifically, I predicted that sex differences in the chemical composition of preen oil would be more common during breeding compared to nonbreeding and in systems with uniparental incubation compared to biparental incubation. To test these predictions, I conducted a meta-analysis on the available literature that tested for sex differences in preen oil secretions.

1.5.1 Methods

I performed literature searches in Google Scholar using the individual search terms “preen oil”, “uropygial”, and “preen wax”, as well the combined terms [“preen oil” OR "uropygial" OR "preen wax" AND "sex"]. I also screened relevant review papers for any
additional references that may have been missed by my Google Scholar searches. I obtained an initial data set of 65 peer reviewed papers. I excluded studies that did not test explicitly for sex differences (N = 8), where the methods were unclear or confounded because the primary study objective was not to test for sex differences (N = 7), and studies that did not use preen oil specifically, including studies that tested for sex differences in real or synthetic feather odour (N = 2), body odour (N = 3), egg odour (N = 1), uropygial gland size or mass (N = 8), and feather or preen gland microbes (N = 3). However, I did include studies that conducted chemical analyses on feathers collected from around the uropygial gland (N = 2), as these would likely contain fresh preen oil secretions. In cases where multiple papers tested the same species at the same breeding stage (N = 9 studies), I selected the first available publication for analysis. Ultimately, I retained data from 24 papers presenting results from 34 species representing 9 phylogenetic orders (Appendix A, Table A1).

For each paper, I recorded the species studied, the time of year at which sampling occurred, and whether or not statistically significant (at $\alpha = 0.05$) sex differences in the composition of preen oil or of feathers surrounding the preen gland were detected. To determine effect sizes from each study, I recorded the number of males and females analyzed and the appropriate test statistics, where possible. I then calculated effect sizes using an online calculator (https://www.psychometrica.de/effect_size.html) and report Cohen’s $d$ (Appendix A, Table A1).

Gas chromatography-mass spectrometry (GC-MS) was the most common analytical method used (22 studies), but chemical analyses also included gas chromatography with flame ionization detection (GC-FID) (2 studies). Many studies coupled GC-MS analyses with GC-FID, thin-layer chromatography, column chromatography, and/or element-specific atomic emission detection. One study (Martín-Vivaldi et al. 2009) did not perform chemical analyses but instead qualitatively examined colour and odour changes in preen oil between the sexes.

I categorized time of year into ‘breeding stage’ (including nest building, egg laying, incubation, and hatching) or ‘nonbreeding stage’ (from fledging through winter, up to nest building of the following year). For studies on free-living birds (N = 19), breeding dates and stages, as well as the incubation type (uniparental versus biparental)
were verified using the Handbook of the Birds of the World (del Hoyo 2009). For studies on captive birds (N = 5), I reviewed the published methods to confirm that birds were brought into breeding condition using appropriate methods (e.g., by using natural light cycles for birds in outdoor aviaries; 4 studies, or by using artificial light to photostimulate birds kept indoors; 1 study).

To test for an effect of breeding stage and incubation type on the probability of detecting significant sex differences in preen oil, I ran a binomial mixed model with a Bayesian Wishart prior probability distribution in R (R Development Core Team 2017) using the package blme (Chung et al. 2013). Species was included as a random factor. Visual assessments of qq-plots and residuals indicated that data and residuals were distributed approximately normally and the residuals showed no evidence of homoscedasticity.

1.5.2 Results

In the 24 articles I retained in my analysis, 34 bird species were studied, including 8 species that were examined during both breeding and nonbreeding stages. With respect to sex differences, only 22.5% (9/40) of the world’s described phylogenetic orders of birds (Donsker and Gill 2020), and fewer than 6% of the species within any of these 9 orders, have been studied (Fig 1.1). Disregarding the proportion of species within a given order, the most well-studied orders are the Charadriiformes (13 species studied) and Passeriformes (11 species studied) (Fig 1.1).

Consistent with predictions derived from the sex semiochemical and olfactory crypsis hypotheses, the probability of detecting sex differences in preen oil was related to both breeding stage and incubation type. Sex differences were more likely when birds were sampled during breeding compared to nonbreeding and in species with uniparental incubation compared to species with biparental incubation (Table 1.1, Fig. 1.2).
Figure 1.1 Percentage (top panel) and number (bottom panel) of bird species in which sex differences in preen oil chemical composition have been studied in each phylogenetic order of the world's birds. Numbers in parentheses indicate the total number of species in each order. Orders for which no data have been collected (31 of the 40 described orders of birds) are not shown.
Table 1.1 Breeding stage and incubation type affect the probability of detecting sex differences in preen oil chemical composition.

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Parameters are estimated from a binomial mixed model fit using a Bayesian Wishart prior.
Figure 1.2 Number of species in which sex differences in preen oil were detected when sampled in breeding (B) versus nonbreeding (NB) stage and with uniparental (Uni) versus biparental (Bi) incubation. Total counts exceed 34 (the number of species studied) because some species were tested during both breeding and nonbreeding stages. See Appendix A, Table A1 for details.
1.5.3 Discussion

The results of my meta-analysis indicate that sex differences in preen oil are indeed more common in breeding stage compared to nonbreeding stage birds, and in species with uniparental compared to biparental incubation. At the proximate level, sex differences in preen oil during the breeding season may be due to physiological changes associated with reproduction (Pollock and Orosz 2002). For example, changes in the chemical composition of preen oil have been associated with the sex steroid hormones estradiol (Bohnet et al. 1991) and testosterone (Whittaker et al. 2011b). Thus, preen oil may function as a reproductive chemical cue (Section 1.6) that originates as a byproduct of physiological processes associated with breeding and reproduction. Other, ultimate level explanations that are not mutually exclusive with physiological explanations are that preen oil changes enhance olfactory crypsis, protecting eggs and young from olfactory-searching predators (Reneerkens et al. 2005), and are involved in chemical (e.g., antimicrobial) defense of eggs and young (Martín-Vivaldi et al. 2014).

Seasonal changes in preen oil composition between breeding and nonbreeding stages have been reported for a number of bird species (Charadriiformes: family Scolopacidae, sandpipers, Reneerkens et al. 2002; crested auklet, Hagelin et al. 2003; and herring gull, Larus argentatus, Fischer et al. 2017; Accipitriformes: black kite, Milvus migrans, Potier et al. 2018; Passeriformes: red-vented bulbul, Pycnonotus cafer, Bhattacharyya and Chowdhury 1995; dark-eyed junco, Soini et al. 2007; gray catbird, Dumetella carolinensis, Shaw et al. 2011; and white-throated sparrow, Zonotrichia albicollis, Tuttle et al. 2014). Such changes have been associated with estradiol (Bohnet et al. 1991), the testicular cycle in males (Bhattacharyya and Chowdhury 1995), testosterone in both sexes (Whittaker et al. 2011b), and with incubation (Reneerkens et al. 2007b).

The olfactory crypsis hypothesis posits that incubating birds switch from monoester (lower molecular weight) to diester (higher molecular weight) secretions during incubation to reduce odour cues at the nest (Reneerkens et al. 2002, 2005). This hypothesis predicts that preen oil changes only occur in incubating birds, leading to sex differences in uniparentally incubating, but not biparentally incubating, species during
breeding. Support for this hypothesis has been found in ground-nesting species in the family Scolopacidae (Order Charadriiformes; Reneerkens et al. 2002; Reneerkens et al. 2005). However, most bird species studied secrete only monoesters (Dekker et al. 2000; Salibian and Montalti 2009). Moreover, in some ground-nesting species such as the dark-eyed junco (Nolan et al. 2002), volatile secretions actually increase during the breeding season (Soini et al. 2007), presumably making birds more, rather than less, odorous, consistent with the sex semiochemical hypothesis.

The antimicrobial properties of preen oil may protect both adults and nestlings against ectoparasites and other pathogens (Martín-Platero et al. 2006; Martín-Vivaldi et al. 2014; Braun et al. 2018), and seasonal changes in preen oil chemical composition may be related to antipathogen defense at high-risk times of year (i.e., during nesting). A more complete understanding of the factors affecting sex differences in preen oil will likely require interdisciplinary collaboration between ecologists, physiologists, biochemists, and microbiologists.

While I restricted my analyses to preen oil, other odour sources are also worth considering. Feather odour did not differ between the sexes in breeding condition crested auklets (Aethia cristatella; Hagelin et al. 2003) or Antarctic prions (Pachyptila desolata; Bonadonna et al. 2007). In crested auklets, both sexes produce odorous, tangerine-scented patches of feathers during breeding (Hagelin et al. 2003). Both species have biparental incubation (Hagelin 2007; del Hoyo 2009), so we might indeed predict that sex differences should not be found during breeding in these birds; however, sex differences were detected in the preen oil of breeding Antarctic prions (Mardon et al. 2010).

Intriguingly, while no sex differences were found in the preen oil secretions of male and female ducklings (Jacob et al. 1979), sex differences have been found in the volatiles emitted from eggs containing male and female embryos, indicating that olfactory cues of sex differences can also be independent of breeding condition and may influence parental investment (Costanzo et al. 2016).

The size of the uropygial gland, which can affect the amount of preen oil secretions, can also differ between the sexes. The uropygial gland is often larger in
females than males (Pap et al. 2010; González 2014; Golüke and Caspers 2017; but see Møller et al. 2009), but can also increase in size during the breeding season, regardless of sex (Vincze et al. 2013; Urvik et al. 2019). Symbiotic microbes associated with feathers or the preen gland also differ between the sexes (Saag et al. 2011; Rodríguez-Ruano et al. 2018; Leclaire et al. 2019b). Such microbes can influence body odour (Whittaker et al. 2019), presumably through the breakdown of preen oil components into different volatiles, depending on the host’s microbial community (following Gorman 1976). Symbiotic microbes may thus contribute to sex differences in chemical cues. I explore sex and seasonal differences in preen oil further in Chapter 4, and test for sex and population differences in preen gland microbes in Chapter 7.

Most of the studies included in my meta-analysis did not test birds’ ability to discriminate between the sexes, but evidence for sex discrimination was found in all three of the studies that did (Zhang et al. 2010; Amo et al. 2012; Van Huyn and Rice 2019). Evidence for sex discrimination was also found in five additional studies that were not included in my meta-analysis (either because sex differences were not measured or they were reported in a prior study). In Galliformes, male domestic chickens (Gallus gallus) prefer females with an intact uropygial gland and male preferences are abolished in anosmic males (Hirao et al. 2009). In Charadriiformes, both sexes prefer male odour in crested auklets (Aethia cristatella); this study used a synthetic odour mimicking two major components of auklet odour (Jones et al. 2004). While this study did not directly test for sex discrimination, in Procellariiformes, Antarctic prions (Pachyptila desolata) recognize both self odour and mate odour, and prefer mate odour over non-mate odour (Bonadonna et al. 2004). In Passeriformes, both sexes prefer male odour in spotless starlings (Sturnus unicolor; Amo et al. 2012) and dark-eyed juncos (Junco hyemalis; Whittaker et al. 2011). Both sexes prefer opposite sex odour in black-capped chickadees (Poecile atricapillus), Carolina chickadees (Poecile carolinensis; Van Huyn and Rice 2019), and song sparrows (Melospiza melodia; Grieves et al. 2019, Chapter 5). In Psittaciformes, female budgerigars (Melopsittacus undulates) prefer male odour (Zhang et al. 2010). Together, these results suggest that the ability to use odour cues to discriminate conspecific sex is widespread in birds.
1.6 Preen oil as a reproductive chemical cue

Birds have long been used as model species for understanding mate choice, primarily through the study of visual and acoustic signals such as plumage and song (Hamilton and Zuk 1982; Nowicki et al. 2002; Searcy and Nowicki 2005; Andersson and Simmons 2006; Gill 2007; Riebel 2009). Reproductive signals or cues should differ among the sexes, and should also reflect reproductive condition (Johansson and Jones 2007). Reproductive signals or cues may also show geographic variation due to population differences in environment, genotype, or their interaction (Johansson and Jones 2007; Whittaker et al. 2010). As outlined in sections 1.2 and 1.3, preen oil represents a rich source of information that may be available to birds in the context of mate choice (Caro et al. 2015). I explore sources of variation in songbird preen oil, and the potential for preen oil to act as a reproductive chemical cue, in Chapter 4. In Chapter 5, I test the ability of songbirds to use these cues in reproductive and other social contexts.

1.6.1 Preen oil as a cue of MHC genotype

The major histocompatibility complex (MHC) gene family is an integral and highly polymorphic component of the immune system of jawed vertebrates (Janeway et al. 2001). MHC genes encode molecules that bind pathogen-derived antigens and present them to T lymphocytes to initiate specific immune responses (Klein 1986). There are two structurally and functionally distinct MHC gene subfamilies, class I and class II, that trigger the immune response against intracellular and extracellular pathogens, respectively (Minias et al. 2019). Thus, MHC genes play an essential role in the adaptive immunity of vertebrates.

Individuals with more MHC alleles can respond to a broader array of pathogens (reviewed in Penn 2002), and evolution should thus favour the ability to assess the MHC genotype of potential mates (Milinski 2006, 2016; Migalska et al. 2019). Choosing an MHC-dissimilar mate with respect to one’s own genotype should confer genetic
(indirect) benefits by maximizing offspring heterozygosity (Penn 2002; Neff and Pitcher 2004), while choosing an MHC-diverse mate is potentially associated with direct benefits, since an MHC-diverse mate likely has greater resistance to disease (Zelano and Edwards 2002). Thus, high heterozygosity at MHC appears to confer a fitness advantage. This appears to be reflected in the high MHC allelic diversity seen in wild populations, particularly in birds (Minias et al. 2019). For example, a sedge warbler (Acrocephalus schoenobaenus) population was found to have over 3500 MHC class I alleles (Biedrzycka et al. 2017) while a common yellowthroat (Geothlypis trichas) population had close to 1000 MHC class II alleles (Bollmer et al. 2012).

The extremely high MHC allelic diversity seen in some bird species is believed to arise from gene duplication, which produces variation in the number of MHC loci (i.e., MHC copy number) (Minias et al. 2019). MHC varies substantially among bird species, with the number of loci ranging from a single dominantly expressed gene at both class I and II in galliforms, birds of prey, and penguins, up to tens of putatively transcribed loci in some passerine species (reviewed in Minias et al. 2019). Indeed, the passerine superfamilies Muscicapoidea and Passeroidea have the highest duplication rates for MHC class II in birds. This extreme level of MHC polymorphism is believed to be maintained primarily by pathogen-mediated balancing selection (Spurgin et al. 2010).

MHC-based mate choice, particularly preferences for MHC-dissimilar or MHC-diverse partners, is widespread among vertebrates, having been demonstrated in fish (Landry et al. 2001; Milinski et al. 2005), amphibians (Bos et al. 2009), reptiles (Olsson et al. 2003), birds (Bonneaud et al. 2006; Strandh et al. 2012), and mammals (Setchell et al. 2010a,b). Mammals and fish assess the MHC through odour cues released by MHC peptides in urine or other secretory products (Milinski et al. 2005; Restrepo et al. 2006), and seabirds have recently been shown to discriminate MHC genotype using odour cues in preen oil (Leclaire et al. 2017). However, despite the prominence of songbirds in studies of mate choice (Coleman 2009), the mechanism by which they might assess the MHC genotype of potential mates has not been explored. Thus, in chapter 6 I experimentally test the ability of songbirds to discriminate the MHC genotype of potential mates using preen oil odour cues.
1.7 Potential mechanisms driving odour cues in preen oil

The fermentation hypothesis of chemical recognition postulates that symbiotic microbes living in specialized glands or other secretory organs (e.g., anal glands and the uropygial gland) produce the odours emanated by their multicellular hosts’ secretions (Gorman 1976). In turn, individual and group differences in symbiotic microbes drive differences in odour, providing hosts with information on individual and group identity (Albone et al. 1974; Gorman 1976; Hepper 1987). Microbes thus have the potential to influence social interactions in vertebrates (Troyer 1984; Lombardo 2008). Symbiotic microbes can be transmitted through the environment, including via social interactions (Archie and Theis 2011). Microbes can also be affected by host genotype. While the mechanisms are not fully understood, MHC genes are thought to influence odour. In particular, because MHC class II molecules are involved in immune defense against extracellular pathogens such as bacteria, an individual’s MHC class II genotype may influence the composition of its symbiotic bacteria, which may in turn affect the individual’s odour (Penn 2002; Kubinak et al. 2015).

Most research on microbially-mediated olfactory signals in vertebrates has focused on mammals (Ezenwa and Williams 2014). However, the presence of odour-producing bacteria in the uropygial gland of birds (e.g., Whittaker and Theis 2016) suggests that microbially-mediated chemical communication is also possible in this taxon. Indeed, preen gland-associated bacteria can produce many of the volatile compounds associated with sex and population differences in dark-eyed junco preen oil (Whittaker and Theis 2016). Recent work has shown that symbiotic bacteria produce volatile compounds in junco preen oil that are known chemical cues involved in social interactions, and juncos’ preen oil volatile profiles are positively associated with the relative abundances of specific preen gland bacteria (Whittaker et al. 2019). However, only a few studies to date have characterized the preen gland microbial communities of birds, and more work is needed to understand the role of microbes in mediating avian chemical communication. In Chapter 7, I explore variation in songbird preen gland microbial communities, and the role of MHC genotype in shaping preen gland microbes and preen oil chemical composition.
1.8 Study species

My study species is the song sparrow (*Melospiza melodia melodia*), a passerine bird that is widespread throughout most of North America. Song sparrows are monomorphic with respect to plumage (Arcese et al. 2002). I chose song sparrows for my research on songbird chemical communication for three main reasons. First, they are abundant, easy to catch, and easy to work with, making them tractable for both field and lab studies. Second, given their monomorphic plumage, visual and behavioural cues of sex may be limited. Additional signal modalities such as chemical cues may thus be important in this species. Third, song sparrows are a well-studied species (Arcese et al. 2002), so I was able to capitalize on a broad background literature.

Song sparrows have been particularly well-studied from the perspective of acoustic communication. Their song plays a role in mate choice (Searcy 1984; O’Loghlen and Beecher 1999; Reid et al. 2004) and parental investment (Reid et al. 2005; Potvin and MacDougall-Shackleton 2010), and is influenced by early life stress (MacDougall-Shackleton 2009; MacDougall-Shackleton et al. 2009b; Schmidt et al. 2014). Female song sparrows tend to mate assortatively, preferring the plumage and song traits of local males, indicating that breeding females assess multiple male traits (Patten et al. 2004). This again suggests that chemical cues may be relevant in this species, but almost nothing is known about chemical communication in song sparrows (Arcese et al. 2002; but see Slade et al. 2016a).

Song sparrows are host to a variety of pathogens, and host-parasite interactions between song sparrows and avian malarial parasites have been studied previously (Kelly et al. 2016; Sarquis-Adamson and MacDougall-Shackleton 2016; Kelly et al. 2018). I was able to leverage this research, particularly the methodologies for experimental infection with *Plasmodium* sp. (Sarquis-Adamson and MacDougall-Shackleton 2016), for my research on odour cues and disease (Chapters 2 and 3). Given the relationship between disease and immune function, prior work in our lab has also explored the role of the MHC in song sparrow immunity, mate choice, and chemical cues (Slade et al.
2016a,b; Slade et al. 2017; Slade et al. 2019). Thus, I was able to use previously developed methods for my work on odour-based discrimination of MHC genotype (Chapter 6).

A better understanding of behavioural, morphological, genetic, and demographic variation among song sparrow populations has been identified as a priority research direction for this species (Arcese et al. 2002). To address this, I explore demographic variation in song sparrow chemical cues, symbiotic microbes, and MHC genotypes in Chapters 2, 4, and 7, while in Chapters 3, 5, and 6 I experimentally test the behavioural responses of song sparrows to chemical cues. Overall, working with such a well-studied species as the song sparrow provided me with a strong foundation for asking fundamental questions about chemical communication in songbirds. Throughout this thesis, I make use of prior knowledge about song sparrows, including laboratory and other research methods, to address new questions in avian chemical ecology.

### 1.9 Dissertation structure

My thesis contains six data chapters, each exploring different components of chemical communication in songbirds. My overarching research objectives were three-fold. First, I aimed to establish what types of information are potentially available in avian preen oil. Second, I experimentally tested whether songbirds are capable of using this information. Third, I explored the relationship between immune genes, symbiotic microbes, and chemical cues, providing a candidate mechanism by which birds might use odour cues to assess MHC genotype.

In Chapter 2, I tested the hypothesis that malarial parasite infection alters preen oil chemical composition. I collected preen oil from song sparrows that had been experimentally infected with *Plasmodium* sp. parasites (Kelly et al. 2018) and compared these samples to those of sham-inoculated controls. I used gas chromatography (GC) and multivariate statistics to quantify changes in preen oil before experimental infection and
during acute infection. The chemical composition of preen oil differed between sham-inoculated birds and birds that were exposed to Plasmodium sp.

In Chapter 3, building on the results of Chapter 2, I used preen oil from infected and uninfected birds to test whether song sparrows can use these odour cues to discriminate between infected and uninfected conspecifics. I used a two-choice design to compare time spent in maze arms containing preen oil either from Plasmodium-infected birds or from uninfected birds. I found no evidence that song sparrows use preen oil odour cues to avoid Plasmodium-infected conspecifics.

In Chapter 4, I explored whether song sparrow preen oil meets the criteria of a reproductive chemical cue. I used GC to test for variation in the chemical composition of preen oil between breeding and nonbreeding seasons, between adults and juveniles, between the sexes, and between two breeding populations. The chemical composition of preen oil differed between breeding and nonbreeding seasons, adults and juveniles, sexes, and breeding populations.

In Chapter 5, I built on findings from Chapter 4 and the primary literature to experimentally test whether song sparrows discriminate among preen oil odour cues from different sexes and species. As in Chapter 3, I used a two-choice design to measure the amount of time song sparrows spend in maze arms containing preen oil from same-sex conspecifics versus no odour, preen oil from same-sex versus opposite-sex conspecifics, and preen oil from heterospecific brood parasites versus no odour. I also used GC and multivariate statistics to test for differences in the preen oil chemical composition of breeding condition male and female song sparrows and between song sparrows and female brown-headed cowbirds (Molothrus ater), a common and costly brood parasite of song sparrows. Song sparrows did not discriminate in time spent near conspecific, same-sex preen oil versus absence of such odour; however, both sexes spent significantly more time with opposite-sex odour than same-sex odour. Finally, males spent significantly more time and females spent significantly less time with heterospecific preen oil.

In Chapter 6, I built on findings from previous research in our lab (Slade et al. 2016a) to test whether song sparrows use preen oil odour cues to discriminate the MHC
similarity and MHC diversity of potential mates (i.e., of opposite sex conspecifics). I used captive birds to replicate findings in wild birds that MHC class II genotype is positively correlated with preen oil chemical composition (Slade et al. 2016a), and I again used a two-choice design to measure the amount of time song sparrows spent with preen oil from MHC-similar versus MHC-dissimilar and more MHC-diverse versus less MHC-diverse potential mates. Song sparrows spent significantly more time with preen oil from MHC-dissimilar and MHC-diverse potential mates.

In Chapter 7, I characterized the preen gland microbiota of song sparrows from three wild populations by amplifying and sequencing the V4 region of the bacterial 16S rRNA gene. I first tested whether preen gland microbiota differ among populations and between the sexes. Then, hypothesizing that variation at MHC underlies variation in preen gland microbiota and that this contributes to variation in preen oil composition, providing a potential mechanism for olfactory assessment of MHC genotype in birds, I tested for correlations between MHC class II genotype, preen gland microbiota, and preen oil chemical composition. Preen gland microbiota differed among populations and between the sexes. MHC genotype was significantly positively correlated with preen gland microbiota and preen oil chemical composition; however, preen gland microbiota were not significantly correlated with preen oil composition.

In Chapter 8, I summarized my findings and discussed how my work advances the field of avian chemical ecology. I also offered directions for future study, focusing on research questions that have yet to be definitively answered and research topics that have yet to be explored.

1.10 References


Audubon JJ. 1826. Account of the habits of the turkey buzzard (Vultur aura) particularly with the view of exploding the opinion generally entertained of its extraordinary power of smelling. Edinb New Philos J. 2:172–184.


Chapter 2

2 Malarial infection alters wax ester composition of preen oil in songbirds: Results of an experimental study

2.1 Introduction

Increased exposure to parasites and other pathogens represents one of the primary costs of group living. In response to this risk, animals have evolved diverse behavioural adaptations to detect and avoid parasitized conspecifics (Hedrick 2017). In mammals, the infection status of conspecifics can be assessed by olfactory cues, because parasitic infection can alter host body odour (Kavaliers et al. 2005a). Rats and mice show odour-based discrimination of and aversion to conspecifics infected with a wide variety of parasites, including the haemosporidian malarial parasite Plasmodium chabaudi (Kavaliers et al. 2005a). Rats and mice also use olfactory cues of conspecific infection status in the context of mate choice (Kavaliers and Colwell 1995; Penn and Potts 1998; Kavaliers et al. 2004) and other social behaviours (Kavaliers et al. 2005a,b). Chemical cues of infection status can be adaptive to the pathogen as well. For example, malaria parasites (Plasmodium spp.) produce volatiles that attract insect vectors when emitted by infected mammalian hosts (mice, De Moraes et al. 2014; humans, Kelly et al. 2015; de Boer et al. 2017; Correa et al. 2017).

In birds, signals of parasitic infection status have been investigated primarily in the context of sexually selected, condition-dependent ornaments and displays (Hamilton and Zuk 1982) involving visual and acoustic signals almost exclusively. Moreover, evidence that sexually selected traits reliably reflect infection status (that is, ornamentation varies negatively with parasite load within a species or population) is mixed (reviewed in Balenger and Zuk 2014). Surprisingly, despite considerable evidence in mammals that infection status alters chemical cues, chemical signaling of infection status in birds remains largely unexplored (but see Kimball et al. 2013).

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Haemosporidian parasites (genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*) infect about 70% of bird species worldwide (Atkinson and Van Riper 1991; Valkiunas 2005) and can negatively affect host fitness by reducing sexually selected trait expression, reproductive success, and survival (Korpimaki et al. 1993; Spencer et al. 2005; Gilman et al. 2007; Asghar et al. 2011, 2015). Although haemosporidia are transmitted indirectly by insect vectors, rather than directly between individuals, close proximity to infected birds is still expected to increase transmission risk because insect vectors have relatively low mobility and likely acquire haemosporidia from infected birds nearby. Thus, selection should favour the ability to identify and avoid conspecifics infected with malarial parasites.

In most bird species the major exocrine organ is the uropygial gland and avian body odour is thought to derive primarily from its sebaceous secretions (Hagelin and Jones 2007; Caro et al. 2015). The uropygial gland produces preen oil, comprised mainly of high molecular mass wax esters. Preen oil, thought to be used primarily in feather maintenance and waterproofing, is comprised of a complex mixture of compounds, including odorous volatile chemicals that are likely involved in intraspecific chemical communication (Caro and Balthazart 2010; Whittaker et al. 2010; Soini et al. 2013; Caro et al. 2015). Although it is not yet certain whether these volatile compounds are derived from the preen oil wax esters themselves (Jacob and Ziswiler 1982; Salibian and Montalti 2009; Soini et al. 2013), based on the chemistry of the volatiles previously described (e.g., 1-alkanols, fatty acids, methyl ketones; Soini et al. 2013), it seems likely that they are.

Regardless of their origin, preen oil compounds are increasingly recognized as candidate substances that mediate chemical signaling in birds. Preen oil composition differs between the sexes (Jacob et al. 1979; Whittaker et al. 2010; Zhang et al. 2010; Tuttle et al. 2014) and among populations (Whittaker et al. 2010), and varies with breeding status (Reneerkens et al. 2002; Whittaker et al. 2011a; Tuttle et al. 2014), diet (Thomas et al. 2010), and major histocompatibility complex (MHC) genotype (Leclaire et al. 2014; Slade et al. 2016). Variation in preen oil chemical composition may be
explained by variation in circulating levels of sex steroids (Whittaker et al. 2011a, 2017) and/or variation in bacterial community composition within the uropygial gland (Reneerkens et al. 2006; Whittaker and Theis 2016; Whittaker et al. 2016).

Sex steroids may also vary with parasitic infection status (Alexander and Stimson 1988; Klein 2000; vom Steeg and Klein 2017), raising the possibility that parasitic infection could affect the chemical composition of preen oil. Moreover, variation in preen oil chemical composition is detectable by birds and can be behaviourally salient. For example, songbirds show species-, sex-, and population-specific preferences for preen oil (Zhang et al. 2009, 2013; Whittaker et al. 2011b), and seabirds appear to use information derived from preen oil in the contexts of mate choice and kin recognition (Bonadonna and Nevitt 2004; Coffin et al. 2011; Bonadonna and Mardon 2013; Leclaire et al. 2017; although see Bonadonna et al. 2009).

In light of the apparent sensitivity of preen oil compounds to variation in individual physiology and condition, I hypothesized that infection with avian malaria alters preen oil wax ester composition, potentially leading to detectable changes in body odour that signal infection status. An experimental approach to this question is crucial because observational studies on naturally infected individuals do not permit disentangling the other factors contributing to variation in preen oil wax ester composition from the effects of infection. I compared the wax ester chemical composition of preen oil in song sparrows (Melospiza melodia) maintained under standardized laboratory conditions then experimentally inoculated with haemosporidian parasites (Plasmodium sp.). I compared preen oil wax ester chemical composition before inoculation and at the period of peak infection intensity. I also compared the chemical composition of preen oil from individuals sham-inoculated with uninfected blood, parasite-inoculated individuals that developed acute parasitemia, and parasite-inoculated individuals that resisted infection. To my knowledge, this is the first experimental investigation of whether parasitic infection alters preen oil wax ester composition (a proxy for body odour) in birds. If such variation induces detectable changes in odour, the chemical composition of preen oil wax esters may honestly signal infection status and
provide birds with a chemical basis for detection and avoidance of parasitized conspecifics as is known to occur in other vertebrate taxa.

2.2 Methods

2.2.1 Study animals and housing

Study subjects were 33 after-hatch-year (i.e., ≥ 1 year old) song sparrows (24 male, 9 female) captured in mist nets between 5 July and 24 August 2016 in London, Ontario, Canada (42.9849 N°, 81.2453° W). Upon capturing each subject, we identified sex based on the presence (male) or absence (female) of a cloacal protuberance, supplemented by measurements of unflattened wing length (measured to the nearest 0.1 mm using dial calipers). We collected a small blood sample (~ 25 µL) via brachial venipuncture at the time of capture to detect existing haematozoan infections (details below). We housed subjects in individual cages at the University of Western Ontario’s Advanced Facility for Avian Research. Rooms were kept free of insect vectors and were maintained between 20 – 22 °C on a light schedule mimicking the natural photoperiod. Birds had ad libitum access to food (parakeet seed mixed with ground Mazuri bird chow) and water.

2.2.2 Detecting naturally occurring infections

To identify birds that were already infected with Plasmodium spp. or other haematozoa at the time of capture, we used microscopy and genetic methods. We used a drop of whole blood collected at the time of capture to prepare a thin-film blood smear for each subject. Smears were air-dried, fixed in 100% methanol, treated with Wright-Giemsa stain, and examined under a light microscope with 100 x objective using oil immersion. We scanned 10 000 erythrocytes for each smear, noting the presence and number of haematozoan parasites.

The remainder of the blood sample was blotted onto high wet-strength filter paper saturated with 0.5 M Na-EDTA (pH 8.0) and allowed to air-dry awaiting genetic analysis.
We extracted DNA from these dried blood blots and used a two-stage, nested PCR approach to amplify a portion of haematozoan cytochrome \( b \) (Hellgren et al. 2004). First-stage PCR used primers HAEMNFI and HAEMNR3 (Hellgren et al. 2004) to amplify a 617 bp fragment of cytochrome \( b \). We used 1 μl of product from the first-stage PCR as template for second-stage PCR, together with the internally nested \textit{Haemoproteus}⁄\textit{Plasmodium}-specific primers HAEMF and HAEMR2 (Hellgren et al. 2004) to amplify a 527 bp region of cytochrome \( b \). PCR was conducted in a total volume of 25 μl with conditions described in Hellgren et al. (2004). We ran second-round PCR products at 100 V for 90 minutes on a 2% agarose gel stained with RedSafe™, then visualized under UV light, excised bands of the expected product size and purified with a Gel/PCR DNA Extraction Kit (FroggaBio). Purified PCR products were sequenced with primer HAEMF on an ABI 3730 Genetic Analyzer, and the resultant sequences were assigned to genus (i.e., \textit{Plasmodium} or \textit{Haemoproteus}) using the BLAST function in GenBank.

Eight song sparrows tested positive for haematozoan infection at the time of capture as assessed by PCR; these infections were also detectable by microscopy (1 – 4 haematozoa detected in the scan of 10 000 erythrocytes). Querying cytochrome \( b \) sequences against BLAST confirmed that all 8 infections were \textit{Plasmodium} spp. (88 – 100% sequence identity to other published \textit{Plasmodium} sequences), and we observed no double peaks indicative of mixed infections. The individual with the heaviest parasite burden as assessed by microscopy (i.e., 4 infected cells per 10 000) was used as the parasite donor. The cytochrome \( b \) sequence from this individual showed 99% sequence identity to lineage P-SOSP 2 previously described for the study population (Sarquis-Adamson and MacDougall-Shackleton 2016; GenBank accession # KT193628).

2.2.3 Inoculation procedures

Following Sarquis-Adamson and MacDougall-Shackleton (2016), we used previously-uninfected individuals as parasite amplifiers: these individuals were inoculated with infected blood, allowed to develop an acute infection, and then euthanized. Their blood was subsequently used to inoculate experimental subjects. Two parasite amplifiers received blood from the parasite donor (inoculation details below). A third sham amplifier, also previously-uninfected, received unparasitized blood from an unparasitized
donor confirmed by microscopy and PCR to have no haematozoan infection. The remaining 30 subjects were assigned to experimental and sham treatments (i.e., inoculated with parasitized and unparasitized blood, respectively; inoculation details below) in a block-randomized fashion such that groups were balanced as best as possible with respect to previous infection status (sham: 2 infected, 9 uninfected; experimental: 5 infected, 8 uninfected) and sex (sham: 8 males, 3 females; experimental: 13 males; 6 females). To account for imperfect infection success, we assigned more birds to the experimental treatment (N = 19) than to the sham treatment (N = 11).

On 31 August 2016 we collected 200 μl of blood from the naturally-infected parasite donor via brachial venipuncture and used this blood to inoculate the two parasite amplifiers. Using a sterile, single-use syringe and 26-gauge needle, we slowly (i.e., over 100 – 15 s) injected 80 μl of fresh collected blood (i.e., collected within 5 min), mixed with 20 μl of 3.7% sodium citrate and 100 μl of 0.9% saline, into the pectoralis muscle of each amplifier. We repeated this procedure to inoculate the sham amplifier with uninfected blood from the unparasitized donor.

Fourteen days later, on 14 September 2016, when parasitemia was expected to be near peak (Sarquis-Adamson and MacDougall-Shackleton 2016), we assessed the infection status of the three amplifiers by collecting 20 μl blood samples and preparing thin-film blood smears. Parasite amplifiers showed one and two infected cells, respectively, in a scan of 10 000 erythrocytes, while the sham amplifier had no detectable parasites. We euthanized all three amplifiers by inhaled overdose of isofluorane, and immediately collected 600 μl of blood from each into a syringe through cardiac puncture. We combined blood from the two parasite amplifiers, then mixed blood with saline/sodium citrate buffer as described above. Subjects in the experimental treatment were inoculated with 200 μl of the infected blood mixture. Subjects in the sham treatment were inoculated with 200 μl of the uninfected blood mixture. After inoculation, subjects were returned to their home cages and maintained under standardized conditions for thirteen days.
2.2.4 Assessing infection success

On 27 September 2016, thirteen days after inoculating experimental and sham-inoculated birds with infected or uninfected blood respectively, we collected 20 μl of blood from each individual via brachial venipuncture. We prepared and scanned thin-film blood smears as described above: smears were examined blind with respect to experimental treatment. Parasite loads of sham-inoculated subjects ranged from 0 – 2 infected cells per 10 000 screened (mean ± SE = 0.46 ± 0.22). Based on these values, which presumably reflect chronic rather than acute-phase infections, we established an arbitrary threshold for infection success of twice the maximum observed chronic-phase parasitemia (Sarquis-Adamson and MacDougall-Shackleton 2016). Thus, birds in the experimental treatment with at least 4 infected cells per 10 000 were considered to have been successfully infected and exhibiting an acute phase of infection. Birds in the experimental treatment with 3 or fewer infected cells per 10 000 were considered to have resisted infection (resistant).

2.2.5 Preen oil collection and analysis

On 12 September 2016, two days before subjects were inoculated with infected or uninfected blood, we collected an initial sample of preen oil from each individual (pre-inoculation). Using a non-heparinized capillary tube, we gently probed the bird’s uropygial gland until a small amount of oil (1 – 3 mg) was expressed into the tube. We then snapped the capillary tube to fit inside a 1.5 mL microcentrifuge tube. Tubes containing preen oil samples were stored at -20 °C until laboratory analysis. We used the same procedure to collect a second sample of preen oil from each subject on 27 September 2016, thirteen days after inoculation with infected or uninfected blood (post-inoculation).

We used gas chromatography with flame ionization detection (GC-FID) to separate and quantify the wax esters of preen oil. In song sparrows, GC-FID peaks are comprised of wax ester mixtures consisting of a homologous series of C18 – C25 fatty alcohols and C12 – C19 fatty acids esterified in different combinations to form C28 –
C39 monoesters (Slade et al. 2016). Capillary tubes containing preen oil samples were transferred to glass vials, then samples were dissolved in 3 mL of chloroform. Following a previously established protocol (Slade et al. 2016), we injected 1 μl of each sample onto a 5% phenyl methyl siloxane column (Agilent Technologies DB-5, 30 m × 0.32 μm ID × 0.25 μm film thickness) on an Agilent 6890N instrument. Samples were injected at 70 ºC and held for 1 min, ramped to 130 ºC at 20 ºC /min, ramped to 320 ºC at 4 ºC /min, then held at 320 ºC for 10 min. Hydrogen was used as a carrier gas at 2.5 mL/min. Each batch of samples included a blank containing only solvent (chloroform) as a negative control, and a sample of known composition previously analyzed by both GC-FID and gas chromatography-mass spectrometry (GC-MS; Slade et al. 2016) to ensure consistency between runs.

Since the volume of preen oil collected varied across samples, we quantified the relative rather than absolute size of each peak, based on peak area relative to that of the full chromatogram. Only peaks that comprised at least 0.1% of the total chromatogram area were retained for analysis (Leclaire et al. 2012; Slade et al. 2016), resulting in 46 unique peaks. Peaks were standardized by total such that within each sample, all peaks add up to 100% (Stoffel et al. 2015).

To test for group differences in preen oil wax ester composition, we transformed peak data from all 46 peaks with a log (x + 1) transformation then constructed a matrix of Bray-Curtis dissimilarity between all pairwise combinations of the 60 samples (30 pre-inoculation, 30 post-inoculation). As large chromatogram peaks could disproportionately affect distance measures, data were normalized using the ‘range’ method in the decostand function in the R package vegan (Dixon and Palmer 2003; Leclaire et al. 2012). We then used nonmetric multidimensional scaling (NMDS) to visually represent each sample on a 2-dimensional scatter plot. This approach preserves ranked distances between samples such that points appearing close together represent samples with similar composition (here, similar composition of preen oil wax esters), whereas points appearing further apart represent more dissimilar samples (Clarke 1999; Stoffel et al. 2015).
To assess the statistical significance of differences between groups we used permutational multivariate analysis of variance (PERMANOVA) derived from Bray-Curtis distance matrices. These analyses were performed in R version 3.3.3 (R Development Core Team 2017) using the *adonis* command in the package vegan (Dixon and Palmer 2003). This permutation-based approach, analogous to a nonparametric MANOVA, does not make assumptions about the data’s distribution and may be less sensitive to group differences in the dispersion of points than other methods such as analysis of similarities (Anderson 2001; Anderson and Walsh 2013).

2.3 Results

Of the 19 experimental birds (i.e., those inoculated with infected blood), 10 resisted infection and 9 became infected using the criteria described above (mean parasite load per 10 000 cells ± SE: resistant = 0.6 ± 0.2, infected = 170.7 ± 162.6). None of the 11 sham-inoculated birds developed acute infections (sham = 0.6 ± 0.3). Thus, my analysis consisted of four groups: pre-inoculation (N = 30), and thirteen days post-inoculation (N = 11 sham inoculation; 10 resistant; 9 infected). Of the five experimental birds that were naturally (chronically) infected prior to inoculation (1 – 3 infected cells per 10 000), three became infected and two resisted infection. I did not find differences in the chemical composition of preen oil between naturally-infected birds and uninfected birds prior to inoculation (PERMANOVA: F = 1.97, R² = 0.07, P = 0.094).

Chemical composition of preen oil differed significantly among the four groups (F = 2.51, R² = 0.12, P = 0.002, Table 2.1, Fig. 2.1). There was no effect of sex (F = 1.35, R² = 0.02, P = 0.23) or a sex by treatment interaction (F = 0.52, R² = 0.02, P = 0.93). I also observed a general shift between pre-inoculation samples and samples collected at 13 days post-inoculation, regardless of treatment type (Fig. 2.2). Accordingly, I tested for differences between preen oil samples collected pre-inoculation (N = 30) and at 13 days post-inoculation (N = 30, pooling all three treatment groups). Wax ester composition differed significantly between these two time points (F = 5.71, R² = 0.09, P < 0.001, Table 2.1, Fig. 2.2). Chromatographic profiles for individuals remained qualitatively
similar between pre- and post-inoculation, in that these time points were not generally associated with appearance or disappearance of peaks. Instead, pre- and post-inoculation profiles for each individual were associated with quantitative changes in relative peak area (Fig. 2.3).

To identify treatment groups in which preen wax ester profiles changed, I compared the pre-inoculation profiles to profiles recovered 13 days post-inoculation for each of the sham, infected, and resistant groups. Pre- and post-inoculation profiles were not significantly different for the sham-inoculated group (N = 11, F = 1.58, $R^2 = 0.07$, $P = 0.157$, Table 2.2, Fig. 2.4a), but did differ for each of the infected and resistant groups (resistant: N = 10, F = 2.91, $R^2 = 0.14$, $P = 0.036$, Table 2.2, Fig. 2.4b; infected: N = 9, F = 2.30, $R^2 = 0.13$, $P = 0.037$, Table 2.2, Fig. 2.4c). I compared the profiles of the infected and resistant groups at the post-inoculation period only and found no significant differences in preen oil wax ester composition (F = 0.62, $R^2 = 0.04$, $P = 0.658$).
Table 2.1 Results of permutational multivariate analysis of variance using distance matrices to test for treatment and sampling time differences in preen oil wax ester composition among groups.

<table>
<thead>
<tr>
<th></th>
<th>Df</th>
<th>Sum of squares</th>
<th>Mean sum of squares</th>
<th>F</th>
<th>R²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment (pre, sham,</td>
<td>3</td>
<td>0.27</td>
<td>0.09</td>
<td>2.51</td>
<td>0.12</td>
<td>0.002</td>
</tr>
<tr>
<td>infected, resistant)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>1</td>
<td>0.05</td>
<td>0.05</td>
<td>1.35</td>
<td>0.02</td>
<td>0.234</td>
</tr>
<tr>
<td>Treatment × Sex</td>
<td>1</td>
<td>0.06</td>
<td>0.02</td>
<td>0.52</td>
<td>0.03</td>
<td>0.930</td>
</tr>
<tr>
<td>Residuals</td>
<td>52</td>
<td>1.89</td>
<td>0.04</td>
<td></td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td>Time (pre/post)</td>
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<td>0.20</td>
<td>0.20</td>
<td>5.71</td>
<td>0.09</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Residuals</td>
<td>58</td>
<td>2.27</td>
<td></td>
<td></td>
<td>1.00</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.2 Results of permutational multivariate analysis of variance using distance matrices to test for differences in preen oil wax ester composition among groups between pre-inoculation and peak-infection time points.

<table>
<thead>
<tr>
<th></th>
<th>Df</th>
<th>Sum of squares</th>
<th>Mean sum of squares</th>
<th>F</th>
<th>R²</th>
<th>P</th>
</tr>
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<tr>
<td>Sham</td>
<td>1</td>
<td>0.12</td>
<td>0.12</td>
<td>1.56</td>
<td>0.07</td>
<td>0.157</td>
</tr>
<tr>
<td>Residuals</td>
<td>20</td>
<td>1.48</td>
<td>0.07</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>1</td>
<td>0.13</td>
<td>0.13</td>
<td>2.30</td>
<td>0.13</td>
<td>0.037</td>
</tr>
<tr>
<td>Residuals</td>
<td>16</td>
<td>0.91</td>
<td>0.06</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resistant</td>
<td>1</td>
<td>0.18</td>
<td>0.18</td>
<td>2.91</td>
<td>0.14</td>
<td>0.036</td>
</tr>
<tr>
<td>Residuals</td>
<td>18</td>
<td>1.32</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.1 Two-dimensional nonmetric multidimensional scaling plot of song sparrow preen oil wax ester composition. Bray-Curtis similarity values were calculated from standardized and log (x + 1) transformed abundance data. Axis scales are arbitrary. The closer the symbols appear on the plot, the more similar the two individuals are.
Figure 2.2 Two-dimensional nonmetric multidimensional scaling plot of song sparrow preen oil wax ester composition. Bray-Curtis similarity values were calculated from standardized and log \((x + 1)\) transformed abundance data. Axis scales are arbitrary.
Figure 2.3 Representative chromatogram showing the GC-FID preen oil wax ester profile of an individual song sparrow sampled pre-inoculation and again at peak-infection (i.e., 13 days post-inoculation) with *Plasmodium* sp. Data were normalized to remove any differences in signal intensity due to differences in sample volume.
Figure 2.4 Two-dimensional nonmetric multidimensional scaling plots showing preen oil wax ester composition of song sparrows sampled prior to inoculation with uninfected blood (sham-inoculated) or blood infected with avian malaria, *Plasmodium* sp. (‘pre-’) and again thirteen days later (‘post-’). A: Sham-inoculated birds; B: Resistant birds were inoculated with *Plasmodium* sp. but resisted infection; C: Birds successfully infected with *Plasmodium* sp. Bray-Curtis similarity values were calculated from standardized and log \((x + 1)\) transformed abundance data. Axis scales are arbitrary.
2.4 Discussion

I experimentally infected song sparrows with avian malaria (*Plasmodium* sp.) to test whether the chemical composition of preen oil wax esters, widely considered to be the main source of avian body odour (Hagelin and Jones 2007; Caro et al. 2015; Moreno-Rueda 2017), would change with infection status. As predicted, I found significant differences in preen oil wax ester profiles among treatment groups (i.e., pre-inoculation, sham inoculation, infected, resistant). Also as predicted, preen oil wax ester profiles changed in individuals that became acutely infected but not in sham-inoculated individuals. Unexpectedly, however, preen oil wax esters were altered not only in infected individuals, but also in individuals that successfully resisted the infection.

Mounting an immune response has been shown to alter body odour in mice (Kimball et al. 2014). A number of innate immunity processes may be activated upon exposure to parasites, including the release of peptides and antimicrobial enzymes, antigen attachment to phagocytes, and the initiation of inflammatory processes. These processes require biochemicals such as lipases, cytokines, and complement protein complexes (Kimball et al. 2014). I observed no visible signs of sickness or distress in our study birds following inoculation with *Plasmodium* parasites. However, if exposure to these parasites elicits an immune response, cellular events involved in immune activation and other metabolic inputs to innate immunity may induce changes in preen oil wax ester composition, regardless of infection outcome. I did not find qualitative differences in preen oil wax ester composition in the post-inoculation period between infected and resistant birds. Preen oil wax esters may thus be a cue of recent exposure and not infection status *per se*, though this remains to be confirmed. Unless an exposed individual is contagious, information on recent immune challenges (i.e., exposure) may not be useful in mate choice or other social contexts if it does not reliably signal the outcome of an infection (i.e., infected or resistant). Conversely, if changes in preen oil chemical composition reliably signal infection status, it may be adaptive for conspecifics to perceive and respond to these cues. From a mate choice perspective, individuals would be expected to avoid an infected potential mate but prefer a mate that is capable of successfully fighting off infection.
A key next step is to determine whether the observed differences between groups are perceptually distinguishable to songbirds. For some signal types, a subtle difference in cue is perceptually quite distinct (like the gas chromatography profiles of lime and lemon oils; Hunter and Moshonas 1966). Alternatively, the chemical differences I observed among groups could be perceptually indistinguishable to these birds. Behavioural experiments, such as the choice tests performed on mice (e.g., Kavaliers et al. 2005b, De Moraes et al. 2014), are needed.

I analyzed whole preen oil wax esters on the reasoning that malaria infection may alter the biosynthesis and/or the breakdown products of them. In assuming that preen oil volatile compounds derive from the wax esters, I can infer the possibility of altered odour cues when wax ester profiles are altered. In other words, different chain length ester components would yield different volatiles, and hence different odour cues. However, I do not exclude the possibility that malaria infection might induce additional changes to preen oil chemistry directly that are not detectable with the analytical method used, such as alterations to short-chain compounds that may be synthesized de novo. In mammals, malaria infection affects the emission of short-chain volatiles: relative to uninfected individuals, infected mice produce more whole-body volatile emissions (De Moraes et al. 2014) and infected humans produce more volatile emissions from the extracellular vesicles of erythrocytes (Correa et al. 2017). Infected human erythrocytes also produce a number of known plant-derived volatile compounds that are produced by the malaria parasites themselves, readily diffuse across the alveolar surface of the lungs, and are apparently recognized by mosquito vectors (Kelly et al. 2015). Future studies using analytical techniques that permit the identification of shorter-chain compounds (e.g., Soini et al. 2005) would be informative.

A variety of infections and disease states are known to alter body odour in mice, rats, and humans (Kavaliers et al. 2005a; Shirasu and Touhara 2011; Olsson et al. 2014). Avian influenza alters mallard (*Anas platyrhynchos*) fecal odour, and is detectable by trained mice (Kimball et al. 2013). Whether conspecifics are capable of detecting these odours remains to be seen, but analysis of fecal odour changes in response to malarial infection may be a promising area for future research. Malarial infection in humans has
been shown to increase attractiveness to mosquito vectors (Lacroix et al. 2005). Species of *Culex* mosquito (a known vector of *Plasmodium* spp.; Gutiérrez-López et al. 2016) are attracted to the odour of avian preen oil (Russell and Hunter 2005), but whether this is related to malarial infection status is unknown.

To my knowledge, this is the first evidence that the preen oil chemistry of songbirds can be affected not only by infection, but also by mere exposure to malarial parasites. However, caution is warranted in interpreting these results due to our modest effect sizes and the need to confirm that these shifts are perceptually salient to song sparrows. Similarly, whereas I observed no significant qualitative differences in wax ester composition between infected and resistant groups, sample sizes were small. Thus, I do not exclude the possibility that song sparrows might be able to detect differences between infected and exposed-but-uninfected (i.e., resistant) conspecifics. Future studies addressing effects of infection on short-chain volatile compounds, testing a wider diversity of host-parasite combinations, and exploring the perceptual salience of the observed shifts in preen oil chemical composition, will help assess the degree to which chemical signaling of infection status occurs in birds.

2.5 References


Whittaker DJ, Theis KR. 2016. Bacterial communities associated with junco preen glands: preliminary ramifications for chemical signaling. Schulte BA, Goodwin


Chapter 3

3 No evidence that songbirds use odour cues to avoid malaria-infected conspecifics²

3.1 Introduction

In addition to altering the physiology of host individuals, parasitic infections can often alter other phenotypic traits such as behaviour, morphology, or odour (Dobson 1988; Penn and Potts 1998; Moore 2013). Such phenotypic alterations can have important effects on disease transmission. For example, transmission rates may increase if infected vertebrate hosts are more attractive or detectable to invertebrate hosts such as biting insects (De Moraes et al. 2014; Kelly et al. 2015), or decrease if conspecifics avoid selecting infected individuals as mates or social partners (Kavaliers et al. 2003; Kavaliers et al. 2005a). In both cases, the main modality involved in recognizing infected individuals (whether by heterospecific vectors or by conspecific individuals) appears to be odour cues (Penn and Potts 1998; Kavaliers et al. 2004). Thus, odour cues of infection status can be an important source of social information.

Some parasites can complete their entire lifecycle within a single species of host, with transmission between host individuals occurring either directly (by contact between an infected and an uninfected conspecific, as in the case of ectoparasites; Kavaliers et al. 2003), or indirectly (moving from an infected host to the external environment to a new host individual, as in the case of fecal-oral transmission; Kavaliers et al. 1998; Poirotte et al. 2017). Within the context of these single-host systems, individuals that are able to identify and avoid parasitized conspecifics should benefit by reducing the risk of contagion. Indeed, many animals have evolved mechanisms to detect and avoid parasitized conspecifics (Kavaliers and Colwell 1995; Kavaliers et al. 2004, 2005b; Poirotte et al. 2017), largely through attending to cues of infection present in body, fecal, or urine odour (Kavaliers et al. 2004; Olsson et al. 2014; Poirotte et al. 2017);

² A version of this chapter is being prepared for submission to the Journal of Parasitology.

Citation: Grieves LA, MacDougall-Shackleton, EA. No evidence that songbirds use odor cues to avoid malaria-infected conspecifics. J Parasitol. In prep.
Gordon et al. 2018). Interestingly, however, avoidance of infected conspecifics may be diminished or abolished when test subjects are themselves infected (Poulin 1994; Poulin and Vickery 1996; Kavaliers et al. 1998).

Many parasites have more complex lifecycles involving multiple host species. Malaria parasites (*Plasmodium* spp.), for example, are vector-borne protozoa that require both an invertebrate host (primarily Culicid mosquitoes; Atkinson and Van Riper 1991) and a vertebrate host (notably mammals, birds, or reptiles; Atkinson 2008; Templeton et al. 2016; Lutz et al. 2016; Perkins and Schaer 2016; Otero et al. 2019) to complete their lifecycle. Sexual reproduction of the parasite occurs in the definitive host (mosquito), asexual reproduction occurs in both host types, and the parasites move between the two hosts during blood feeding (Cox 2010).

Vector-borne parasites such as *Plasmodium* are particularly interesting from the standpoint of alterations to host phenotype because there are multiple potential audiences. First, parasites may manipulate host phenotype to enhance transmission to the other species of host (Prugnolle et al. 2009). *Plasmodium* parasites produce volatile compounds that attract mosquitoes when emitted by the infected mammalian host (mice: De Moraes et al. 2014; humans: Kelly et al. 2015; Correa et al. 2017). In birds, *Plasmodium* infection may either increase attractiveness to biting insects (Cornet et al. 2013) or reduce attractiveness (Lalubin et al. 2012); the latter pattern may suggest that insects prefer to take blood meals from uninfected hosts (Tomás et al. 2008; Martínez-de la Puente et al. 2009). However, individuals of the infected host’s own species may also attend to cues of infection and use this information to inform mate choice or other social behaviour. Direct contagion is not an issue in multiple-host systems without direct transmission of parasites between conspecifics, but selection might still favour avoiding parasitized conspecifics. Close proximity to infected conspecifics may increase the likelihood of encountering infected insects (Aron and May 1982). In the context of mate choice, preferences for uninfected individuals likely confer direct or indirect benefits (Hamilton and Zuk 1982; Balenger and Zuk 2014). Additionally, merely mounting an immune response can alter body odour in some species (e.g., mice; Kimball et al. 2014) and conspecifics may simply
avoid such odour cues, regardless of whether they result from a directly transmissible parasitic infection. For example, mice and rats avoid the odour of conspecifics infected with *Plasmodium* parasites (Kavaliers et al. 2005b).

Most examples of parasitic infection altering host phenotype and of conspecific or heterospecific responses to the infected individual involve odour cues. In birds, the primary source of body odour is preen oil, a waxy secretion of the uropygial gland (Hagelin and Jones 2007). Preen oil is composed of a complex mixture of high molecular weight wax esters together with lower molecular weight volatiles (Caro and Balthazart 2010; Soini et al. 2013). In addition to its role in feather maintenance and waterproofing, preen oil also appears to function as an infochemical. The chemical composition of preen oil varies between species (Soini et al. 2013), between the sexes (Whittaker et al. 2010), and across populations (Whittaker et al. 2010; Van Hynh and Rice 2019). Moreover, this variation appears to be detectable to birds and used in contexts including mate choice (Bonadonna and Nevitt 2004; Leclaire et al. 2017), species recognition (Zhang et al. 2013; Van Huynh and Rice 2019), and kin recognition (Coffin et al. 2011).

Recently, I found significant changes in the preen oil chemical composition of song sparrows (*Melospiza melodia*) exposed to avian *Plasmodium* parasites. Among birds that were experimentally inoculated with *Plasmodium*, the wax ester composition of preen oil changed significantly from pre-infection to two weeks post-infection (the timeframe of maximum parasitemia), regardless of whether infections succeeded or were cleared by the birds. No significant changes to preen oil were seen over this timeframe in sham-inoculated birds’ blood (Grieves et al. 2018, Chapter 2). Song sparrows discriminate behaviourally based on other cues available in preen oil, for example spending more time with preen oil from conspecifics with dissimilar than similar genotypes at the major histocompatibility complex (MHC) (Grieves et al. 2019a, Chapter 6) and more time with odour cues from opposite-sex than same-sex conspecifics (Grieves et al. 2019b, Chapter 5). Based on the apparent ability of song sparrows to detect and respond to information available in preen oil (Grieves et al. 2019a,b), and the finding that exposure to *Plasmodium* alters preen oil composition (Grieves et al. 2018, Chapter 2), I hypothesized that song sparrows would avoid odour cues from conspecific individuals
infected with *Plasmodium* sp. To test this hypothesis, I presented breeding-stage song sparrows with preen oil from conspecifics that had been either experimentally-infected with *Plasmodium* sp. or sham-inoculated with uninfected blood. Using a two-choice experimental design, I monitored time spent by males and females with each sample type (infected or sham-inoculated). Because some test subjects were naturally infected with haematozoan parasites at the time of capture and testing, I also compared responses of *Plamosodium*-exposed versus unexposed focal birds to odour cues of *Plasmodium*-infected versus sham-inoculated conspecifics.

### 3.2 Methods

#### 3.2.1 Collection and preparation of preen oil samples

Preen oil samples used in this experiment were collected as part of a prior study investigating the effects of malarial infection on preen oil chemical composition (Grieves et al. 2018, Chapter 2): full details of experimental infections, preen oil collection, and sample processing are described therein. In brief, I collected preen oil from adult song sparrows captured in London, Ontario, Canada (42.9849 N°, 81.2453° W) during July and August 2016 and kept the birds on an ambient photoperiod until September 2016. Sparrows were assigned to either the experimental or the sham-inoculation group in a block-randomized fashion, such that groups were balanced as best as possible with respect to previous infection status and sex. Birds in the experimental group were inoculated by intramuscular injection with whole blood from song sparrows infected with *Plasmodium* (99% sequence identity to lineage P-SOSP2, GenBank accession no. KT193628); birds in the sham-inoculation group were inoculated with whole blood from uninfected song sparrows. Thirteen days after inoculation, small blood samples were collected by brachial venipuncture and thin-film blood smears were prepared. Smears were stained and examined under a light microscope and infection success of birds in the experimental group was assessed (i.e., whether the infection had succeeded or, conversely, whether it had been cleared or otherwise failed to establish).
Details of preen oil collection and storage are reported elsewhere (Grieves et al. 2018, Chapter 2). For the present study, I used preen oil from 8 successfully-infected birds (5 males, 3 females) and from 9 sham-inoculated birds (7 males, 2 females), but not from birds in the experimental treatment that cleared or resisted infection. Samples were collected thirteen days after inoculation, near the timing of maximum expected parasitemia (Sarquis-Adamson and MacDougall-Shackleton 2016). I expressed preen oil from the uropygial gland into a non-heparinized capillary tube, snapped the tube to fit into a microcentrifuge tube, and stored at -20 °C for 2 months. Samples were later thawed and transferred to glass vials, dissolved in 3 mL of organic solvent (pure chloroform, CHCl₃), then held at 4 °C for 15 months.

To prepare preen oil samples for use in behavioural trials, I allowed them to just dry by loosening the caps under a fume hood at room temperature, checking frequently to re-cap the samples once dry. When all samples were dry, I re-dissolved each sample in 250 μL of CHCl₃. This method ensured that preen oil samples would be presented at a comparable concentration to that used in other two-choice odour studies using a similar experimental design (Grieves et al. 2019a,b, Chapters 5, 6). I then pooled samples within each treatment group to create two cocktails, one from the 8 infected birds and one from the 9 sham-inoculated birds. Average (± SE) parasite loads (parasites per 10 000 cells examined) of birds contributing to the infected and sham-inoculated cocktails were 170.7 ± 162.6, and 0.6 ± 0.3, respectively.

### 3.2.2 Study subjects and housing

Study subjects were 36 adult song sparrows (27 male, 9 female), captured by mist net in August and September 2017 in London, Ontario. I determined sex by morphological measurements and later confirmed by PCR amplification using primers P2 and P8 (Griffiths et al. 1998). I housed subjects in individual cages in a single room at the University of Western Ontario’s Advanced Facility for Avian Research. Birds had ad libitum access to water and food (Mazuri Small Bird Maintenance chow and parakeet seed), and weekly supplements of greens, mealworms, and cooked egg.
The room was maintained at 20 ± 1 °C, and the light schedule mimicked the natural photoperiod until February 2018. On 22 February 2018, when the natural photoperiod is approximately 11 L:13 D at this latitude, I increased the light phase of the photoperiod to 14 L:10 D to photostimulate the subjects and bring them into breeding condition (Wingfield 1993); birds were maintained on this photoperiod throughout the experiment. Male song sparrows began singing on 13 February 2018 and continued to sing throughout the duration of behavioural experiments; thus, I considered it likely that all birds were in breeding condition at the time of this experiment.

3.2.3 Parasite screening of test subjects

To screen for prior exposure history to malarial parasites in my captive song sparrows (study subjects), I used PCR as this method can rapidly and reliably detect even low-level malarial infections (Perkins et al. 1998; Richard et al. 2002). I collected approximately 20 µL of blood via brachial venipuncture from all 36 birds at time of capture. I extracted DNA using a salt extraction protocol, then used a two-stage nested PCR approach to amplify parasite cytochrome b (Hellgren et al. 2004). I used the first-stage primers HAEMNFI and HAEMNR3 (Hellgren et al. 2004) to amplify an initial 617 bp fragment of cytochrome b from genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*. Using 1 µL of first-stage product as template, I then performed two separate second-stage reactions: one used the internally nested primers HAEMF and HAEMR2 to amplify a 478 bp fragment of *Plasmodium* and *Haemoproteus* cytochrome b, and the other used primers HAEMFL and HAEMRL to amplify a 480 bp fragment of *Leucocytozoon* cytochrome b.

PCR reactions were conducted in a total volume of 10 µL and included 50 ng total genomic DNA as template (or 1 µL of first-stage product for the second-stage PCR), 0.2 mM dNTPs, 2.0 mM MgCl₂, 1X Buffer, 0.6 mM of each primer and 0.5 units *Taq* DNA polymerase. Thermocycling conditions included an initial step of 94 °C for 3 min; 20 cycles (first-stage) or 35 cycles (second-stage) of 94 °C for 30 sec, 50 °C for 30 sec and 72 °C for 45 sec; and a final extension step of 72 °C for 10 min. I ran 5 µL of second-stage products on a 2% agarose gel including a water-only negative control and a
positive control for each of the two second-stage primer sets. I inferred infection status from the presence (infected) versus absence (uninfected) of a band in the second stage reactions for each primer set. Eight of 36 birds (6 males, 2 females) were infected with *Plasmodium* and/or *Haemoproteus* at the time of capture and no *Leucocytozoon* infections were detected.

### 3.2.4 Behavioural trials of study subjects

Behavioural trials began on 26 March 2018 and ended on 29 March 2018. I conducted trials in a Plexiglas Y-maze using a design similar to Whittaker et al. (2011) (arms: 20 cm H × 40 cm L × 20 cm W; central area: 20 cm H × 35 cm L × 20 cm W). I placed a perch near the end of each maze arm and placed each odour stimulus (see below) on a cotton ball taped into a dish at the end of each arm (8 cm from the perch). The maze contained a start chamber (20 cm H × 14 cm L ×µ 20 cm W) separated by an opaque Plexiglas barrier that could be slid open and closed to release the bird into the maze. I made the side walls opaque by taping brown Kraft paper to the outside of the maze and placed a wire screen on top of the maze so that birds could detect the ceiling. I used a vacuum pump (Neptune DynaPump, Thermoscientific) to circulate air from the odour stimulus (dissolved preen oil applied to clean cotton balls) down the arms of the maze while preventing mixing in the central area. This was achieved by connecting equal lengths of air tubing near the base of each arm (5.5 cm H × 9 cm from the central area) to the vacuum pump. Because the vacuum pump produced noise, I habituated subjects to the sound by running the pump in their holding room for 1 hr/d from 22 February 2018 to 1 March 2018. Birds had also participated in additional odour preference trials in this apparatus during the previous three weeks (Grieves et al. 2019a,b, Chapters 5 and 6), so they were familiar with the testing apparatus.

The maze was placed in an observation room such that each side of the maze was equidistant from the wall and the maze was positioned evenly between two overhead lights. All trials were video recorded with an Activeon CX high-definition camera.
At the start of each testing day, I removed preen oil stimuli from 4 °C storage and warmed them to room temperature for approximately 5 min. I conducted trials from 0800 h to 1130 h daily. Before each test, I transported the focal bird in an opaque cloth bird bag from its home cage to the observation room. From 2 – 5 min before each trial began, I used a Hamilton syringe to apply 50 µL of odour stimulus onto a clean cotton ball affixed to each arm of the maze. I used a random number generator to determine the order in which birds would be tested. I flipped a coin to assign stimulus type to maze arm for the first trial, then alternated stimulus locations for each subsequent trial.

Trials lasted 20 min in total and began with the focal bird being placed into a start chamber separated from the rest of the maze by a slidable opaque barrier for a 5 min acclimation period. After this period, the barrier was opened and closed immediately after the bird entered the maze. Most birds entered the maze as soon as the barrier was opened, and all birds entered within a few seconds. The next 5 min constituted the exploration period. For trials to be considered successful, the focal bird was required to enter both maze arms or to enter one arm and also orient towards the other arm (defined as standing within one body width of the arm with bill oriented toward that arm for at least 10 sec) during this exploration period. The final 10 min were considered the choice period. In the case of unsuccessful trials (9 birds were re-trialed) I tested the focal bird one to two days later up to a maximum of two trial attempts. Most birds investigated the maze during the exploration period prior to the start of the trial, such that 75% (27/36) of trials were ultimately successful.

For successful trials (as defined above), I scored the time within the 10 min choice period that the focal bird spent in or orienting towards each arm of the maze. Trials were scored blind with respect to bird and stimulus identity.

3.2.5 Statistical analysis

I tested for differences in time spent with stimulus (odour) type by fitting a restricted maximum likelihood (REML) linear mixed model using the R package lme4 (Bates et al.
Fixed effects included sample type (sham-inoculated versus malaria-inoculated preen oil), sex of the focal bird, malaria exposure history of focal bird, and the relevant two-way interactions (sample type × sex, and sample type × exposure history). Focal bird ID was included as a random effect and the dependent variable was time spent in or approaching (as defined above) a maze arm. Visual assessments of qq-plots and residuals confirmed that data and residuals were distributed approximately normally and the residuals showed no evidence of homoscedasticity. P-values were obtained using Wald tests (using the Anova function in the R package car). All analyses were performed in R version 3.2.3 (R Development Core Team 2017).

3.3 Results

There was no significant difference in the amount of time song sparrows spent with preen oil from malaria-infected versus uninfected birds. I found no main effect of sample type, sex, or focal bird’s malaria exposure history on time spent with odour cues from infected versus uninfected birds, nor were there any significant interactions (Table 3.1, Fig 3.1).
Table 3.1 Song sparrows did not discriminate in time spent with preen oil samples from uninfected (sham-inoculated) or malaria-infected conspecifics in a two-choice Y-maze test. N = 54 observations on 27 birds.

<table>
<thead>
<tr>
<th>Fixed effects</th>
<th>Estimate</th>
<th>SE</th>
<th>t</th>
<th>$\chi^2$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>149.1</td>
<td>69.0</td>
<td>2.16</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Stimulus type</td>
<td>126.1</td>
<td>97.6</td>
<td>1.29</td>
<td>1.05</td>
<td>0.30</td>
</tr>
<tr>
<td>Sex of focal bird</td>
<td>49.1</td>
<td>84.5</td>
<td>0.58</td>
<td>0.09</td>
<td>0.92</td>
</tr>
<tr>
<td>Exposure history of focal bird</td>
<td>61.9</td>
<td>195.1</td>
<td>0.32</td>
<td>-0.35</td>
<td>0.66</td>
</tr>
<tr>
<td>Type × sex</td>
<td>-76.2</td>
<td>119.5</td>
<td>-0.64</td>
<td>0.70</td>
<td>0.40</td>
</tr>
<tr>
<td>Type × infection status</td>
<td>-10.1</td>
<td>275.9</td>
<td>-0.04</td>
<td>0.61</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Parameters are estimated from a linear mixed model fit by REML; P-values are derived from type II Wald chi square tests.
Figure 3.1 Time spent by 27 song sparrows with preen oil from either uninfected (filled circle) or malaria-infected (open circle) conspecifics in two-choice Y-maze experiments. Values reported are mean ± SE. Filled and open circles connected by black lines are mean ± SE, values in gray show paired data for each individual. A: All focal individuals, B: male and female focal individuals, C: unexposed and malaria-exposed individuals.
3.4 Discussion

I tested whether song sparrows would avoid the preen oil odour of malaria-infected conspecifics. Contrary to my prediction, I found no evidence that song sparrows discriminated between preen oil from malaria-infected versus uninfected (sham-inoculated) birds. Similarly, malarial parasite exposure history of the focal bird was not significantly related to the amount of time birds spent with preen oil from infected versus uninfected conspecifics, although birds with no prior exposure spent about one and a half times more time with preen oil from uninfected than infected conspecifics. Similarly, while not statistically significant, female song sparrows spent nearly twice as much time with preen oil from uninfected compared to infected conspecifics, a pattern generally consistent with findings that mice and rats use olfaction to avoid infected individuals (Kavaliers and Colwell 1995; Penn et al. 1998; Kavaliers et al. 2005a,b).

I expected both sexes to avoid the odour of parasitized conspecifics. Recently, I found that male and female song sparrows both spend more time with preen oil odour of opposite sex conspecifics (Grieves et al. 2019b, Chapter 5) and with preen oil odour of MHC-dissimilar and MHC-diverse potential mates (i.e., opposite sex conspecifics; Grieves et al. 2019a, Chapter 6), indicating that both sexes can and do use preen oil odour cues of sex and genotype. While it is unclear why I did not detect evidence of odour-based discrimination of preen oil from malaria-infected birds, I propose several potential explanations.

The lack of avoidance may be an artefact of my experimental design. First, pooling the stimulus preen oil samples from multiple individuals may have disrupted the ability of focal (test) birds to process chemical cues of infection status. Second, although test subjects had been photostimulated and were presumably in breeding condition, odour stimuli were collected from post-breeding birds. It is possible that such stimuli are non-stimulating to breeding-condition birds, especially given that preen oil chemical composition differs between breeding and post-breeding stages in song sparrows (Grieves et al. 2019c, Chapter 4) and other species (Bhattacharyya and Chowdhury 1995; Reneerkens et al. 2002; Fischer et al. 2017). However, by using samples collected from birds in nonbreeding condition, I aimed to reduce the likelihood that preen oil cues of
sex, known to be salient to song sparrows (Grieves et al. 2019b,c, Chapters 4 and 5), might confound or otherwise influence focal subjects.

Third, my samples were collected during acute-stage infection (Sarquis-Adamson and MacDougall-Shackleton 2016; Grieves et al. 2018, Chapter 2). Mosquitos (Culex pipiens) are more attracted to chronically-infected than to either acutely-infected birds (i.e., at peak parasitemia as in this study) or to uninfected birds (Cornet et al. 2013), and gametocytes (capable of infecting mosquitoes) are produced and enter red blood cells of the vertebrate host during the chronic, not the acute, phase of infection (Valkiunas 2005; Rivero and Gandon 2018). Although a prior study conducted on the same samples used here as test stimuli detected significant changes in the preen oil chemical profiles of acutely-infected song sparrows compared to sham-inoculated controls (Grieves et al. 2018, Chapter 2), it is possible that chronic-stage infection is more biologically relevant to both hosts and vectors, as this is the time during which the disease can be spread.

Alternatively, birds may be unable to detect cues of infection status. Vectors such as mosquitoes may be the sole audience of infection-related shifts in preen oil chemical composition (Robinson et al. 2018). Finally, I cannot exclude the possibility that birds may be able to detect cues of Plasmodium infection, but do not behaviourally discriminate in their response to infected and uninfected conspecifics. Because Plasmodium parasites are not transmitted directly from bird to bird or by environmental contamination, the risks of proximity to infected conspecifics may not be particularly high. More work is needed to determine the extent to which vectors may be using chemical cues of infection status in birds, identify the specific chemical cues, and determine whether they are universal across host and vector species and to confirm whether or not avian and other hosts are able to detect and use these cues.

3.5 References


Chapter 4

4 Wax ester composition of songbird preen oil varies seasonally and differs between sexes, ages, and populations

4.1 Introduction

Despite early controversy surrounding avian olfaction, there is no longer any doubt that birds possess a fully functional olfactory system (Balthazart and Taziaux 2009; Caro and Balthazart 2010; Caro et al. 2015). Indeed, it is now widely accepted that birds use olfaction in a variety of contexts including navigation, food location, predator detection, nest location, and conspecific, kin, and mate recognition (Bonadonna and Nevitt 2004; Balthazart and Taziaux 2009; Caro and Balthazart 2010; Caspers and Krause 2013; Caro et al. 2015; Moreno-Rueda 2017). The role of olfaction in avian reproduction and mate choice is of particular interest, and a growing body of evidence supports the importance of this previously overlooked area of research (Caro et al. 2015).

In birds, the major source of body odour is preen oil, a complex mixture of waxy secretions produced by the uropygial gland and consisting of low boiling (low molecular) and high boiling (high molecular) components (Hagelin and Jones 2007; Caro et al. 2015). The chemical composition of preen oil can differ between the sexes, among individuals, and among species (Jacob et al. 1979; Soini et al. 2007, 2013; Whittaker et al. 2010; Tuttle et al. 2014). Moreover, some birds exhibit sex, population, and conspecific odour preferences (Bonadonna and Nevitt 2004; Whittaker et al. 2010, 2011; Amo et al. 2012a), indicating that birds can detect and respond to chemical information available in preen oil.

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While avian chemical communication is enjoying a surge in research interest, much remains to be discovered (Hagelin and Jones 2007; Moreno-Rueda 2017), particularly concerning the role of chemical signaling in mate choice. In particular, although the order Passeriformes comprises over half of all extant bird species (Gill 2007) and has been well-represented in studies of avian mate choice (Andersson 1994; Andersson and Simmons 2006), we are just beginning to study reproductive chemical communication in this diverse group (Whittaker et al. 2010; Caro et al. 2015). Reproductive chemical cues should differ between the sexes and may also vary seasonally, reflecting reproductive condition (Johansson and Jones 2007). Further, reproductive cues may vary geographically due to population differences in environment (e.g., diet), genotype, or their interaction (Johansson and Jones 2007; Whittaker et al. 2010).

I used gas chromatography with flame ionization detection (GC-FID) to characterize the wax ester composition of preen oil from song sparrows (Melospiza melodia), a sexually monomorphic and geographically widespread songbird. Wax esters are comprised of a fatty alcohol and fatty acid linked by an ester bond. I treated variation in the chemical composition of wax esters as a proxy for variation in the composition of preen oil-derived volatiles. That is, I expect variation in the wax ester composition to contribute to variation in odour. I tested for differences between sexes, age classes, two geographically distinct populations, and between breeding and post-breeding stages. I used gas chromatography-mass spectrometry (GC-MS) to identify the wax ester components of song sparrow preen oil and assessed which compounds likely contribute most to the group differences I observed.

I report differences in wax ester composition of preen oil from breeding stage males versus females, between post-breeding adults versus juveniles, between breeding populations, and between breeding versus post-breeding stages. My results show that preen oil wax esters in this species vary between sexes, age classes, populations, and seasons, and therefore could be precursors to volatiles that convey information salient to reproductive decision making. My findings provide the foundation for future behavioural
experiments that will test whether passerine birds attend to the information available in preen oil.

4.2 Methods

4.2.1 Field methods

I captured song sparrows using seed-baited Potter traps and mist nets at two breeding locations in Ontario, Canada: a northeastern site on land owned by the Queen’s University Biological Station near Newboro (43.008°N, 81.291°W; hereafter Newboro) and a southwestern site at the rare Charitable Research Reserve near Cambridge (43.383°N, 80.357°W; hereafter Cambridge). These two sites are separated by 390 km, well beyond the mean range of juvenile dispersal for this species, which is estimated as about 6 km (Zink and Dittmann 1993). At each site, I captured song sparrows during the early part of the breeding stage (hereafter breeding), which encompasses nest building and early egg laying (Newboro: 12 April – 5 May 2016 and 8 April – 3 May 2017; Cambridge: 3 April – 1 May 2017), and during late summer (Newboro: 15 – 28 July 2016; Cambridge: 8 – 28 August 2016) after most chicks have fledged and juveniles are largely independent (hereafter post-breeding).

In the field, I determined the age class and sex of each song sparrow captured. I used wing length, plumage, and gape characteristics to distinguish juveniles (hatch-year) from adults (after-hatch-year) and used wing length, together with the presence versus absence of a cloacal protuberance (male) or brood patch (female), to distinguish males from females. From each bird, I collected a small blood sample through brachial venipuncture for genetic analysis, and later confirmed sex for all birds using the P2/P8 PCR protocol described by Griffiths et al. (1998). I collected preen oil by gently probing the uropygial gland with an unheparinized capillary tube until ~1 – 5 mg was expressed into the tube. Samples were kept on ice in the field and stored at -20 °C pending analysis. I fitted each bird with a numbered aluminum leg band (Canadian Wildlife Service: 10691) to enable identification of previously captured birds.
In all, I collected 356 samples of preen oil. Breeding stage samples were collected from 49 males and 41 females at Newboro in 2016; 48 males and 28 females at Newboro in 2017; and 48 males and 36 females at Cambridge in 2017. In 2016, I collected post-breeding samples from 26 adults (24 males, 2 females) and 28 juveniles (5 males, 18 females, and 5 that were not successfully sexed and were excluded from sex-specific analysis) at Newboro and from 16 adults (11 males, 5 females) and 36 juveniles (17 males, 17 females, and 2 that were not successfully sexed and were excluded from sex-specific analysis) at Cambridge.

4.2.2 Laboratory methods

I dissolved preen oil samples in 1 – 3 mL chloroform (CHCl₃) and analyzed them using an Agilent 7890A gas chromatograph with flame ionization detector (GC-FID), fitted with a 5% phenyl methyl siloxane column (Agilent Technologies DB-5, 30 m × 0.32 μm ID × 0.25 μm film thickness) as described previously (Slade et al. 2016). Briefly, 1 μL samples were injected with a 30 psi pressure pulse (1 min) and, after an initial 1 min hold at 70 ºC, eluted with the following temperature profile: increase to 130 ºC at 20 ºC /min, then to 320 ºC at 4 ºC /min. The injector and FID temperatures were 200 ºC and 310 ºC, respectively. Hydrogen was used as a carrier gas at 2.5 mL/min. Each batch of GC-FID runs (typically 20 – 24) included a blank sample containing solvent only (CHCl₃) and a sample of known composition (i.e., previously analyzed with both GC-FID and GC-MS; Slade et al. 2016). Because the volume of preen oil collected varied across individuals, peak sizes were quantified based on the proportional peak size relative to total chromatogram peak area. Peaks that were at least 0.1% of the total chromatogram area were retained for analysis, while peaks that were < 0.1% were counted as zero. Peaks were then standardized by total peak area per individual (Stoffel et al. 2015).

To provide preliminary identification of the wax esters present in preen oil, I performed GC-MS on a subset of 21 samples, balanced across groups (male versus female, adult versus juvenile, Newboro versus Cambridge, breeding versus post-breeding). GC-MS was performed on a Varian 3800 gas chromatograph connected to a
Varian MS220 ion trap mass spectrometer. The GC parameters were the same as for GC-FID, except that helium was used as a carrier gas at 1 mL/min. I identified monoesters based on the \( m/z \) of the protonated fatty acid fragments and parent ions (Thomas et al. 2010; Slade et al. 2016) and determined the fatty alcohol part by subtraction. I used GC-MS to compare the preen oil profiles of breeding males (N = 3) and females (N = 3), post-breeding males (N = 3) and females (N = 2), and post-breeding males, females, and juveniles (N = 4) from Newboro. To assess population differences, I also compared breeding males (N = 3) and females (N = 3) from Cambridge with those from Newboro. Due to low sample sizes per group, I did not perform statistical analyses but instead visually identified peaks that might contribute to the group differences found using GC-FID, and quantified peak sizes based on the proportional peak area relative to total chromatogram peak area. Peaks that were at least 0.1% of the total chromatogram area were retained for analysis, while peaks that were < 0.1% were counted as zero.

4.2.3 Statistical analyses

To prevent large chromatogram peaks from disproportionately affecting distance measures, I normalized chemical data using the range method in the `decostand` function in the R package vegan (Dixon and Palmer 2003) following previous studies (Leclaire et al. 2012; Slade et al. 2016). I then log \((x + 1)\) transformed the GC-FID data on preen oil wax ester composition, and constructed pairwise matrices of Bray-Curtis dissimilarity. Chemical distances (i.e., Bray-Curtis dissimilarities) between samples were visualized using nonmetric multidimensional scaling (NMDS). This approach places each sample on a two-dimensional scatter plot, preserving ranked pairwise distances such that two points close together represent two individuals with relatively similar chemical composition while points further apart represent individuals that are more dissimilar (Clarke 1999; Stoffel et al. 2015).

To assess the statistical significance of differences between groups (males versus females, adults versus juveniles, Newboro versus Cambridge, breeding- versus post-breeding), I used nonparametric analysis of similarities (ANOSIM), implemented in the
R package vegan (Dixon and Palmer 2003) with 10,000 iterations. This permutation approach does not make assumptions about the data’s distribution (Clarke 1999; Stoffel et al. 2015). To minimize effects of year-to-year variation within an analysis, our analyses of sex and population differences were restricted to samples from breeding-stage adults collected in 2017. Similarly, I tested for adult versus juvenile differences using samples collected during post-breeding 2016; and for breeding versus post-breeding differences using samples collected at Newboro in 2016.

All analyses were performed in R version 3.2.3 (R Development Core Team 2017). As noted above, for the GC-MS dataset I report qualitative rather than quantitative differences among groups.

4.3 Results

My GC-MS and GC-FID analysis on 21 samples revealed that the wax esters comprising song sparrow preen oil represented at least two homologous series of differently methyl branched fatty alcohols (C18 – C25) and differently methyl branched fatty acids (C12 – C19) esterified in different combinations to form monoesters with a total carbon number of C30 – C38. I characterised 53 unique wax esters and detected a characteristic pattern of clearly separated doublet peaks (denoted A and B; Fig. 4.1) having the same total number of carbons. For a given carbon number and molecular weight, peaks of series A and B were largely comprised of distinct mixtures of up to 13 isomeric monoesters, with some variation in the proportions of each component (Appendix B, Table B1).

4.3.1 Sex differences

Among adult song sparrows sampled during breeding, I found significant sex differences in the wax ester composition of preen oil at both sites (ANOSIM; Newboro: Global R = 0.31, P < 0.0001; Fig. 4.2; Cambridge: Global R = 0.25, P < 0.0001; all samples collected in 2017). By contrast, sex differences were not detected in post-breeding adults at either
site (ANOSIM; Newboro: Global $R = 0.20$, $P = 0.178$; Cambridge: Global $R = 0.06$, $P = 0.269$; all samples collected in 2016). However, it should be noted that post-breeding sample sizes for adult females were very low ($N = 2$ and 5 for Newboro and Cambridge respectively) and this reduces statistical power.

In my qualitative GC-MS comparison of breeding-stage males versus females (samples collected at Newboro), I noted certain low abundance compounds that appeared elevated in males relative to females. These compounds were C18 – C22 and C25 fatty alcohols esterified to C12 fatty acids (hereafter denoted by C#alcohol:C#acid; e.g., C18 – C22:C12, C25:C12). In contrast, relative to breeding stage males, breeding stage females appeared to have elevated C17:C13 – C15 wax esters. I also observed sex differences, albeit less dramatic, in C18 – C19:C13 esters (elevated somewhat in breeding stage females relative to males) and C17:C13 and C17 – C18:C17 esters (elevated somewhat in breeding stage males relative to females; Appendix B, Figs. B2, B3).

4.3.2 Age differences

At both sites, preen oil wax ester composition was significantly different between adults and juveniles (ANOSIM; Newboro: Global $R = 0.07$, $P = 0.016$, Fig. 4.3; Cambridge: Global $R = 0.25$, $P < 0.001$; all samples collected during post-breeding, 2016). However, despite these statistically significant differences in preen oil composition, my review of GC-MS profiles showed no obvious candidate substances differing in relative abundance between adults and juveniles (Appendix B, Fig. B4).

4.3.3 Population differences

Comparing the wax ester composition of males and females from Newboro and Cambridge identified significant differences between these four groups (ANOSIM: Global $R = 0.27$, $P < 0.0001$, Fig. 4.4; all samples collected from breeding stage adults, 2017). To disentangle sex differences from site differences, I repeated this analysis on the same dataset but pooled sexes within each site. Again, I observed significant differences
in wax ester composition of breeding stage adults from Newboro versus Cambridge (ANOSIM: Global R = 0.08, P < 0.0001; all samples collected in 2017). In contrast, population differences were not observed in adults sampled post-breeding (ANOSIM: Global R = 0.01, P = 0.381; all samples collected in 2017).

In my qualitative GC-MS comparison of breeding stage samples for Newboro versus Cambridge, I noted that the C17:C13 ester was elevated in the Newboro females relative to Newboro males and both sexes from Cambridge. Similarly, a C19:C15 ester was elevated in the Cambridge females only. Cambridge males showed elevated levels of C18 – C19:C13 esters relative to all other groups but lacked a number of esters (C22:C13, C22:C15, C23:15) that were present at low abundance in Newboro males. The C17:C14 ester was elevated in males from both populations, relative to females (Appendix B, Figs. B4, B5).

4.3.4 Seasonal differences

Breeding versus post-breeding stage adults differed significantly in wax ester composition of preen oil (sexes pooled, ANOSIM: Global R = 0.84, P < 0.0001, Fig. 4.5; all samples collected in Newboro, 2016). In my qualitative GC-MS comparison of breeding versus post-breeding stage adults at the Newboro site, I noted the low abundance compounds that were elevated in males relative to females during breeding (C18 – C22:C12 and C25:C12 wax esters) were absent in both males and females post-breeding. Similarly, levels of the C17:C13 wax ester (elevated in females relative to males during breeding) were dramatically lower post-breeding, especially in females. Levels of the C17:C14 – C15 esters also decreased in females from breeding to post-breeding stages. In both sexes, levels of C20:C14, C20:C16, C19:C16, and C19:C18 wax esters were higher post-breeding than during breeding (Appendix B, Figs. B7, B8).
Figure 4.1 Representative GC-MS chromatogram of preen oil from a breeding stage song sparrow (male, sampled at Newboro). Peaks A and B denote two peaks for monoesters of the same total carbon number, labeled for C33 as an example.
Figure 4.2 Wax ester composition of song sparrow preen oil from breeding stage males and females. The figure shows a two-dimensional nonmetric multidimensional scaling (NMDS) plot indicating Bray-Curtis chemical similarity: each symbol represents an individual (sampled at Newboro, 2017), and points appearing closer together are more chemically similar. Axis scales are arbitrary. 2D stress represents the amount of disagreement between the 2D configuration and predicted values from the multivariate regression (values closer to zero are better).
**Figure 4.3** Wax ester composition of song sparrow preen oil from post-breeding stage adults and juveniles (sexes pooled within each age class, sampled at Newboro). The figure shows an NMDS plot indicating Bray-Curtis chemical similarity (see Fig. 4.2 for details). For complete wax ester composition see Appendix B, Fig. B4.
Figure 4.4 Wax ester composition of song sparrow preen oil from different populations (breeding stage adults sampled at Newboro and Cambridge, 2017). The figure shows an NMDS plot indicating Bray-Curtis chemical similarity (see Fig. 4.2 for details).
Figure 4.5 Wax ester composition of song sparrow preen oil from different times in the season (sexes pooled, breeding and post-breeding samples collected at Newboro, 2016). The figure shows an NMDS plot indicating Bray-Curtis chemical similarity (see Fig. 4.2 for details).
4.4 Discussion

Preen oil wax ester profiles differed significantly between breeding stage males and females, adults and juveniles, breeding populations, and between breeding and post-breeding stages.

4.4.1 Sex differences

I observed significant sex differences in the wax ester profiles of song sparrows during the breeding stage (April through early May), a time period corresponding at these sites to birds returning from spring migration, establishing territories, pairing, constructing nests, and laying eggs for their first brood. In particular, I noted an increase in shorter chain fatty acids (especially C12) in the wax esters of male song sparrows. These differences were replicated across two breeding sites, but were no longer evident in the post-breeding stage (July to August), corresponding to the post-fledging period when juveniles are becoming independent. Sample sizes (particularly for females) were low during post-breeding, and this latter result should thus be interpreted with caution. That said, in a separate study conducted at a third breeding site and with a larger sample size, I similarly found no sex differences in preen oil wax ester composition for song sparrows sampled during late summer (Grieves et al. 2018, Chapter 2).

Evidence for sex differences in preen oil and feather chemical composition is mixed across bird species. Sex differences have been reported for domestic ducks, Anas platyrhynchos (Jacob et al. 1979); Sandpipers, Scolopacidae (Reneerkens et al. 2002); dark-eyed juncos, Junco hyemalis (Whittaker et al. 2010); budgerigars, Melopsittacus undulatus (Zhang et al. 2010); black-legged kitiwakes, Rissa tridactyla (Leclaire et al. 2011); house finches, Carpodacus mexicanus (Amo et al. 2012a); and spotless starlings, Sturnus unicolor (Amo et al. 2012b), but not for crested auklets, Aethia cristatella (Hagelin et al. 2003); rock pigeons, Columba livia (Salibian and Montalti 2009); New Zealand silvereyes, Zosterops lateralis (Azzani et al. 2016); Cory's shearwaters, Calonectris borealis and Scopoli's shearwaters, C. diomedea (Gabirot et al. 2016). My finding that the wax ester composition of song sparrow preen oil differs between the
sexes during breeding but not post-breeding indicates that seasonal effects are important to consider.

4.4.2 Age differences

I detected subtle but statistically significant differences in the wax ester profiles of post-breeding adults compared to juvenile song sparrows. However, GC-MS did not reveal any candidate compounds dramatically elevated in one age class relative to the other.

4.4.3 Population differences

I detected significant differences in the wax ester profiles of two geographically distinct breeding populations of song sparrow. Chemical stimuli are important in maintaining reproductive isolation in many taxa, but evidence for this function in birds is lacking (Smadja and Butlin 2009; Caro et al. 2015). However, differences in preen oil chemistry have been detected between two recently diverged populations of dark-eyed junco (Whittaker et al. 2010), suggesting that chemical stimuli may function as isolating mechanisms in birds as in other taxa (LeMaster and Mason 2003; Martín and López 2006; Smadja and Butlin 2009; Whittaker et al. 2010).

4.4.4 Seasonal differences

I found significant differences in preen oil when I compared samples collected during breeding versus post-breeding, consistent with several other studies: domestic duck (Jacob et al. 1979); Sandpipers, Scolopacidae (Reneerkens et al. 2002); Emberizidae (7 species), Corvidae (2 species), Mimidae (1 species) (Haribal et al. 2005); dark-eyed junco (Soini et al. 2007); white-throated sparrow, Zonotrichia albicollis (Tuttle et al. 2014); and herring gull, Larus argentatus (Fischer et al. 2017). This result may also support my finding that sex differences diminish by the end of breeding and may help to explain why some studies have failed to detect sex differences in preen oil. Sex differences in preen
oil may be driven by seasonal changes in female physiology (Jacob et al. 1979); however, findings from sandpipers (Scolopacidae) suggest that chemical changes in preen oil may be influenced by the incubating sex rather than females specifically (Reneerkens et al. 2002). Additionally, seasonal changes in preen oil composition may play a role in nest defense via chemical crypsis (Reneerkens et al. 2002, 2005).

Avian preen oil is commonly made up of mixtures of large monoester waxes comprised of straight chain and methyl branched fatty acids esterified to long-chain monohydroxy fatty alcohols (which can also be straight chain or branched). The diversity of carbon chain lengths and methylation patterns can lead to mixtures containing hundreds of compounds (Campagna et al. 2012). The wax esters I detected in song sparrows consisted of monoesters with both even and odd total carbon numbers (hereafter “even-numbered” and “odd-numbered”, respectively). Even-numbered waxes included both even-numbered alcohols esterified to even-numbered fatty acids and odd-numbered alcohols esterified to odd-numbered fatty acids. Conversely, odd-numbered waxes included even-numbered alcohols esterified to odd-numbered fatty acids, as well as odd-numbered alcohols esterified to even-numbered fatty acids. These patterns are similar to those reported by Thomas et al. (2010) for the closely related white-throated sparrow. In all, I characterized four even-numbered and six odd-numbered alcohols and four even-numbered and four odd-numbered fatty acids, esterified in the combinations described above.

The presence of odd-numbered alcohols and fatty acids suggests that the chemical components of song sparrow preen oil may contain multiple methyl branches. In some avian families, for example in the red knots (Calidris canutus, order Charadriiformes), odd-numbered esters are predominantly composed of even-numbered alcohols esterified to odd-numbered fatty acids (Dekker et al. 2000). In the case of even-numbered carbon waxes, odd- and even-numbered carbon alcohols were roughly equal in number, and various isomers of branched fatty acids were detected with increasing molecular mass (Dekker et al. 2000). In songbirds, branched alcohols have been found in several species, including northern mockingbirds (Mimus polyglottos) and Carolina chickadees (Poecile carolinensis) (Soini et al. 2013). Thus, my findings are not unprecedented for songbirds.
Due to the complexity of avian preen oil, the structural identification of each individual component is rarely achieved (Campagna et al. 2012). Consequently, it is common to instead qualitatively analyze chromatographic profiles (Campagna et al. 2012). While I was unable to pursue detailed structural analysis of each preen wax ester, I used GC-MS and proportional analysis to measure the chromatographic profiles of avian preen oil for a subset of my data. Because of sample size constraints, I did not perform any statistical analysis on these data.

Using thermal desorption-cooled injection gas chromatography-mass spectrometric analysis of organic compounds extracted from preen oil by solid phase extraction, Soini et al. (2013) identified many of the same fatty acids and fatty alcohols I found in intact preen oil wax esters, but especially the lower molecular weight compounds (e.g., C12-C18 1-alkanols and C12, C14 and C16 fatty acids). My analysis allowed me to measure intact preen oil using standard GC-FID equipment. The column and temperature profile I used are suitable for both volatile components and intact wax esters; however, I found no direct evidence for the presence of low-boiling components in our preen oil samples.

4.4.5 Conclusion

The wax ester composition of song sparrow preen oil differs between the sexes, between adults and juveniles, between populations, and breeding stages. This variation, together with the identification of specific compounds that vary among these groups, provides the foundation for future behavioural experiments on chemosignaling in this and other songbird species, particularly with respect to mate choice and reproduction.

4.5 References


Chapter 5

5 Behavioural responses of songbirds to preen oil odour cues of sex and species

5.1 Introduction

Chemical communication is the oldest form of communication and is widespread across animal taxa (Searcy and Nowicki 2005; Caro et al. 2015). Much of our current theory on mate choice and communication in birds has involved examining visual and acoustic signals such as plumage and song (Hamilton and Zuk 1982; Nowicki et al. 2002; Searcy and Nowicki 2005; Andersson and Simmons 2006; Gill 2007; Riebel 2009). Recently, however, advances in chemical ecology have begun to shift our understanding of the role of chemical signaling in avian mate choice and communication.

Although birds were long considered to have little or no sense of smell (Audubon 1826; Stager 1967), they are now known to have fully functional olfactory systems and to use odour cues in a variety of contexts (Caro et al. 2015; Hagelin and Jones 2007; Wenzel 1971). Birds use smell to find food (Healy and Guilford 1990; Nevitt et al. 2008; Potier et al. 2019), to avoid predators (Amo et al. 2008), and in many social contexts including the recognition of mates (Bonadonna and Nevitt 2004), kin (Coffin et al. 2011; Bonadonna and Sanz-Aguilar 2012; Krause et al. 2012), and species (Krause et al. 2014).

Most bird species possess a uropygial gland, which secretes preen oil. Preen oil is a complex mixture of volatile and nonvolatile compounds that function in feather protection, but is also thought to be the major source of avian body odour (Caro et al. 2015; Jacob 1978). The chemical composition of preen oil varies among species (Soini et al. 2013), among individuals (Leclaire et al. 2011; Potier et al. 2018), between the sexes

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(Whittaker et al. 2010; Tuttle et al. 2014; Grieves et al. 2019, Chapter 4), with genotype at the major histocompatibility complex (MHC) (Leclaire et al. 2014; Slade et al. 2016), and between age classes (Shaw et al. 2011; Grieves et al. 2019, Chapter 4). Preen oil composition also varies seasonally (Bhattacharyya and Chowdhury 1995; Fischer et al. 2017; Grieves et al. 2019, Chapter 4), and with diet (Thomas et al. 2010), microbiome (Jacob et al. 2014), and parasitic infection status (Grieves et al. 2018, Chapter 2). Thus, preen oil represents a rich source of information that may be available to birds and other receivers in the contexts of intra- and interspecific communication (Hagelin and Jones 2007; Caro et al. 2015).

Avian chemical communication was first examined in seabirds (Nevitt 1994; Wenzel 1986), a group already known to use olfaction in navigation and foraging contexts (Mardon et al. 2010; Nevitt 1994; Nevitt et al. 2008; Wenzel 1986). By contrast, passerine birds (Passeriformes), comprising over half of all extant bird species, have small olfactory bulbs relative to total brain size (Bang and Cobb 1968), and thus were long assumed to have little to no olfactory capabilities. However, olfactory bulb size is now known to be a poor predictor of olfactory acuity in passerine birds, and odour detection thresholds in this group are now considered comparable to those of macrosmatic mammals such as rabbits and rats (Clark et al. 1993).

Evidence is accumulating rapidly that passerine birds, like other vertebrate groups, are capable of using odour cues in social and interspecific contexts. For example, zebra finches (*Taeniopygia guttata*) spend more time with the odour of conspecifics compared to that of closely related diamond firetails (*Stagonopleura guttata*; Krause et al. 2014). Fledgling zebra finches use odour cues to discriminate between kin and non-kin (Krause et al. 2012), and newly hatched chicks recognize and respond to parental odour, particularly that of their mother, even after cross-fostering (Caspers et al. 2017). Both male and female dark-eyed juncos (*Junco hyemalis*) spend more time with preen oil of male conspecifics than females, and female juncos also spend more time with preen oil from smaller males than larger males (Whittaker et al. 2011). Similarly, male and female spotless starlings (*Sturnus unicolor*) spend more time with preen oil from male starlings.
than females (Amo et al. 2012a). In house finches (*Carpodacus mexicanus*), males in poor condition spend less time with the odour of conspecific males, whereas males in good condition spend more time with this odour (Amo et al. 2012b).

The studies reviewed above support a role for preen oil odour cues in mediating chemical communication in seabirds, and in gregarious species of passerine (Nolan et al. 2002; del Hoyo 2009, 2010, 2011). By contrast, with a few exceptions (e.g., in asocial diamond firetails females do not distinguish between odours of female conspecifics and heterospecifics; Krause et al. 2014), little is known about how nonsocial passerines respond to odour cues of sex and species identity.

I examined the responses of song sparrows (*Melospiza melodia*), which are socially monogamous, relatively asocial, and frequently parasitized by brood-parasitic brown-headed cowbirds (*Molothrus ater*) (Arcese et al. 2002), to odour cues of sex and species. The chemical composition of preen oil in song sparrows differs between the sexes, between breeding and nonbreeding seasons, between age classes, and between populations (Grieves et al. 2019, Chapter 4); varies with genotype at the major histocompatibility complex (MHC) (Slade et al. 2016); and varies with exposure to malarial parasites (Grieves et al. 2018, Chapter 2). Given this wealth of potential information that receivers may obtain from preen oil odour cues, behavioural experiments are required to address whether song sparrows can use the information available in preen oil, and in what contexts.

I conducted chemical analysis of preen oil to confirm that its composition differs between breeding-condition male and female song sparrows, as previously described (Grieves et al. 2019, Chapter 4), and between song sparrows and female brown-headed cowbirds. I then hypothesized that song sparrows use odour cues derived from preen oil in the contexts of intraspecific and interspecific interactions. To test this hypothesis, I conducted a series of two-choice behavioural experiments using a Y-maze. First, I compared time spent with preen oil odour from same-sex conspecifics relative to the absence of such odour. Provided that song sparrows can detect preen oil odour cues, I predicted that they should be attracted to conspecific odours, as has been reported in
seabirds (e.g., Bonadonna and Nevitt 2004; Coffin et al. 2011) and gregarious passerines (Krause et al. 2014). Second, I compared time spent with preen oil odour from opposite-sex relative to same-sex conspecifics. Provided song sparrows can detect sex differences in preen oil, I predicted that breeding-condition adults would prefer the odour of opposite-sex over same-sex individuals. However, I note that in some passerines, both sexes spend more time with preen oil odour of males than females (Amo et al. 2012a; Whittaker et al. 2011). Finally, I tested song sparrow responses to (heterospecific) odour of their major brood parasites, female brown-headed cowbirds. Song sparrows actively exclude adult cowbirds from their territories (Arcese et al. 2002), but only rarely reject cowbird eggs from their nests (Rothstein 1975; Lowther 1993). As an exploratory test of whether song sparrows can detect the odour of brown-headed cowbirds, I compared the time spent with preen oil from female cowbirds relative to the absence of such odour.

5.2 Methods

5.2.1 Study animals and housing

Study subjects were 36 adult song sparrows (27 male, 9 female) captured by mist net in August and September 2017 in London, Ontario, Canada (42.9849 N°, 81.2453° W). I determined sex by morphological measurements, and later confirmed using the P2/P8 genotyping assay (Griffiths et al. 1998). I housed song sparrows in a single room held at 20 ± 1 °C. Each bird was in an individual cage (45.7 cm × 45.7 cm × 45.7 cm) containing 3 – 5 perches of varying materials and thicknesses (wooden dowel, textured plastic, natural sterilized branches, and rubber tubing) in a single room. Birds had ad libitum access to water and food (Mazuri Small Bird Maintenance chow and parakeet seed supplemented weekly with mealworms, cooked egg, and greens).

Until February 2018, the lighting schedule of the holding room mimicked the natural photoperiod (approximately 11 L:13 D in this area during February). Male song sparrows began singing on 12 February 2018 and continued to sing throughout the duration of behavioural experiments (2 – 18 March 2018). To increase the likelihood that all subjects would come into breeding condition, on 22 February 2018 I increased the
light phase of the photoperiod to 14 L:10 D (photostimulatory conditions; Wingfield 1993) and held birds at this photoperiod throughout the trials.

5.2.2 Odour stimuli

I collected conspecific (song sparrow) preen oil samples from the same set of birds that participated in behavioural trials. Conspecific samples (Experiments 1 and 2) were collected after 1 – 2 weeks of photostimulation and 2 – 3 weeks after the onset of spontaneous male song. Heterospecific (cowbird) preen oil (Experiment 3) was collected from 24 adult female brown-headed cowbirds that had been group-housed in mixed-sex flocks in outdoor aviaries in Flamborough, Ontario (Davies and White 2018). Preen oil used in Experiments 1, 2, and 3 was collected on 1, 7, and 11 March 2018 respectively.

I applied gentle pressure to the uropygial gland to express a small sample (1 – 5 mg) of preen oil into an unheparinized capillary tube, which I snapped to fit inside a 1.5 mL borosilicate glass vial with polytetrafluoroethylene (PTFE) lined cap suitable for use with chloroform (CHCl₃). I dissolved the preen oil in 0.1 – 0.5 mL of CHCl₃, scaled for the mass of oil collected, then stored it at 4 ºC awaiting use in experiments. Dissolved samples were kept refrigerated for up to 18 d during experiments, then, after the experiments were completed, the remainder of all preen oil samples were stored at -20°C awaiting chemical analysis.

5.2.3 Chemical analysis of preen oil

I dissolved a portion of each preen oil sample used as odour stimuli in an additional 1 – 3 mL of CHCl₃. I conducted chemical analysis using an Agilent 7890A gas chromatograph with flame ionization detector (GC-FID) fitted with a 5% phenyl methyl siloxane column (Agilent Technologies DB-5, 30 m × 0.32 µm ID × 0.25 µm film thickness). Briefly, 1 µL samples were injected with a 30 psi pressure pulse (1 min) and, after an initial 1 min hold at 70 ºC, eluted with the following temperature profile: increase to 130 ºC at 20 ºC/min, then to 320 ºC at 4 ºC/min. Injector and FID temperatures were 200 ºC and 310 ºC,
respectively. Hydrogen was used as a carrier gas at 2.5 mL/min. Each batch of GC-FID runs (typically 20) included a blank sample containing solvent only (CHCl₃) and a sample of known composition (i.e., previously analyzed by both GC-FID and gas chromatography-mass spectrometry (GC-MS) (Slade et al. 2016).

5.2.4 Behavioural trials

All subjects participated in all choice trials, in the order outlined below. I avoided testing an individual with its own preen oil, that of an individual that had been housed in an adjacent cage, or that of a likely previous mate (i.e., an opposite-sex individual captured from the same territory as the focal individual the previous summer). Further, I ensured that each focal bird received a unique same-sex preen oil sample for each of Experiments 1 and 2. Odour stimuli were prepared fresh each day. I first removed preen oil samples from 4 °C storage and warmed to room temperature for 5 min, then 2 – 5 minutes before the trial began, I applied 50 µL of odour stimulus (i.e., 0.5 mg of preen oil dissolved in 50 µL CHCl₃ or 50 µL CHCl₃ alone) onto a clean cotton ball affixed to each arm of the maze. Two to five minutes was sufficient time for the CHCl₃ to completely evaporate, though I cannot rule out the presence of residual solvent in either preen oil or carrier solvent treatments.

5.2.5 Experiment 1: Conspecific preen oil versus absence of preen oil odour cues

To test whether song sparrows spend more time with conspecific odour cues than with the absence of such cues, I presented subjects with a two-choice test involving same-sex preen oil in one maze arm, and residual solvent only in the other arm.
5.2.6 Experiment 2: Opposite-sex *versus* same-sex conspecific preen oil

To test whether song sparrows spend more time with preen oil from opposite-sex than same-sex conspecifics, I presented subjects with a two-choice test involving opposite-sex preen oil in one maze arm and same-sex preen oil in the other arm.

5.2.7 Experiment 3: Cowbird preen oil *versus* absence of preen oil odour cues

To test whether song sparrows spend more or less time with preen oil from female cowbirds than with no preen oil, I presented subjects with a two-choice test involving preen oil from a female cowbird in one maze arm and residual solvent only in the other arm.

5.2.8 Behavioural trial methodology

I conducted trial in a Plexiglas Y-maze following the design of Whittaker et al. (2011) (arms: 20 cm H × 40 cm L × 20 cm W; central area: 20 cm H × 35 cm L × 20 cm W). A wooden perch was positioned near the end of each arm, and an odour stimulus (described above) was placed on a cotton ball taped into a dish at the end of each arm (8 cm from the perch). The maze contained a starting chamber (20 cm H × 14 cm L × 20 cm W) separated by an opaque Plexiglas barrier that could be slid open and closed to release the bird into the maze. Side walls were made opaque by taping brown Kraft paper to the outer surface and a wire screen was placed atop the maze to prevent subjects from colliding with the maze ceiling. The maze was positioned evenly between two overhead lights in an observation room. I recorded all trials using a camera (Activeon CX) mounted on a tripod positioned above the start chamber. I used a vacuum pump (Neptune DynaPump, Thermoscientific) connected to two equal lengths of air tubing to circulate air from the odour stimulus down the arms of the maze while preventing mixing in the
central area. I habituated subjects to the sound of the vacuum pump by running the pump in their home room for 1 hr/d for seven days preceding behavioural trials.

Trials were conducted between 0800 h and 1130 h daily from 2 – 18 March 2018. For each experiment, I used a random number generator to determine the order in which birds would be tested. I flipped a coin to assign stimulus type to maze arm for the first trial of each experiment then alternated stimulus location for each subsequent trial.

Immediately before each trial, I transported the focal bird in an opaque cloth bird bag from its home cage to the observation room (travel time < 2 min). The bird was then placed into the start chamber for a 5 min acclimation period under dim lighting. After 5 min, video recording began, and the barrier to the start chamber was opened then closed immediately behind the bird. Birds typically emerged from the start chamber as soon as the barrier was opened and none remained in the start chamber for more than a few seconds. I then left the room, turned on the observation room light, and allowed the subject to explore the maze for 15 min.

The first 5 min after the bird left the start chamber was treated as an *exploration period*. After the 5 min exploration period, video recording continued for another 10 min, the *choice period*. At the end of this choice period, I gently tapped the walls of the maze to guide the subject back into the start chamber, placed the subject in a bird bag, and returned it to its home cage. The maze was cleaned using 70% ethanol and allowed to air dry between trials.

I considered a trial to be ‘successful’ if during the initial 5 min exploration period the subject either entered both arms of the maze, or entered one arm and approached the other (defined as standing continuously for at least 10 sec within one body width of the non-entered maze arm with its head and bill oriented toward the non-entered arm). Trials in which the subject remained in the central area of the maze (i.e., did not enter or approach either arm), and trials in which one of the maze arms was neither entered nor approached within the initial 5 min exploration period were excluded. In these cases, I tested the subject with the same stimuli 24 – 36 hours later, up to 2 more times during a given experiment. If a subject did not respond by its third trial, it was excluded from that
experiment. Overall, 87% (94/108) of trials were successful. Ten birds (8 male, 2 female) were responsible for the 14 unsuccessful trials, indicating that most birds (26/36) successfully investigated the maze during the exploration period prior to the start of each trial.

Successful trials were scored from videos, with file names scrambled so that I was blind to the stimulus type in each maze arm. I tabulated the time that the focal bird spent in or approaching each arm of the maze (as described above) during the 10 min choice period.

5.2.9 Data analysis: Sex and species differences in preen oil

All analyses were performed in R version 3.2.3 (R Development Core Team 2017). To confirm sex and species differences in the chemical composition of preen oil (male *versus* female song sparrows; song sparrows *versus* cowbirds), I quantified the relative size of each chromatogram peak identified by GC-FID, retaining for analysis only peaks that comprised ≥ 0.1% of the total chromatogram area (Leclaire et al. 2012; Slade et al. 2016). To prevent large peaks from disproportionately influencing distance measures (Leclaire et al. 2014), I normalized the data using the ‘range’ method in the *decostand* function in the R package vegan (Dixon and Palmer 2003). I log (x + 1) transformed the normalized dataset then constructed pairwise matrices of Bray-Curtis dissimilarity, which we interpret as chemical distances between samples.

To visualize these pairwise chemical distances, we used nonmetric multidimensional scaling (NMDS). This approach places each sample on a two-dimensional scatter plot, preserving ranked pairwise distances such that two points close together represent two individuals with relatively similar chemical composition while points further apart represent individuals that are more dissimilar (Clarke 1999; Stoffel et al. 2015). To assess the statistical significance of chemical differences between the sexes and between species, I used nonparametric analysis of similarities (ANOSIM) implemented in vegan (Dixon and Palmer 2003) with 10 000 iterations. This permutation
approach does not make assumptions about the data’s distribution (Clarke 1999; Stoffel et al. 2015).

5.2.10 Data analysis: Behavioural trials

For each of the three behavioural experiments, I tested for differences in time spent with stimulus (odour) type by fitting a restricted maximum likelihood (REML) linear mixed model using the lme4 package (Bates et al. 2015). Fixed effects included sample type (e.g., same-sex versus opposite-sex preen oil), sex of the focal bird, and their interaction; bird ID was included as a random effect; and the dependent variable was time spent in or approaching (as defined above) a maze arm. For all experiments, visual assessments of qq-plots and residuals confirmed that data and residuals were distributed approximately normally and the residuals showed no evidence of homoscedasticity. P-values were obtained using Wald tests (using the Anova function in the R package car).

5.3 Results

5.3.1 Chemical analysis of preen oil

The chemical composition of preen oil differed significantly between male and female song sparrows (ANOSIM: Global R = 0.26, P = 0.01, Fig. 5.1a), and between female brown-headed cowbirds and song sparrows (sexes pooled; ANOSIM: Global R = 0.94, P = < 0.0001, Fig. 5.1b).

5.3.2 Experiment 1: Conspecific preen oil versus solvent

Song sparrows did not appear to behaviourally discriminate between the presence and absence of conspecific same-sex odour cues. I found no main effect of sample type or sex of the focal bird in predicting time spent near conspecific same-sex preen oil as opposed to the absence of such odour, nor did I observe a significant interaction (Table 5.1, Fig.
5.3.3 Experiment 2: Opposite-sex *versus* same-sex conspecific preen oil

Song sparrows spent more time with preen oil from opposite-sex than same-sex conspecifics. I observed a main effect of sample type (i.e., opposite *versus* same-sex) in predicting time spent near a stimulus, but no effect of focal bird sex or the interaction term (Table 5.2, Fig. 5.3). Thirty-four of 36 tests (26 of 27 tests on males and 8 of 9 tests on females) were successful using the criteria described above.

5.3.4 Experiment 3: Cowbird preen oil *versus* solvent

In comparing time spent with preen oil from female brown-headed cowbirds *versus* absence of such odour, I found no main effect of sample type or of focal bird sex on time spent with each sample. However, there was a significant interaction between sample type and focal bird sex: male song sparrows spent almost twice as much time with preen oil from female cowbirds than with solvent, while female song sparrows showed the reverse pattern (Table 5.3, Fig. 5.4). Thirty of 36 trials (23 of 27 trials on males and 7 of 9 trials on females) were successful using the criteria described above.
Figure 5.1 Two-dimensional nonmetric multidimensional scaling (NMDS) plot of song sparrow and brown-headed cowbird preen oil wax ester composition based on Bray-Curtis distances. In panel A, each symbol represents an individual song sparrow (9 females, 27 males). In panel B, each symbol represents an individual song sparrow (36 birds, sexes combined) or brown-headed cowbird (24 females). Points appearing closer together are more chemically similar. Axis scales are arbitrary. 2D stress represents the amount of disagreement between the 2D configuration and predicted values from the multivariate regression (values closer to zero are better).
Table 5.1 Song sparrows did not discriminate in time spent near conspecific, same-sex preen oil *versus* absence of such odour in a two-choice Y-maze test.

<table>
<thead>
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<th>Fixed effects</th>
<th>Estimate</th>
<th>SE</th>
<th>t</th>
<th>$\chi^2$</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
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<td>60.6</td>
<td>3.31</td>
<td>3.3</td>
<td>0.001</td>
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<tr>
<td>Sample type</td>
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<td>104.9</td>
<td>-0.46</td>
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<td>Sex of focal bird</td>
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<td>0.2</td>
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<tr>
<td>Type × sex</td>
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<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.811</td>
</tr>
</tbody>
</table>

Parameters are estimated from a linear mixed model fit by REML; $P$-values are calculated from type II Wald chi square tests. $N = 60$ observations on 30 birds.
**Figure 5.2** Time spent by song sparrows with preen oil from same-sex conspecifics in two-choice Y-maze experiments. Large symbols denote the mean (± SE). Small gray symbols are individual responses to each stimulus type. Sample sizes are reported in parentheses.
Table 5.2 Song sparrows spent more time near preen oil from opposite-sex than same-sex conspecifics, in a two-choice Y-maze test.

<table>
<thead>
<tr>
<th>Fixed effects</th>
<th>Estimate</th>
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<th>$\chi^2$</th>
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<tbody>
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<td>73.0</td>
<td>2.94</td>
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<td>–</td>
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<tr>
<td>Sample type</td>
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<td>103.2</td>
<td>-0.37</td>
<td>6.50</td>
<td>0.011</td>
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<tr>
<td>Sex of focal bird</td>
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<td>83.5</td>
<td>1.49</td>
<td>1.3</td>
<td>0.260</td>
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<tr>
<td>Type × sex</td>
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<td>118.1</td>
<td>-0.99</td>
<td>1.0</td>
<td>0.324</td>
</tr>
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</table>

Parameters are estimated from a linear mixed model fit by REML; P-values are calculated from type II Wald chi square tests. N = 60 observations on 30 birds.
Figure 5.3 Time spent by song sparrows with preen oil from either same-sex or opposite-sex conspecifics in two-choice Y-maze experiments. Large symbols denote the mean (± SE). Small gray symbols are individual responses to each stimulus type. Sample sizes are reported in parentheses.
**Table 5.3** Sex and sample type interact to affect time spent by song sparrows near preen oil from female brown-headed cowbirds *versus* absence of such odour in a two-choice Y-maze test.

<table>
<thead>
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<th></th>
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<th>$\chi^2$</th>
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<td>Type × sex</td>
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<td>103.8</td>
<td>-1.99</td>
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<td>0.047</td>
</tr>
</tbody>
</table>

Parameters are estimated from a linear mixed model fit by REML; P values are calculated from type II Wald chi square tests. N = 60 observations on 30 birds.
Figure 5.4 Time spent by song sparrows with preen oil from female brown-headed cowbirds in two-choice Y-maze experiments. Large symbols denote the mean (± SE). Small gray symbols are individual responses to each stimulus type. Sample sizes are reported in parentheses.
5.4 Discussion

Chemical analysis of preen oil wax esters confirmed significant differences between the sexes (male versus female song sparrows in breeding condition) and between species (song sparrows versus female brown-headed cowbirds). More importantly, behavioural trials suggest that song sparrows are capable of using olfactory cues to assess this information, and show behavioural discrimination in social and other ecologically relevant contexts (spending more time with preen oil from opposite-sex than from same-sex conspecifics; males spending more time with preen oil from female cowbirds than with solvent alone, and females showing the opposite pattern).

Passerine birds have small olfactory bulbs relative to their overall brain size (Bang and Cobb 1968) and thus were long assumed to have little to no olfactory capabilities. However, a growing body of research now indicates that passerines do attend to odour cues, including those derived from preen oil of conspecifics (Amo et al. 2012a,b; Whittaker et al. 2011) and heterospecifics (Krause et al. 2014). My findings add to the growing body of research on chemical communication in birds and indicate that even relatively nonsocial species are capable of using olfactory cues in social and interspecific contexts.

Perhaps surprisingly, song sparrows did not spend more time with conspecific (same-sex) preen oil odour when provided the choice between this and no odour. In isolation, this finding could suggest either that song sparrows cannot detect conspecific preen oil odour cues, or that such cues are detectable but not inherently attractive. Given the differential responses I observed to sex and species identity, I favour this second interpretation. Song sparrows are not a particularly social species, especially during the breeding season when they actively exclude same-sex conspecifics from territories (Arcese et al. 2002). I think it likely that song sparrows are simply neither attracted to nor repulsed by the odour of same-sex conspecifics. The lack of attraction to same-sex conspecific odour relative to absence of odour is also consistent with findings from female diamond firetails, which showed no preference for same-sex conspecific odour relative to that of heterospecifics (Krause et al. 2014). In light of my findings, I suggest
that the patterns seen in diamond firetails may similarly reflect a lack of preference for same-sex conspecific odour, rather than an inability to recognize this odour.

In my study, song sparrows were housed in a common room for several months before testing and presumably became familiar with one another’s odour. Behavioural responses to odour cues of sex (i.e., spending more time with opposite-sex odour, Experiment 2) and species identity (i.e., males spending more time and females spending less time with cowbird odour, Experiment 3) were observed for both a conspecific experiment (in which the stimulus odours were likely familiar) and a heterospecific experiment (in which the stimulus odours were not familiar). Thus I think it unlikely that my findings are affected by the fact that conspecific but not heterospecific stimuli were familiar. Future experiments comparing responses to odour from novel versus familiar individuals should help to disentangle how familiarity and individual recognition interact with odour cues of sex and species identity. I am aware of only one other study in birds that compared time spent with an ecologically relevant odour to time spent with a control odour such as solvent or water (Amo et al. 2008). The finding that song sparrows do not discriminate in their behavioural response to (same-sex, familiar) conspecific odour versus solvent illustrates the risks of conflating absence of discrimination with an inability to detect a given stimulus.

Because subjects were in breeding condition and presumably motivated to pursue mating opportunities I predicted that, provided song sparrows are capable of detecting conspecific odour cues, they would spend more time with opposite-sex than same-sex odour. This prediction was supported, as both males and females preferentially associated with odour cues from opposite-sex rather than same-sex individuals. However, while I observed a significant main effect of sample type overall, I note that preference for opposite-sex odour was more pronounced in males than in females and that the larger sample size for males may have driven the overall effect observed. Regardless, my findings suggest that not only does preen oil chemical composition differ between the sexes during the breeding season, song sparrows (at least males, and potentially females) are capable of perceiving this information and using it to guide behaviour. Odour cues may thus be an important cue of sex recognition during the breeding season in this
species. Song sparrows are sexually monomorphic with respect to plumage (Arcese et al. 2002), and although usually only males sing, females occasionally sing during the breeding season (Arcese et al. 1988) meaning that visual and behavioural cues of sex may be limited.

Song sparrows also showed behavioural discrimination in their responses to odour from female cowbirds, albeit with sex-specific responses. Males spent nearly twice as much time, on average, with female cowbird odour when presented with a choice between this and no odour; conversely, females spent approximately one and a half times more time, on average, in the Y-maze arm with no odour than with female cowbird odour. Interestingly, the apparent ability of song sparrows to recognize cowbird odour cues does not generally lead to a rejection of cowbird eggs in the wild. Although brown-headed cowbirds have been reported to use over 200 species of host, song sparrows are among the most commonly parasitized (Lowther 1993). Song sparrows are classified as “acceptors” of cowbird parasitism (Rothstein 1975), meaning that they eject, abandon, or bury cowbird eggs less than 20% of the time (Lowther 1993). This lack of rejection could reflect an inability to recognize cowbird eggs; for example, odour cues from the female cowbird’s preen oil may not be transferred to eggs. Chemical and headspace analysis (Webster et al. 2015) of the surfaces of host versus cowbird eggs would help to address this possibility. Alternatively, the costs to song sparrows of rejecting cowbird eggs (e.g., the risk of a cowbird retaliating by destroying the clutch; Hoover and Robinson 2007) may outweigh the costs of accepting such eggs.

Although song sparrows do not typically reject cowbird eggs, they do respond behaviourally to adult female cowbirds. In the wild, song sparrows of both sexes give alarm calls in the presence of cowbirds, and adult females stop nest-building (Arcese et al. 2002; Smith et al. 1984). Similarly, female song sparrows produced more alarm calls, made more flights, and approached more closely to a taxidermied female cowbird than to a control taxidermied dark-eyed junco mount, and male song sparrows spent more time near the cowbird than the junco mounts (Smith et al. 1984). If such aggressive responses can deter cowbirds from parasitizing song sparrow nests, this may reduce the need for egg rejection (Robertson and Norman 1976).
Preen oil chemical profiles of brown-headed cowbirds are distinct from those of other species (Soini et al. 2013; this study), but whether and how song sparrows and other hosts use this information in the wild remains to be determined. Female cowbirds use a variety of tactics to find host nests, including perching quietly and watching for nest building activity; alternating short flights with noisy landings, as if intentionally trying to flush potential hosts from their nests; and walking on the ground (Norman and Robertson 1975; Lowther 1993). For ground-nesting, ground-foraging species such as song sparrows, the “walking” tactic in particular may provide localized olfactory cues that cowbirds are present in an area, potentially influencing the selection of territories or nest site selection.

My findings suggest that song sparrows attend to preen oil odour cues from both conspecifics (e.g., preferences for opposite-sex preen oil) and heterospecifics (e.g., males spending more time and females spending less time with preen oil from brood parasitic cowbirds than with the absence of such odour). Overall, my findings suggest that even relatively nonsocial species with small olfactory bulbs are capable of using olfactory stimuli for chemical communication both within and between species.

5.5 References


Audubon JJ. 1826. Account of the habits of the turkey buzzard (Vultur aura) particularly with the view of exploding the opinion generally entertained of its extraordinary power of smelling. Edinb New Philos J. 2:172–184.


Chapter 6

6 Songbirds show odour-based discrimination of similarity and diversity at the major histocompatibility complex

6.1 Introduction

The major histocompatibility complex (MHC), a family of genes encoding receptors that recognize and bind to invading antigens in jawed vertebrates, is unusually polymorphic (Janeway et al. 2001). High levels of population and individual genetic diversity at MHC are due in part to pathogen-mediated selection, such that individuals with particular alleles (e.g., rare alleles) or allelic combinations (e.g., heterozygote advantage) are more resistant to infectious disease (Migalska et al. 2019; Milinski 2006; but see Minias et al. 2018). However, MHC-based mate choice is also thought to play a role in maintaining diversity at these loci (Milinski 2006). Choosing mates that are optimally dissimilar at MHC (i.e., compatible; Neff and Pitcher 2004) should optimize the MHC diversity of the resultant offspring. Moreover, choosing mates who are themselves optimally diverse at MHC may enhance access to parental care or other material benefits (Zelano and Edwards 2002).

MHC-based mate choice or mate preferences have been demonstrated in all major groups of jawed vertebrates, including mammals (Setchell et al. 2010), birds (Bonneaud et al. 2006; Strandh et al. 2012), reptiles (Olsson et al. 2003), amphibians (Bos et al. 2009), and fish (Milinski et al. 2005). Thus, some mechanism must exist by which animals can assess their potential mates’ MHC profile. In mammals and fish, fragments of MHC glycoproteins are secreted into bodily fluids such as urine, and can be smelled by conspecific receivers (Milinski et al. 2005; Restrepo et al. 2006); but in birds, the possibility of odour-based signaling has historically been discounted (Stager 1967).

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Recently, however, secretions of the uropygial gland (preen oil) have emerged as a candidate source of chemical cues in birds (Whittaker et al. 2010, 2011). Preen oil chemical makeup varies among species and populations (Zhang et al. 2013), among populations within a species (Gries et al. 2019a, Chapter 4), between the sexes (Zhang et al. 2009; Whittaker et al. 2010; Gries et al. 2019a, Chapter 4), and changes seasonally (Fischer et al. 2017; Gries et al. 2019a, Chapter 4) and with exposure to disease (Gries et al. 2018, Chapter 2). Notably, the chemical composition of preen oil covaries with MHC class II genotype in both seabirds (black-legged kittiwakes, Rissa tridactyla; Leclaire et al. 2014) and songbirds (song sparrows, Melospiza melodia; Slade et al. 2016). Seabirds appear to use odour cues from preen oil to mate nonrandomly at MHC (Leclaire et al. 2017). However, despite the prominence of songbirds in studies of mate choice and communication (Coleman 2009), their ability to assess MHC remains uncertain, much less the mechanism by which this might be accomplished.

For animals to use phenotypic cues to assess MHC compatibility or diversity, two conditions must be met: the cues must covary with MHC genotype, and be perceptible by the animals. I tested both these requirements in songbirds, focusing on odour cues derived from preen oil. Pairwise similarity in preen oil chemistry predicts similarity at the hypervariable second exon of MHC class II in wild song sparrows (Slade et al. 2016), so I first confirmed that this relationship also holds in captivity. I then conducted a two-choice odour preference experiment, asking whether song sparrows could distinguish between preen oil samples from opposite-sex individuals (i.e., potential mates) with greater MHC dissimilarity and/or diversity. Song sparrows are socially monogamous, and both sexes invest heavily in parental care (Arcese et al. 2002). Thus, both sexes may obtain genetic (indirect) and/or material (direct) benefits through choosing mates that are MHC-dissimilar and/or MHC-diverse, respectively. Accordingly, I predicted that both sexes should prefer the odour of preen oil from opposite-sex individuals with MHC genotypes dissimilar to their own and from opposite-sex individuals that are more diverse at MHC.
6.2 Methods

6.2.1 Study subjects and housing

Study subjects were 36 adult song sparrows (27 male, 9 female) captured in mist nets on their breeding territories in London, Ontario, Canada (42.9849 N°, 81.2453° W) between 8 August – 1 September 2017. I housed birds in the same holding room over winter in individual cages with ad libitum access to water and food (Mazuri Small Bird Maintenance chow and parakeet seed; supplemented weekly with mealworms, cooked egg, and greens) under a simulated natural photoperiod. Males began singing on 13 February 2018 and on 22 February 2018, I increased the photoperiod to 14 L:10 D to speed the onset of breeding condition for all birds. All males continued singing in their home cages throughout the duration of behavioural trials (20 – 24 March 2018), suggesting subjects were in breeding condition during these trials.

6.2.2 Genetic analysis

I collected a small blood sample from each bird via brachial venipuncture for genetic confirmation of sex (Griffiths et al. 1998) and MHC characterization. I amplified the hypervariable second exon of MHC class II (338 – 350 bp) using primers SospMHCint1f (Slade et al. 2016) and Int2r.1 (Edwards et al. 1998), which bind within introns 1 and 2 respectively. Each primer included an Illumina MiSeq adaptor sequence, four wobble bases, and an individually-unique ‘barcode’ of eight bases. I performed PCR in a total volume of 35 µL containing 12.5 µL of GoTaq® Hot Start Green Master Mix (Promega), 0.2 µM of each primer, and 60 ng of template genomic DNA. The thermocycling profile consisted of 3 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 62 °C, and 45 s at 72 °C, and a final extension step of 10 min at 72 °C. I confirmed amplification by agarose gel electrophoresis.

I pooled PCR products into a single library and sequenced with 300 bp paired-end reads on an Illumina MiSeq at the London Regional Genomics Centre. I used a pipeline (Gloor et al. 2010) to collapse sequences into clusters of identical reads and assign
recovered sequences to individuals. To identify a threshold frequency below which sequences are likely due to PCR errors rather than to true alleles, I amplified MHC class II exon 2 for two individual song sparrows using the primers and PCR conditions described above. Using cloning (Promega pGEM-T Easy Vector System), I generated multiple colonies, each presumably containing a single allele. I included PCR products from 8 colonies (5 from one individual and 3 from the other) in the Illumina flow cell run along with the pooled library. Each colony should yield only one sequence in the absence of PCR or sequencing errors; thus, I used the frequency of rare secondary sequences in each colony as an estimate of PCR and sequencing error rate. Based on the observed frequencies of secondary sequences across the 8 colonies (median = 0.011, variance = 2.0 × 10^{-5}, range = 0.006 – 0.018), I established a threshold error rate of 0.01 and thus retained sequences comprising at least 1% of an individual’s reads (mean ± SE retained reads per individual = 20 736 ± 1 939).

I aligned amino acid sequences in MEGA v.7.0 (Kumar et al. 2016) and trimmed based on comparison to conspecific sequences in GenBank (Benson et al. 2005). Trimming resulted in alleles of 73 – 86 amino acids, corresponding to most of exon 2. In all, I recovered 186 unique amino acid sequences (mean ± SE per individual = 15.5 ± 0.5 amino acid alleles; Appendix C, Table C1). I calculated MHC allelic diversity for each individual as the number of unique amino acid sequences.

To assess pairwise genetic dissimilarity, I first constructed a maximum-likelihood phylogeny of all alleles using a WAG model (Whelan and Goldman 2001) with five discrete gamma categories in MEGA. I then calculated amino acid distances between all male-female dyads using the UniFrac phylogenetic comparison tool (Lozupone and Knight 2005) implemented in the R package GUniFrac (Chen et al. 2012). This method uses a phylogeny of all detected alleles to calculate the branch length distance between the translated MHC genotype of individuals, such that two individuals with an identical set of alleles would have a UniFrac distance of zero, while two individuals with alleles derived from completely different clades in the reference tree would have a UniFrac distance of one (Lozupone and Knight 2005). Genotypic data were binary (allele presence or absence), so I calculated unweighted rather than weighted UniFrac distances.
Because this metric can be sensitive to cutoff thresholds and other methodological
decisions (Lozupone et al. 2010; Wong et al. 2016), in addition to calculating distances
for the full dataset I also generated nine additional phylogenies, each removing one of the
nine alleles with the longest branch lengths. I calculated unweighted UniFrac distances
for each of the ten phylogenies, then used the average of all analyses. These mean
pairwise distances (hereafter “amino acid” distances) ranged from 0.28 to 0.69 for
opposite-sex dyads.

The functional properties of amino acids may also explain MHC-mediated mate
choice (Leclaire et al. 2017; Strandh et al. 2012). Therefore, as a complementary analysis
to the approach described above, I also calculated MHC distances between individuals
based on the chemical binding properties of each amino acid. For this analysis, I trimmed
alleles to 70 amino acids and removed any alleles containing indels (5.4% of sequences).
I then assigned five z-score descriptors to each amino acid, describing its
physicochemical properties ($z_1$: hydrophobicity, $z_2$: steric bulk, $z_3$: polarity, $z_4$ and $z_5$: electronic properties; Sandberg et al. 1998). With the resulting matrix, I constructed an
alternative phylogeny (functional tree) in PHYLIP 3.695 (Felsenstein 2005) using
‘ContmL’. As described above, I also generated nine additional phylogenies, each
removing one of the nine alleles with the longest branch lengths, and calculated mean
unweighted UniFrac distances across all ten functional trees. These mean pairwise
distances (i.e., based on physicochemical differences; hereafter “functional” distances)
ranged from 0.43 to 0.75 for opposite-sex dyads. Functional distances were weakly but
significantly correlated with amino acid distances (Spearman’s $r_{1295} = 0.09$, $P = 0.003$).

6.2.3 Chemical analysis of preen oil

To confirm previous findings that chemical similarity of preen oil reflects genetic
similarity at MHC class II (Slade et al. 2016), I separated and quantified the chemical
components of preen oil using gas chromatography with flame ionization detection (GC-
FID) following previously established methods (Slade et al. 2016). I diluted preen oil
samples in 1 – 3 mL of solvent (chloroform, CHCl$_3$; see Odour stimuli below for
collection details). Then, I injected 1 μL of each sample onto a 5% phenyl methyl siloxane column (Agilent Technologies DB-5, 30 m × 0.32 μm ID × 0.25 μm film thickness) on an Agilent 6890N instrument. Samples were injected at 70 ºC and held for 1 min, ramped to 130 ºC at 20 ºC/min, ramped to 320 ºC at 4 ºC/min, then held at 320 ºC for 10 min. I used hydrogen as a carrier gas at 2.5 mL/min. To ensure consistency between runs, I included a solvent blank and a sample of known composition previously analyzed by GC-FID and gas chromatography-mass spectrometry (GC-MS) in each batch of samples (Slade et al. 2016).

I quantified the relative size of each chromatogram peak based on its area relative to that of the individual’s full chromatogram, and retained only those peaks representing at least 0.1% of the total chromatogram area (Leclaire et al. 2012; Slade et al. 2016). Song sparrow preen oil is composed of a series of different chain length fatty alcohols and fatty acids esterified in different combinations to form monoesters (Grieves et al. 2019a, Chapter 4). In all, I noted 44 preen oil wax ester peaks (29.1 ± 0.2 peaks per individual), similar to previous reports on preen oil collected from free-living song sparrows during the breeding season (30 ± 0.5 peaks per individual; Slade et al. 2016). To prevent large peaks from disproportionately influencing distance measures, I normalized peak area using the decostand function in the R package vegan (Dixon and Palmer 2003).

6.2.4 Odour stimuli

I collected preen oil for odour stimuli from the same set of birds in which we tested odour preferences. On 18 March 2018 (after 24 days of photostimulation; two days before trials began), I applied gentle pressure to each bird’s uropygial gland to express 1 – 5 mg of preen oil into an unheparinized capillary tube, then snapped the tube into a glass vial. I dissolved samples in CHCl₃ (0.1 – 0.5 mL, scaled for the mass of oil collected) and stored at 4 ºC awaiting use in behavioural trials.

I did not test birds with preen oil from cage neighbours (i.e., housed in an adjacent home cage), or with oil from their previous social mate (inferred from capture locations).
I identified for each focal individual the opposite-sex individuals that were most similar and most dissimilar at MHC class II, based on mean unweighted amino acid distance. Within the constraints noted above (i.e., excluding cage neighbours and previous mates), I tested each bird with preen oil from the most similar and the most dissimilar opposite-sex individual (Table 6.1). The odour stimuli used in behavioural trials were collected from 19 individuals, selected as described above; each of these was used as a stimulus in 3.2 ± 0.6 (mean ± SE; range = 1 – 9) behavioural trials. Of the 19 stimulus individuals, ten were used at least once as the “similar” stimulus and at least once as the “dissimilar” stimulus (based on amino acid distance). Another eight were used as stimulus only once (four as “similar”, four as “dissimilar”), and one was used twice as “dissimilar” but never as “similar”.

I conducted a post hoc analysis of MHC functional distances for stimuli used in behavioural trials (Table 6.1). In 90% of trials (27 of 30), the “similar” stimulus based on amino acid distance was also the more similar of the two stimuli presented based on functional distance; in the remaining three trials, the “similar” stimulus based on amino acid distance was the less similar of the two stimuli presented based on functional distance.

I also conducted post hoc comparisons of MHC diversity for both stimulus birds used in each trial. First, I compared the number of MHC alleles for each stimulus bird (allelic diversity; Table 6.1). As a complementary measure of MHC diversity, I calculated Faith’s phylogenetic diversity index (Faith 1992) for each stimulus individual, based on unweighted UniFrac functional branch-lengths averaged over the ten MHC functional trees; this was conducted using the R package picante (Kembel et al. 2019) (functional phylogenetic diversity; Table 6.1). If focal birds were tested with preen oil from two individuals that were equally MHC-diverse (identical allelic or functional phylogenetic diversity), the trial was excluded from analysis of preferences for MHC diversity.
Table 6.1 A) MHC distances, calculated by mean unweighted UniFrac, between focal birds and stimulus birds, based on amino acid distance and functional distance at MHC. B) MHC diversity, based on allelic diversity and Faith’s phylogenetic diversity, of more-diverse vs less-diverse stimulus birds. Values reported as mean ± SE.

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<tr>
<td><strong>A) MHC distance</strong></td>
<td></td>
<td></td>
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<tr>
<td>Amino acid distance from focal</td>
<td>0.37 ± 0.01</td>
<td>0.65 ± 0.01</td>
</tr>
<tr>
<td>Functional distance from focal</td>
<td>0.43 ± 0.02</td>
<td>0.75 ± 0.02</td>
</tr>
<tr>
<td><strong>B) MHC diversity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allelic diversity of stimulus</td>
<td>14.1 ± 0.4</td>
<td>17.6 ± 0.8</td>
</tr>
<tr>
<td>Faith’s phylogenetic diversity of stimulus</td>
<td>15.6 ± 0.4</td>
<td>21.2 ± 0.4</td>
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</tbody>
</table>
6.2.5 Behavioural trials

I conducted two-choice behavioural trials in a Y-maze using a design similar to that of Whittaker et al. (2011). Each arm of the maze had dimensions 20 cm H × 40 cm L × 20 cm W, with a central area 35 cm L × 20 cm W and a wire screen placed on top of the maze so that birds could visually detect the ceiling and not fly into it. I placed perches near the end of each maze arm and placed each preen oil stimulus on a cotton ball taped into a dish at the end of each arm 8 cm in front of the perch. I used brown Kraft paper on the outer surface of each side wall to make the maze opaque. The maze was housed in an observation room such that each side of the maze was equidistant from the wall and the maze was positioned evenly between two overhead lights.

I used a vacuum pump (Neptune DynaPump, Thermoscientific) to pull air from the odour source (dissolved preen oil applied to cotton balls) down the arms of the maze while preventing mixing in the central area. This was achieved by connecting equal lengths of air tubing near the base of each arm (5.5 cm H × 9 cm from the central area) to the vacuum pump. As the vacuum pump produced noise, I acclimated the birds to the sound by running the pump in their holding room for 1 hr/d from 22 February 2018 to 1 March 2018.

I used a random number generator to determine the order in which focal birds were to be tested, tossed a coin to determine which maze arm would receive MHC-similar versus MHC-dissimilar preen oil for the first trial, then alternated stimulus location for subsequent trials. At the start of each testing day, I warmed preen oil samples to room temperature for a minimum of 5 min and transported the focal birds individually in opaque cloth bird bags to the test room. At the start of each trial I applied 50 µL of each stimulus sample (1 mg preen oil dissolved in 100 µL CHCl₃) to cotton balls affixed to the left and right arms of the maze.

Trials lasted 20 min and began with the focal bird being placed into a start chamber, separated from the rest of the maze by an opaque barrier, for a 5 min acclimation period. After this, the barrier was slid open then closed immediately after the bird entered the maze. Most birds entered the maze as soon as the barrier was opened,
and all birds entered within a few seconds. The next 5 min constituted the exploration period. For a successful trial, the focal bird was required to enter both maze arms, or to enter one arm and also orient towards the other arm (defined as standing within one body width of the arm with bill oriented toward that arm) for at least 10 sec during the exploration period. The final 10 min were the choice period. In the case of unsuccessful trials, I tested the focal bird one to four days later with the same stimuli, up to a maximum of two attempts. The maze was cleaned using 70% ethanol and allowed to air dry between each trial.

Overall, 22 of 27 males and 8 of 9 females completed a trial successfully. Of these, one male was not successfully genotyped, and one female could not be genotyped until after behavioural trials, preventing me from identifying appropriately similar and dissimilar stimuli with respect to amino acid distance (i.e., post-hoc genotyping of this female revealed that she had been tested with two samples of nearly identical amino acid dissimilarity). I thus excluded these two focal birds from the analysis of amino acid dissimilarity, resulting in data from 21 focal males and 7 focal females. For the post hoc analysis of functional dissimilarity, I used data from 21 males and 8 females (including the female that was genotyped post-hoc). For post-hoc analyses of allelic diversity, three of the 8 focal females were excluded because they had been tested with stimuli from two males with identical allelic diversity. Therefore, I used data from all 22 males that completed a successful trial, but only 5 of the 8 females. For post-hoc analyses of functional phylogenetic diversity, three of the 22 focal males were excluded because they had been tested with stimuli from two females with identical functional phylogenetic diversity. Therefore, I used data from all 8 focal females that completed a successful trial, but only 19 of the 22 males.

All trials were video recorded with an Activeon CX high-definition camera. For successful trials, I scored the time within the 10 min choice period that the focal bird spent in, or orienting towards, each arm of the maze. Trials were scored blind with respect to bird and stimulus identity.
6.2.6 Data analysis

All analyses were performed in R version 3.2.3 (R Development Core Team 2017). To validate that chemical similarity of preen oil reflects similarity at MHC as previously reported for free-living song sparrows (Slade et al. 2016), I calculated pairwise Aitchison distances for all opposite-sex dyads (27 males × 9 females) based on preen oil chemical composition. Aitchison distance is appropriate for compositional data, but to maintain comparability with previous work in this species (Slade et al. 2016), I also calculated chemical distance using Bray-Curtis dissimilarity (implemented in the R package vegan; Dixon and Palmer 2003). I compared the resultant pairwise matrices of chemical distances to the matrix of amino acid distances at MHC. Because numbers of males and females were unequal, the pairwise matrices were not square. Following previous work in this area (Leclaire et al. 2014; Slade et al. 2016), I thus ran a correlation test (perm.cor.test in the R package jmuOutlier; Garren 2018) using 10 000 permutations and Spearman’s r to assess correlations between chemical dissimilarity and genetic distance.

To investigate whether the chemical diversity of an individual’s preen oil might reflect its diversity at MHC, I used simple linear regression. Specifically, I tested whether allelic and/or functional phylogenetic diversity at MHC predicted preen oil chemical richness (number of chromatogram peaks), and/or Shannon’s diversity (calculated using diversity in vegan; Dixon and Palmer 2003).

I tested for differences in time spent with stimulus (odour) type by fitting restricted maximum likelihood (REML) linear mixed models using the R package lme4 (Bates et al. 2015). Fixed effects included sample type (e.g., MHC-dissimilar versus MHC-similar preen oil), sex of the focal bird, and their interaction; bird ID was included as a random effect; and the dependent variable was time spent in or approaching (as defined above) an arm. For all experiments, visual assessments of qq-plots and residuals confirmed that data and residuals were distributed approximately normally and the residuals showed no evidence of homoscedasticity. P-values were obtained using Wald tests (using the Anova function in the R package car).
6.3 Results

Pairwise chemical dissimilarity of preen oil (Aitchison distance) was positively correlated with genetic distance at MHC, albeit weakly (amino acid distance; correlation permutation test, Spearman’s $r_s = 0.13$, $P = 0.045$). Calculating chemical dissimilarity via Bray-Curtis yielded similar findings (Spearman’s $r_s = 0.13$, $P = 0.041$). Individual allelic diversity at MHC did not predict chemical richness ($r^2 = 0.01$, $P = 0.24$), or Shannon’s diversity ($r^2 = -0.01$, $P = 0.42$) of preen oil. However, phylogenetic diversity weakly but significantly predicted preen oil chemical richness ($r^2 = 0.09$, $P = 0.047$): individuals with higher phylogenetic diversity at MHC had fewer chromatogram peaks. A similar trend was observed for Shannon’s diversity of preen oil, but this relationship was not statistically significant ($r^2 = 0.07$, $P = 0.059$).

Song sparrows spent more time with preen oil from opposite-sex conspecifics that were MHC-dissimilar than with those that were MHC-similar, regardless of whether amino acid or functional similarity was considered (Table 6.2, Fig. 6.1). Song sparrows also spent more time with preen oil from opposite-sex conspecifics that were more MHC-diverse than with those that were less-diverse, for both allelic and functional phylogenetic measures of MHC diversity (Table 6.3, Fig. 6.2).
Table 6.2 Song sparrows in a two-choice Y-maze test spent more time with preen oil from MHC-dissimilar than from MHC-similar opposite-sex conspecifics in a two-choice Y-maze test. A) Amino acid distance (56 observations on 28 birds), B) Functional distance (58 observations on 29 birds).

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<thead>
<tr>
<th></th>
<th>Estimate</th>
<th>SE</th>
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<th>$\chi^2$</th>
<th>P</th>
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<tr>
<td><strong>A. Amino acid distance</strong></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td>Intercept</td>
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<td>63.2</td>
<td>3.55</td>
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<td>–</td>
</tr>
<tr>
<td>Sample type (similar)</td>
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<td>-0.74</td>
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<td>Sex of focal bird</td>
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<td>73.0</td>
<td>0.61</td>
<td>0.2</td>
<td>0.67</td>
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<td>Type × sex</td>
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<td>103.3</td>
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<td><strong>B. Functional distance</strong></td>
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<tr>
<td>Intercept</td>
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<td>22.8</td>
<td>3.93</td>
<td>–</td>
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<tr>
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<td>Type × sex</td>
<td>-126.3</td>
<td>92.7</td>
<td>-1.4</td>
<td>1.9</td>
<td>0.17</td>
</tr>
</tbody>
</table>

Parameters are estimated from a linear mixed model fit by REML; P-values are calculated from type II Wald chi square tests.
Figure 6.1 Time spent by song sparrows with preen oil from MHC-dissimilar, compared to MHC-similar, opposite-sex conspecifics. A) Amino acid distance, B) Functional distance. Total time scored was 10 min. Large symbols denote the mean (± SE). Small gray symbols are individual responses to each stimulus type.
Table 6.3 Song sparrows in a two-choice Y-maze test spent more time with preen oil from MHC-diverse than less-diverse opposite-sex conspecifics. A) Allelic diversity (54 observations on 27 birds), B) Faith’s phylogenetic diversity (54 observations on 27 birds).

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<tr>
<th></th>
<th>Estimate</th>
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<th>t</th>
<th>$\chi^2$</th>
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<td><strong>A. Allelic diversity</strong></td>
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<tr>
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<td>3.49</td>
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</tr>
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</table>

Parameters are estimated from a linear mixed model fit by REML; P-values are calculated from type II Wald chi square tests.
**Figure 6.2** Time spent by song sparrows with preen oil from more MHC-diverse, compared to less MHC-diverse, opposite-sex conspecifics. A) Amino acid allelic diversity, B) Faith’s phylogenetic diversity. Total time scored was 10 min. Large symbols denote the mean (± SE). Small gray symbols are individual responses to each stimulus type.
6.4 Discussion

Song sparrows spent more time with preen oil odour cues from opposite-sex conspecifics that were more dissimilar, and more diverse, at MHC. Despite the central role that studies of passerine birds have had in shaping sexual selection and signaling theory, the study of chemical communication in this taxon is still in its infancy. Nonrandom mating at MHC has been demonstrated in passerines (Bonneaud et al. 2006; Griggio et al. 2011; Winternitz et al. 2015), as it has in other vertebrates, but my findings provide the first evidence to suggest that passerines may use odour cues to achieve this result.

My findings confirm previous findings from wild-caught song sparrows that the chemical composition of preen oil corresponds to genetic similarity at MHC (Slade et al. 2016). Moreover, chemical richness of preen oil (although not chemical diversity) decreased with increasing individual phylogenetic diversity at MHC. I note that in both cases, the relationships observed were weak in magnitude. However, the apparent preferences I observed for odour cues of MHC-dissimilar and MHC-diverse conspecifics suggest that song sparrows are capable of detecting these cues. MHC diversity has been linked to microbial community structure on feathers and skin (Pearce et al. 2017; Leclaire et al. 2019), suggesting that effects on microbial communities within the uropygial gland are also possible. I speculate that individuals with greater MHC diversity may have reduced microbial diversity within the uropygial gland, potentially resulting in reduced chemical richness of preen oil if different microbes alter wax esters in different ways. Song sparrow preen oil consists of many different wax esters, a small subset of which appear to drive the relationship between MHC and preen oil similarity (Slade et al. 2016). Similarly, it remains to be determined whether the observed relationship between MHC diversity and chemical richness is driven primarily by a subset of alleles and/or of chemical compounds. Finally, I do not exclude the possibility that other components of preen oil (e.g., volatile compounds) may also reflect MHC diversity, perhaps more strongly than the whole wax esters analyzed here.

In song sparrows, as in most passerines, both sexes provide extensive care to offspring (Arcese et al. 2002). Thus, both sexes likely exercise some degree of mate choice, particularly when selecting social mates. Although mutual mate choice is likely to
be widespread in socially monogamous species, most experiments have focused on female choice for male ornaments (Fitzpatrick and Servedio 2018). Unlike many visual and acoustic ornaments, preen oil is produced by both sexes, and my findings suggest that both males and females attend to its associated odour cues. Indeed, song sparrows’ preen oil preferences aligned with predictions of both compatible-genes (i.e., dissimilarity) and direct-benefit (i.e., diversity) models of mate preference.

Importantly, in this study I investigated odour preferences (which I suggest reflect mating preferences): I did not investigate mate choice. Although preference functions are expected to influence mate choice in the wild, preference and choice may not correspond fully because the latter is typically constrained by competition, the costs of choosiness, and other factors (Zandberg et al. 2017). Supporting this idea, female song sparrows perform more copulation solicitation displays in response to males with larger song repertoires, suggesting that females in this species prefer larger repertoires (Searcy 1984). However, this preference does not translate to enhanced pairing success of males with large repertoires in the wild (Searcy 1984).

Relatively, although I interpret the observed preference for odour of MHC-dissimilar and MHC-diverse opposite-sex conspecifics as a mating preference, an alternative possibility is that these patterns represent social rather than mating preferences. However, song sparrows are not a particularly social species, especially during the breeding season when they actively exclude same-sex conspecifics from territories (Arcese et al. 2002). As well, as part of another experiment, the same study subjects spent more time with preen oil from opposite-sex than from same-sex conspecifics, and indeed showed no preferences for same-sex preen oil relative to the absence of preen oil (Grieves et al. 2019b, Chapter 5). Thus, I think it likely that our results reflect a mating preference, rather than a more general social preference, for MHC-dissimilar and MHC-diverse individuals.

Establishing that songbirds can perceive the cues of MHC dissimilarity and diversity present in preen oil represents a major advance to our understanding of MHC-mediated mating preferences in this taxon. Remaining to be discovered are the
mechanisms by which MHC genotype affects the chemical composition of preen oil. Thus, although odour-based assessment of MHC is taxonomically widespread, the mechanisms by which birds achieve such discrimination remains an open question.

6.5 References


Pearce DS, Hoover BA, Jennings S, Nevitt GA, Docherty KM. 2017. Morphological and genetic factors shape the microbiome of a seabird species (Oceanodroma leucorhoa) more than environmental and social factors. Microbiome. 5:146.


descriptors relevant for the design of biologically active peptides. A multivariate

Searcy WA. 1984. Song repertoire size and female preferences in song sparrows. Behav


Slade JWG, Watson MJ, Kelly TR, Gloor GB, Bernards MA, MacDougall-Shackleton
EA. 2016. Chemical composition of preen wax reflects major histocompatibility


Strandh M, Westerdahl H, Pontarp M, Canback B, Dubois M-P, Miquel C, Taberlet P,
Bonadonna F. 2012. Major histocompatibility complex class II compatibility, but
not class I, predicts mate choice in a bird with highly developed olfaction. Proc R

from multiple protein families using a maximum-likelihood approach. Mol Biol

Songbird chemosignals: volatile compounds in preen gland secretions vary among

Whittaker DJ, Richmond KM, Miller AK, Kiley R, Bergeon Burns C, Atwell JW,
Ketterson ED. 2011. Intraspecific preen oil odor preferences in dark-eyed juncos


Chapter 7

7 Preen gland microbiota of songbirds differ between sexes and populations and covary with MHC class II genotype

7.1 Introduction

Microbes (microscopic organisms including bacteria, protozoa, and fungi) are fundamentally and ubiquitously associated with plants and animals (Zilber-Rosenberg and Rosenberg 2008). While microbes such as bacteria are responsible for many diseases, the majority of symbiotic bacteria exist in mutually-beneficial relationships with their hosts. In vertebrates, symbiotic bacteria facilitate nutrient uptake, produce vitamins, promote development of the immune system, and even affect the behaviour of their vertebrate hosts (Archie and Theis 2011). Intriguingly, microbes can also affect the behaviour of non-hosts by mediating olfactory communication (Ezenwa et al. 2012; Carthey et al. 2018).

The fermentation hypothesis of chemical recognition posits that metabolic byproducts produced by symbiotic microbes affect the odour of their vertebrate hosts (Gorman 1976). In turn, differences among host individuals and groups in the community composition of these symbiotic microbes drive individual and group differences in odour. Thus, microbially-mediated odours may provide hosts with information on individual and group identity as well as kinship (Albone et al. 1974; Gorman 1976; Hepper 1987).

Symbiotic microbes can be transmitted through both the physical and social environment (i.e., via social interactions) (Archie and Theis 2011). Microbes acquired from the physical environment can be derived from anywhere in an animals’ home range (e.g., watering holes, hunting and foraging patches, dens, burrows, nests, and roost sites) and by diet (Carthey et al. 2018). Microbes can be acquired from the social environment by vertical transmission from parents to offspring and by horizontal transmission among conspecifics and even heterospecifics (Carthey et al. 2018). Many mammals engage in scent marking behaviour and, particularly in species with specialized scent glands such as
the anal glands of hyenas, bacteria inhabiting these glands are believed to contribute to host odour cues (Archie and Theis 2011). In social mammals, chemical signatures of group identity can arise through cross-infection due to cohabitation and frequent scent marking at shared locations (Albone et al. 1974).

In addition to these physical and social environmental sources of microbes, host microbial communities can be shaped by host genotype. The major histocompatibility complex (MHC) is a highly polymorphic family of genes in jawed vertebrates that encode receptors that recognize and bind to invading antigens (Janeway et al. 2001). While the mechanisms are not fully understood, MHC genes are believed to influence odour. MHC molecules and/or the antigens that bind to them may themselves be odorous (Hinz et al. 2013; Milinski et al. 2013). Given that MHC class II molecules are involved in immune defense against extracellular pathogens such as bacteria, an individual’s MHC class II genotype may influence host bacterial communities, shaping host odour indirectly (Penn 2002; Kubinak et al. 2015).

To date, most research on microbially-mediated olfactory signals in vertebrates has focused on mammals (Ezenwa and Williams 2014). In spotted hyenas (Crocuta crocuta), for example, the volatile profiles of anal gland secretions and gland-associated bacterial communities covary with sex and reproductive state (Theis et al. 2013). Further, anal gland bacterial communities are more similar in hyenas from the same social group compared to hyenas from different groups (Theis et al. 2012). Similarly, in meerkats (Suricata suricata), both the volatile profiles of anal pouch secretions and pouch-associated bacterial communities differ between sexes and among social groups (Leclaire et al. 2014a, 2017a). Moreover, similarity of volatile profiles predicts similarity of bacterial community composition in male meerkats (Leclaire et al. 2017a).

Evidence for a role of microbes in chemical communication has also been found in humans. Human armpit odours act as individual recognition cues and even convey information about kinship and genotype (reviewed in Havlicek and Roberts 2009; Archie and Theis 2011). Sebaceous secretions of the armpit are initially odourless, suggesting that human armpit odours are not synthesized de novo. Instead, the main components of
armpit odour are known products of bacterial metabolism (Archie and Theis 2011; Fredrich et al. 2013).

Although the study of chemical communication in birds has lagged behind that of mammals and other taxa, it is now clear that birds produce, detect, and respond to odours in the context of intraspecific communication. The major source of body odour in birds is preen oil, produced by the uropygial (preen) gland (Jacob 1978; Caro et al. 2015). The chemical composition of preen oil secretions varies among species (Soini et al. 2013), among individuals (Leclaire et al. 2011; Potier et al. 2018), among populations (Whittaker et al. 2010; Grieves et al. 2019a, Chapter 4), between the sexes (Whittaker et al. 2010; Grieves et al. 2019a, Chapter 4), between age classes (Shaw et al. 2011; Grieves et al. 2019a, Chapter 4), and with MHC class II genotype (Leclaire et al. 2014b; Slade et al. 2016a; Grieves et al. 2019b, Chapter 6). Furthermore, birds are capable of using these odour cues to discriminate species (Grieves et al. 2019c, Chapter 5), sexes (Whittaker et al. 2011a; Grieves et al. 2019c, Chapter 5), populations (Whittaker et al. 2011a; Van Huynh and Rice 2019), kin (Coffin et al. 2011), and the MHC class II genotype of potential mates (Leclaire et al. 2017b; Grieves et al. 2019b, Chapter 6).

Similar to mammalian scent glands, the uropygial gland provides a warm, moist environment that is rich in sebaceous secretions and thus potentially favourable to bacteria (Moreno-Rueda 2017; Maraci et al. 2018). Indeed, diverse bacterial communities have been documented in the preen gland (e.g., Whittaker and Theis 2016; Pearce et al. 2017; Leclaire et al. 2019), suggesting that chemical communication in birds may be microbiially-mediated, as it is in mammals. Preen gland-associated bacteria are capable of producing many of the volatile compounds associated with sex and population differences in dark-eyed junco (Junco hyemalis) preen oil (Whittaker and Theis 2016); however, no significant covariation was detected between preen oil volatiles and preen gland microbes in this species (Whittaker et al. 2016).

Social environment influences both preen oil odour and preen gland microbiota in juncos (Whittaker et al. 2016) but not in Leach’s storm petrels (Oceanodroma leucorhoa); there was no effect of nest burrow microbiota on preen gland microbiota, and
there were no differences in the microbiota of mates compared to non-mates (Pearce et al. 2017). However, in Leach’s storm petrels, preen gland microbiota significantly differ between the sexes. Moreover, in males, preen gland bacterial community structure differs between MHC-homozygous and MHC-heterozygous individuals (Pearce et al. 2017). Similarly, microbiota on feathers surrounding the preen gland and MHC genotype covary in blue petrels (*Halobaena caerulea*), such that individuals who are more similar at MHC class II also have more similar feather bacteria (Leclaire et al. 2019).

While the studies reviewed above are correlative, there is mounting experimental evidence for a causal relationship between olfactory cues and symbiotic microbes. In mice, the bacterially-derived chemosignal trimethylamine (TMA) is an attractant excreted in urine that is involved in social communication. Mice treated with antibiotics excrete about 90% less TMA, and the resultant TMA-depleted urine is less attractive to conspecifics (Li et al. 2013). In birds, most evidence for a causal relationship between odour cues and microbes comes from studies of the antimicrobial properties of preen oil. Green woodhoopoes (*Phoeniculus purpureus*) produce malodorous preen oil secretions believed to be involved in chemical defense. The preen gland bacteria *Enterococcus phoeniculicola* alter the colour, viscosity, and odour of woodhoopoe preen oil secretions. Injecting antibiotics into the preen gland kills preen gland bacteria and alters the chemical composition of green woodhoopoe preen oil (Law-Brown 2001). Volatile compounds in the preen oil of both green woodhoopoes and European hoopoes (*Upupa epops*) have antimicrobial effects, and injecting antibiotics into the preen gland alters preen oil composition in these species by obliterating most of the volatile compounds and antimicrobial properties that characterize the preen oil of untreated birds (Martín-Vivaldi et al. 2010).

Microbes can also alter social cues in birds. In dark-eyed juncos, specific volatile compounds have been established as chemical cues involved in social interactions, and bacteria in the phyla Actinobacteria, Firmicutes, and Proteobacteria produce these compounds (Whittaker et al. 2019). Moreover, volatile profiles in junco preen oil are associated with the relative abundances of specific bacteria inhabiting the preen gland (Whittaker et al. 2019). Finally, antibiotics injected into the preen gland alter both the
microbial composition of the preen gland and the chemical composition of preen oil volatiles (Whittaker et al. 2019).

Although there is growing evidence to suggest that microbes mediate chemical communication in birds, as has been found in mammals, most of our knowledge to date comes from just a few studies focused on a small number of bird species (i.e., dark-eyed juncos, Whittaker et al. 2019; Leach’s storm petrels, Pearce et al. 2017; and blue petrels, Leclaire et al. 2019). More work is needed to understand sources of variation in preen gland microbiota and, crucially, whether or not birds can detect and respond to changes in their symbiotic microbes and associated odour cues.

I sequenced a portion of the 16S rRNA gene to characterize the preen gland microbiota of song sparrows (*Melospiza melodia*), songbirds that are abundant and widespread across North America. I tested for population and sex differences in their preen gland microbiota. I also hypothesized that variation at MHC underlies some of the variation in preen gland bacterial communities, and that this in turn contributes to variation in preen oil composition. If supported, this sequence of events would provide a potential mechanism for how birds assess MHC similarity and diversity through olfactory cues (Leclaire et al. 2017b, Grieves et al. 2019b, Chapter 6). To explore this hypothesis, I tested for correlations between MHC class II genotype, preen gland microbiota, and preen oil chemical composition.

### 7.2 Methods

#### 7.2.1 Study subjects and sample collection

Our field team captured adult song sparrows using seed-baited Potter traps and mist nets at three breeding locations: on Western University property in London, Ontario, Canada (42.9849 N°, 81.2453° W; hereafter London), at the rare Charitable Research Reserve in Cambridge, Ontario, Canada (43.383°N, 80.357°W; hereafter Cambridge), and on land owned by the Queen’s University Biological Station near Newboro, Ontario, Canada (43.008°N, 81.291°W; hereafter Newboro). These locations are separated by 100 – 490
km, well beyond the mean range of juvenile dispersal for this species, which is estimated as about 6 km (Zink and Dittmann 1993). In total, 153 song sparrows were captured in 2017 (41 in London captured between 2 – 5 May and between 8 August – 1 September; 54 in Cambridge between 3 April – 1 May; and 55 in Newboro between 8 April – 3 May).

From each bird, we collected preen oil by gently probing the uropygial gland with an unheparinized capillary tube to express ~1 – 5 mg of oil into the tube. The preen gland was swabbed for bacteria immediately after preen oil collection to ensure collection of microbes from both inside and outside the gland. This was achieved by dipping a sterile medical grade cotton swab into sterile molecular grade water then firmly rubbing around the gland three times in each direction using a continuous motion: clockwise, counterclockwise, and up and down. Samples were kept on ice in the field and stored at -20 °C pending analysis. Each bird was handled using a fresh pair of nitrile gloves to minimize contamination. Due to overlapping timing of the field seasons at the three sites, each bird was captured, sampled, and swabbed by one of three different researchers on the team: I sampled in London (in both May and August) and in Cambridge in April and May; a second researcher sampled in London in August and in Newboro in April and May; and a third researcher sampled in Newboro in April and May.

We also collected a small blood sample (~ 20 µL) from each bird through brachial venipuncture for genetic analysis. Following the field season, I sexed all birds using the P2/P8 PCR protocol described by Griffiths et al. (1998). After collecting preen oil, preen gland swabs, and blood, each bird was banded to ensure individuals were only sampled once then released at the site of capture.

7.2.2 16S genetic analysis

I extracted bacterial DNA from swabs using Qiagen DNeasy PowerSoil DNA isolation kits with some modifications to the manufacturer’s recommended protocol (see Appendix D for detailed modified extraction protocol). Extractions were carried out in 14 batches,
each consisting of 23 samples plus a swab-only negative control (this count includes additional samples that were part of a separate study). Samples were chosen haphazardly from among the three sampling locations such that roughly equal numbers of samples were extracted from each location in each batch. After completing all extractions, I used a Qubit Fluorometer to measure the DNA concentration of 14 samples (1 sample selected haphazardly from each of the 14 batches). To screen for background DNA contamination, I also carried out DNA extractions from fresh swabs dipped in the sterile water I used in the field (N = 4).

I amplified the V4 region of the 16S rRNA gene using the universal primers F518 (Lane et al. 1985) and R806 (Caporaso et al. 2011). Each primer included an Illumina MiSeq adaptor, four wobble bases, and an individually-unique barcode of eight bases. I performed PCR in a total volume of 25 µL, including 10 µL of 5PRIME HotMasterMix (Quantabio), 0.2 µM of each primer, and 2 µL of template (\( \bar{x} \) concentration = 0.1 ng/µL, range = 0.01 – 0.12 ng/µL). The thermocycling profile consisted of 2 min at 94 °C; 35 cycles of 45 s at 94 °C, 60 s at 50 °C, and 90 s at 72 °C; and a 10 min final extension at 72 °C.

I confirmed amplification by running samples on a 2% agarose gel. 11 of the 18 water and swab-only negative controls showed a band of the expected product size (~ 300 bp) of comparable or weaker intensity to my samples, indicating some source of contamination in the PCR product. I sequenced contaminated controls along with the target samples so I could subtract likely contaminant sequences from subsequent processing stages (see below).

I pooled PCR products into a library and sequenced with 250 bp paired-end reads on an Illumina MiSeq at the London Regional Genomics Centre. I used a pipeline (Gloor et al. 2010) to collapse sequences into clusters of identical reads and assign sequences to individuals. I used a second pipeline (Bian et al. 2017) and the R package dada2 (Callahan et al. 2016) to overlap reads, remove ambiguous reads, and screen for chimeras. Singleton (i.e., sequences that appeared only once in the dataset) sequence variants (SVs) and SVs rarer than 0.1% in any sample were excluded, resulting in an
initial dataset containing 5243 SVs from across 205 samples. Following this, taxonomic assignments were made by clustering at ≥ 97% sequence identity (following Gloor et al. 2010) using the naïve Bayesian Ribosomal Database Project (RDP) Classifier (Wang et al. 2007; Callahan et al. 2016).

High throughput sequencing data are relative abundance data and are thus compositional (Gloor and Reid 2016; Gloor et al. 2017; Quinn et al. 2018; Quinn and Erb 2019). Therefore, I used a compositional data (CoDa) analysis approach (Pincus and Aitchison 1986) that examines the ratios between SVs. Most data sets do not actually contain all possible components; often, small values, including values below an instrument’s detection limit (e.g., the Illumina MiSeq and GC equipment used in this study), are rounded off to zero. However, such zero counts are assumed to be due to limited sampling or to equipment limitations (Palarea-Albaladejo and Martin-Fernandez 2015). So, following Bian et al. (2017), I used Bayesian-multiplicative replacement to impute values for zero count SVs using the R package zCompositions (Palarea-Albaladejo and Martin-Fernandez 2015). I then applied a centred log-ratio transformation to the zero replaced data set, which renders the use of Euclidean distances meaningful in subsequent analyses (Gloor and Reid 2016; Bian et al. 2017; Quinn et al. 2018).

Next, I filtered sequences by the minimum proportion, minimum occurrence, and minimum sample count of reads. Thus, sequences found in fewer than 0.5% of reads, sequences found in fewer than 10% of samples, and sequences with fewer than 5000 reads were removed from the initial dataset. Then, I conducted a principal components analysis (PCA) of the centred log-ratio transformed data using zero centered, rotated variables and the prcomp function in base R. This allowed me to visually assess and remove any remaining sequences that were likely due to contamination. To do this, I plotted principal components 1 and 2 (PC1, PC2) and identified SVs that were associated specifically with the contaminated control samples (N = 6 putative contaminant SVs, Appendix D, Table D1). Next, I plotted all possible pairwise combinations of the 11 contaminated controls against each other, to double check that these 6 SVs were shared among controls (i.e., SVs that fell on or near the 1:1 line of each biplot were considered
likely to be contaminants). All 6 SVs were indeed shared among the contaminated control samples, so they were removed from the dataset.

As noted above, multiple researchers collected swabs, and swabbing technique or contamination with researcher-specific microbiota could cause samples to cluster artificially by location, given that different researchers sampled in different study sites. Accordingly, I also checked the PCA plot to ensure that samples were not clustering by researcher. I found no evidence that London samples I collected clustered with Cambridge samples that I collected, or that London samples collected by the second researcher clustered with Newboro samples collected by that researcher. These patterns suggest that researcher identity was not an issue in this dataset.

### 7.2.3 MHC genetic analysis

Due to resource constraints, I only sequenced MHC for a subset of birds (N = 31) captured from London (N = 19) and Cambridge (N = 12) for which I also had 16S data. Detailed MHC sequencing methods are described elsewhere (Grieves et al. 2019b, Chapter 6). Briefly, I amplified the hypervariable second exon of MHC class II (~ 350 bp) using primers SospMHCint1f (Slade et al. 2016a) and Int2r.1 (Edwards et al. 1998), which bind within introns 1 and 2 respectively and amplifies all of exon 2. Each primer included an Illumina MiSeq adaptor, four wobble bases, and an individually-unique barcode of eight bases. I performed PCR in a total volume of 35 µL and each reaction included 12.5 µL of GoTaq® Hot Start Green Master Mix (Promega), 0.2 µM of each primer, and 60 ng of genomic DNA. The thermocycling profile consisted of 3 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 62 °C, and 45 s at 72 °C; and a 10 min final extension at 72 °C. I confirmed amplification by electrophoresis on a 2% agarose gel.

I pooled PCR products into a library and sequenced with 300 bp paired-end reads on an Illumina MiSeq at the London Regional Genomics Centre. I used a pipeline (Gloor et al. 2010) to collapse sequences into clusters of identical reads and assign sequences to
individuals. Following Grieves et al. (2019b), I retained sequences comprising at least 1% of an individual’s reads (mean ± SE retained reads per individual = 20 736 ± 1939).

Using previously described information in this system (Slade et al. 2016a; Grieves et al. 2019b) (i.e., our lab’s database of song sparrow MHC class II exon 2 alleles), I assigned each retained sequence to its corresponding protein sequence. I then applied zero count multiplicative and centred log-ratio transformations to the data to allow comparison to the 16S dataset. In some cases, different DNA sequence reads translated to the same amino acid sequence. For these, I calculated the average log-ratio value so that only unique protein sequences were included in further analysis. Finally, I removed any putative pseudogenes, nonfunctional DNA segments that resemble functional genes, as identified in Slade et al. (2016a).

### 7.2.4 Preen oil chemical analysis

I dissolved preen oil samples (N = 153) in 1 – 3 mL chloroform (CHCl₃; scaled for the volume of preen oil collected for a final concentration of 1 mg preen oil/mL CHCl₃) and analyzed them using an Agilent 7890A gas chromatograph with flame ionization detector (GC-FID), fitted with a 5% phenyl methyl siloxane column (Agilent Technologies DB-5, 30 m × 0.32 μm ID × 0.25 μm film thickness). I used hydrogen as a carrier gas at 2.5 mL/min, injected 1 μL of sample with a 30 psi pressure pulse for 1 min and, after an initial 1 min hold at 70 ºC, eluted with this temperature profile: increase to 130 ºC at 20 ºC/min, then to 320 ºC at 4 ºC/min. The injector and FID temperatures were 200 ºC and 310 ºC, respectively. Each batch of GC-FID runs (typically 20 – 24) included a blank sample containing solvent only (CHCl₃) and a sample of known composition (i.e., previously analyzed with both GC-FID and GC-MS; Slade et al. 2016a). Because the volume of preen oil collected varied across individuals, I quantified peak sizes based on the proportional peak size relative to total chromatogram peak area. Then, I applied zero count multiplicative and centred log-ratio transformations to these proportional data, to maintain comparability with the 16S and MHC genetic datasets.
7.2.5 Data analysis

All statistical analyses were performed in R version 3.3.3 (R Development Core Team 2017). I used the centred log-ratio data to construct Euclidean distance matrices for each data set (16S, MHC, preen oil). Distances were calculated between all available pairwise dyads. Of the 153 song sparrows I sequenced at the V4 region of the 16S rRNA bacterial gene, I retained usable data from 61 birds. Of these 61 birds, I had preen oil chemical data for 60 and MHC class II genotype data for 31. Thus, to assess correlations between preen gland microbial genetic distance and preen oil chemical distance, I used 60 birds for which I had both 16S sequencing data and preen oil GC-FID data (60 × 60 matrix, 3600 pairwise combinations). To assess correlations between microbial genetic distance and MHC class II genetic distance, I used data from 31 birds for which I had both 16S and MHC genetic sequencing data (31 × 31 matrix, 961 pairwise combinations). To assess correlations between MHC genetic distance and preen oil chemical distance, I used data from the same 31 birds that were genotyped at MHC class II (31 × 31 matrix, 961 pairwise combinations).

To assess the statistical significance of differences in preen gland microbial community composition among populations and between the sexes, I conducted permutational multivariate analysis of variance on the pairwise Euclidian distance matrices using the adonis command in the vegan package (Dixon and Palmer 2003). This permutation-based approach is analogous to a nonparametric MANOVA, does not make assumptions about the data’s distribution, and may be less sensitive to group differences in the dispersion of points compared to other methods (Anderson 2001; Anderson and Walsh 2013). To visualize pairwise microbial distances between samples, I conducted a PCA of the centred log-ratio transformed data using zero centered, rotated variables and the prcomp function in base R. To further explore population and sex differences, I conducted three two-way ANOVAs using population and sex as the predictor variables and the PCA factor scores from each of PC1, PC2, and PC3 as the dependent variables. Visual assessments of qq-plots and residuals indicated that data and residuals were distributed approximately normally and the residuals showed no evidence of
homoscedasticity. Finally, as a preliminary exploration of whether differences in MHC allelic diversity and preen oil chemical diversity may partially explain population or sex differences in preen gland microbial communities, I conducted unpaired t-tests to compare populations (London versus Cambridge) and sexes in their MHC genetic diversity (number of MHC amino acid alleles per individual) and chemical diversity (number of preen oil peaks per individual).

Using the pairwise Euclidean distances calculated for all song sparrow dyads based on preen gland microbial community composition, MHC amino acid distance, and preen oil chemical composition, I compared the resultant pairwise distance matrices in three separate tests. I ran Mantel tests in the R package vegan (Dixon and Palmer 2003) with 10,000 permutations to assess correlations (Spearman’s $r$) between 1) MHC amino acid distance and preen gland microbial distance, 2) preen gland microbial distance and preen oil chemical distance, and 3) MHC amino acid distance and preen oil chemical distance.

7.2.6 Data accessibility

Pipelines used for next generation sequencing data processing can be found at: github.com/ggloor/miseq_bin/blob/dada2/Illumina_SOP.pdf; github.com/ggloor/miseq_bin

7.3 Results

After all data filtering and removal steps were completed, I retained data from 49 SVs and 61 birds (London: 29 [10 females, 19 males]; Cambridge: 13 [4 females, 9 males], Newboro: 19 [7 females, 12 males], mean ± SE retained reads per individual = 8540 ± 1552). Across the 31 birds genotyped at MHC class II, I detected 151 unique amino acid alleles (mean ± SE amino acid alleles per individual = 16.23 ± 0.61). For these same birds, I detected 72 unique preen oil peaks.
7.3.1 Preen gland microbiota

The 49 preen gland microbial SVs I identified in this study were assigned to six phyla: Actinobacteria (class Actinobacteria), Bacteroidetes (class Flavobacteria), Cyanobacteria (class Chloroplast), Firmicutes (classes Bacilli and Clostridia), Gemmatimonadetes (class Gemmatimonadetes), and Proteobacteria (classes Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria). Three SVs assigned to the Cyanobacteria were identified as chloroplasts and likely resulted from natural environmental contamination, as song sparrows are predominantly ground foragers (Arcese et al. 2002). These SVs likely reflect plant material or plant residue on the birds that was picked up during swabbing and subsequently amplified, rather than photosynthetic microbes living symbiotically within the preen gland. However, I took a conservative approach and retained these SVs for further analysis as they nevertheless represented a valid taxonomic group sampled from the preen gland of song sparrows. Of the remaining 46 SVs, 5 could not be assigned below the level of family. The remaining 41 SVs were assigned to 30 different genera. Of these 30 genera, 19 (63%) have previously been identified in birds by other studies that sampled feathers of the body or rump, feathers around the preen gland, or the preen gland itself (Appendix D, Table D2).

7.3.2 Population and sex differences in preen gland microbiota

The preen gland microbiota of song sparrows differed significantly among populations and between the sexes (Table 7.1; Fig. 7.1). Based on visual analysis of the PCA scree plot, I retained the first three principal components, which accounted for 20.1%, 9.1%, and 7.5% of the variance respectively. The rotated component matrix is shown in Table 7.2. PC1 was positively associated with several families in the bacterial phylum Firmicutes and also negatively associated with a single family in the Firmicutes (Table 7.2). PC2 was positively associated with the phyla Cyanobacteria and Proteobacteria and negatively associated with Firmicutes and Proteobacteria (Table 7.2). PC3 was positively associated with the phyla Gemmatimonadetes and Proteobacteria and negatively associated with Cyanobacteria, Firmicutes, and Proteobacteria (Table 7.2).
The SVs most strongly associated with PC1 and PC2 were also broadly associated with population differences in the preen gland microbiota of free-living adult song sparrows. Birds in the London population had a higher relative representation of Sphingomonadaceae (SV_17), Comamonadaceae (SV_23), Enterobacteriaceae (SV_31), Lachnospiraceae (SV_36) and Methylobacteriaceae (SV_6; lower portion of Fig. 7.1). Birds in the Cambridge population had a higher relative representation of Enterococcaceae (SV_11), Clostridiaceae (SV_18, SV_20), Family XI (class Clostridiales; SV_14, SV_24), and Bacillaceae (SV_38; upper right portion of Fig. 7.1), and birds in the Newboro population had a higher relative representation of Bacillaceae (SV_45), Staphylococcaceae (SV_29), Pseudomonadaceae (SV_16), and chloroplast DNA (SV_9; upper left portion of Fig. 7.1). I found a significant difference between populations based on factor scores from PC2, but not from PC1 or PC3 (Table 7.3).

In contrast, there was no clear separation of the sexes based on the SVs associated with population differences. The sexes were relatively evenly clustered in the Cambridge and Newboro populations, but females tended to separate from males along PC1 in the London population (more males than females in the lower left portion of Fig 7.1). Overall, males were dispersed relatively evenly throughout the plot, while females clustered weakly in the positive half of PC1 (Fig. 7.1). I found no significant differences between the sexes based on factor scores from PCs 1, 2, or 3 (Table 7.3).

I did not have MHC genotypic data for Newboro birds, but relative to London birds, Cambridge birds had, on average, more MHC amino acid alleles (mean ± SE; London: 14.95 ± 0.73; Cambridge: 18.25 ± 0.78; unpaired t_{1,29} = 2.95, P = 0.006; Appendix D, Figure D1A) and more preen oil peaks (London: 21.11 ± 0.59; Cambridge: 30.00 ± 1.66; unpaired t_{1,29} = 5.92, P < 0.0001; Appendix D, Figure D1B). Males and females did not differ in the mean number of MHC amino acid alleles (mean ± SE; females: 15.83 ± 0.77; males: 16.47 ± 0.88; unpaired t_{1,29} = 0.50, P = 0.618) or preen oil peaks (females: 21.83 ± 1.08; males: 25.58 ± 1.51; unpaired t_{1,29} = 1.80, P = 0.083) between the sexes.
7.3.3 Preen gland microbiota, MHC genotype, and preen oil chemical composition

Pairwise preen gland microbial distance was significantly positively correlated with MHC genetic distance (Mantel test, Spearman’s $r_{465} = 0.23$, $P = 0.011$; Fig. 7.2A) but not with preen oil chemical distance (Mantel test, Spearman’s $r_{1770} = 0.08$, $P = 0.057$; Fig. 7.2B). MHC genetic distance was significantly positively correlated with preen oil chemical distance (Mantel test, Spearman’s $r_{465} = 0.38$, $P < 0.0001$; Fig. 7.2C), as has been previously reported in both free-living and captive song sparrows (Slade et al. 2016a; Grieves et al. 2019b).
Table 7.1 Results of permutational multivariate analysis of variance using Euclidean distance matrices to test for differences in preen gland microbial community composition among populations and between the sexes in free-living adult song sparrows.

<table>
<thead>
<tr>
<th>Group</th>
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<th>Mean sum of squares</th>
<th>F</th>
<th>R²</th>
<th>P</th>
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</thead>
<tbody>
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<td>745.6</td>
<td>2.71</td>
<td>0.08</td>
<td>&lt; 0.001</td>
</tr>
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<td>Sex</td>
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<td>484.1</td>
<td>1.76</td>
<td>0.03</td>
<td>0.034</td>
</tr>
<tr>
<td>Population × Sex</td>
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<td>258.8</td>
<td>0.94</td>
<td>0.03</td>
<td>0.546</td>
</tr>
<tr>
<td>Residuals</td>
<td>55</td>
<td>15149.8</td>
<td>275.5</td>
<td>—</td>
<td>0.86</td>
<td>—</td>
</tr>
</tbody>
</table>
Figure 7.1 PC1 and PC2 scores derived from preen gland microbe sequence variant (SV) relative abundances in free-living adult song sparrows from three populations. Arrows indicate loadings based on SV relative abundances that were most strongly associated with PC1 and PC2 (see Table 7.2 for loadings). PCA was based on Euclidean distances calculated from centred log-ratio transformed 16S read abundance data.
Table 7.2: Eigenvalues, percentage of variance explained, and rotated component matrix for the first three principal components extracted from PCA analysis of preen gland microbial community data. Taxonomic assignment based on the Bayesian Ribosomal Database Project for each sequence variant (SV) is shown at the level of phylum and family. Bold text indicates SVs strongly associated with each principal component. For complete taxonomic information see Appendix D, Table D2.

<table>
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<tr>
<th></th>
<th>PC1</th>
<th>PC2</th>
<th>PC3</th>
<th>Phylum</th>
<th>Family</th>
</tr>
</thead>
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<td>20.2</td>
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<td>-0.133</td>
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<td><strong>Bacillaceae</strong></td>
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<td><strong>Bacillaceae</strong></td>
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<td>Clostridiales, family XI</td>
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<td>Lachnospiraceae</td>
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<td>Lachnospiraceae</td>
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<td>-0.017</td>
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</table>
Table 7.3 Results of analysis of variance tests using factor scores from the first three principal components of PCA to test for differences in preen gland microbial community composition among populations and between the sexes in free-living adult song sparrows. PC1 was associated with Firmicutes. PC2 was associated with Cyanobacteria, Proteobacteria, and Firmicutes. PC3 was associated with Gemmatimonadetes, Proteobacteria, Cyanobacteria, and Firmicutes (see Table 7.2 for details).

<table>
<thead>
<tr>
<th>Group</th>
<th>df</th>
<th>Sum of squares</th>
<th>Mean sum of squares</th>
<th>F</th>
<th>P</th>
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<tr>
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<td>2885.6</td>
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<td>—</td>
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<td><strong>PC2</strong></td>
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<td>Population</td>
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<td>Residuals</td>
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<td>11.4</td>
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<td>70.3</td>
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Figure 7.2  A) Preen gland microbial distance is significantly positively correlated with MHC class II genetic distance, but B) not with preen oil chemical distance. C) Preen oil chemical distance is significantly positively correlated with MHC class II genetic distance. Distances were calculated from all pairwise dyads: A) N = 31 birds (465 pairwise combinations), B) N = 60 birds (1770 pairwise combinations), C) N = 31 birds (465 pairwise combinations). Solid lines show least-squares regression. Note: the x-axis scale in B differs from that in A and C.
7.4 Discussion

7.4.1 Preen gland microbiota

The preen gland of song sparrows contains diverse microbial communities. The 49 sequence variants I detected were distributed among six bacterial phyla and comprised at least 30 different genera (not all sequence variants were classified to the genus level). Of these 30 genera, about two-thirds (63%) have been previously identified in and around the preen gland or on body and wing feathers of bird species spanning six phylogenetic orders (Anseriformes, Braun et al. 2018; Galliformes, Shawkey et al. 2006; Procellariformes, Pearce et al. 2017; Bucerotiformes, Martín-Platero et al. 2006; Charadriiformes, Shawkey et al. 2006; Passeriformes, Whittaker and Theis 2016; Appendix D, Table D2).

Preen oil serves multiple functions including waterproofing, feather maintenance, thermoregulation (Jacob and Ziswiler 1982; Salibian and Montalti 2009), parasite and pathogen defense (Martín-Platero et al. 2006; Martín-Vivaldi et al. 2010), and chemical communication (Bonadonna et al. 2007; Whittaker et al. 2011a; Grieves et al. 2019a, b, c). Symbiotic bacteria inhabiting the preen gland may contribute to all of these functions. While there are host-specific bacteria (e.g., in green woodhoopoes; Law-Brown and Meyers 2003) as well as bacteria associated with specific environments (e.g., the ocean-associated bacteria found on Leach’s storm petrels; Pearce et al. 2017), the overlap in shared bacterial genera across taxonomically diverse avian hosts suggests that there is some underlying consistency in the symbiotic microbial communities of birds. This overlap may be related to the shared functions of preen oil across bird species, but more work is required to disentangle the relationships between preen oil and preen gland microbes, and the role of microbes in avian ecology and behaviour.

7.4.1.1 Potentially novel preen gland microbiota

To the best of my knowledge, about one-third of the genera I detected in the preen gland of song sparrows have not been previously reported in birds’ preen glands or on their
feathers. These genera are primarily associated with either soil and plants or the vertebrate digestive tract. *Actinomycetospora* was proposed as a new genus in 2008 (Jiang et al. 2008), and the first species assigned to this genus was isolated from tropical rainforest soil in northern Thailand (Jiang et al. 2008). Since then, new species have been identified in lichens (Yamamura et al. 2011) and in association with the roots of various plant species (e.g., He et al. 2015; Kaewkla et al. 2019). The genus *Tardiphaga* is associated with root nodules of black locust (*Robinia pseudoacacia*; De Meyer et al. 2012) and the legume *Vavilovia formosa* (Safronova et al. 2015). The genus *Neorhizobium* was proposed in 2014 (Mousavi et al. 2014) and is also associated with legumes, being involved in nitrogen fixation (Österman et al. 2015). The genus *Dyella* contains species found in soil and associated with the rhizosphere of several plant species (Weon et al. 2009; Anandham et al. 2011). Species in the genera *Xylophilus* and *Pantoea* are primarily plant pathogens (Dreo et al. 2007; Cruz et al. 2007); however, some *Pantoea* species can also cause disease in humans (Cruz et al. 2007), and some species are found in the gut of wild birds (Davidson et al. 2019). With the exception of *Pantoea*, which may occur naturally in preen oil or be a result of cross contamination (e.g., with gut bacteria excreted into the cloaca then subsequently collected on preen gland swabs), these genera were likely picked up by song sparrows from their environment, much like the chloroplast DNA I extracted from preen gland swabs.

The genus *Eubacterium* is commensal in the vertebrate gut (Razzauti et al. 2015). In humans, various species inhabit the oral cavity (Zhou and Li 2015) and the intestinal tract (Actor 2012), and are opportunistic pathogens in the female genital tract (Mandell et al. 2015). Anaerobic *Eubacterium* sp. inhabit the avian caecum, producing lactic acid, succinic acid, acetic acid, and ethanol from glucose (Barnes and Impey 1974). Species in the *Anaerosphaera* genus are anaerobic and have been isolated from animal waste reactors. These species are aminolytic, fermenting amino acids into volatile fatty acids (Ueki et al. 2009). Another anaerobic genus, *Oscillibacter*, contains species that have been identified in both invertebrates (e.g., in the alimentary canal of corbicula clams, *Corbicula japonica*; Iino et al. 2007) and vertebrates (e.g., in the rumen of Korean native cattle, *Bos taurus coreanae*; Lee et al. 2012). Species in the genus *Oscillibacter* produce pentanoic acid, also called valeric acid, which is a low molecular weight straight-chain
carboxylic acid that produces a strong odour. Volatile esters of valeric acid are often used in perfumes and cosmetics (PubChem database 2020). *Eubacterium, Anaerospaera,* and *Oscillibacter* are thus genera that may be involved in producing volatile chemical cues that could be involved in song sparrow chemical communication. Culturing these bacteria and comparing the volatiles they produce to those found in song sparrow preen oil, as well as culturing these bacteria using preen oil as a substrate, would help to determine whether this is the case.

### 7.4.1.2 Previously reported preen gland microbiota

With respect to microbially-mediated chemical communication, bacteria from the family *Pseudomonadaceae* have been identified in the preen gland secretions and/or on feathers of several passerine species, including dark-eyed juncos (Whittaker and Theis 2016), house finches (*Carpodacus mexicanus*; Shawkey et al. 2003), eastern bluebirds (*Sialis sialis*; Shawkey et al. 2005), and song sparrows (this study). *Pseudomonadaceae* contains species that are known odour producers (Lemfack et al. 2018), and bacteria from this family produce several volatile compounds found in junco preen oil that are involved in intraspecific chemical communication (Whittaker et al. 2019).

Bacteria from the family *Burkholderiaceae* have also been identified in the preen gland secretions of juncos, particularly the odour producing genus *Burkholderia* sp. While I did not find this genus in song sparrows, I detected one genus from the family *Burkholderiaceae: Ralstonia*. Species in this genus also produce volatile compounds (Spraker et al. 2014; Lemfack et al. 2018) and at least one species uses volatile fatty acids as a substrate (Chakraborty et al. 2009), but whether this genus influences avian chemical communication remains to be determined. Other odour producing genera reported in both juncos (Whittaker et al. 2019) and song sparrows (this study) include *Bacillus, Staphylococcus,* and *Lactococcus.* Together, these observations suggest that the fermentation hypothesis of chemical recognition, originally developed for mammals, extends to birds as well, but more experimental studies are needed to test this hypothesis.
7.4.2 Population and sex differences in preen gland microbiota

I found significant population and sex differences in the preen gland microbial communities of adult song sparrows. The microbial communities of London birds were distinguishable from those of Cambridge and Newboro primarily along axis PC2 (with London birds having higher relative representation of Sphingomonadaceae, Comamonadaceae, Enterobacteriaceae, Lachnospiraceae, and Methylobacteriaceae), whereas the microbial communities of Cambridge and Newboro birds were distinguishable from one another primarily along axis PC1 (with Cambridge birds having a higher representation of Enterococccaeae, Clostridiaceae, Family XI in the class Clostridiales, and Bacillaceae, and Newboro birds having a higher representation of Bacillaceae, Staphyloccocccaeae, Pseudomonadaceae, and chloroplast DNA). Thus, these population differences appear to be driven by differences in the ratios of certain subsets of microbes, which may be a result of environmental and/or genetic differences among populations.

The physical and social environment influences microbial profiles in spotted hyenas (Theis et al. 2012), meerkats (Leclaire et al. 2014a), European hoopoes (Martínez-García et al. 2016), blue petrels (Leclaire et al. 2019), and dark-eyed juncos (Whittaker et al. 2016), but not Leach’s storm petrels (Pearce et al. 2017). In meerkats, members of the same social group have more similar anal pouch microbiota. Meerkats breed cooperatively, sharing burrows and engaging in allogrooming, allogrooming, and babysitting behaviours that likely increase microbial transmission (Leclaire et al. 2014a), suggesting that social environment plays an important role in shaping host microbiota in this species. In dark-eyed juncos, the cloacal and preen gland microbiota of nestlings are more similar to their mother than to other adult females in the population, and genetic relatedness among nestlings does not influence the similarity of their microbiota (Whittaker et al. 2016). These results suggest that, in juncos, nestling microbiota is shaped by both the physical environment (the nest) and the social environment (mother and nest mates).

Population differences in preen gland microbiota may also be explained in part by population differences in MHC genotype. I found significant differences in the mean
number of MHC class II alleles between London and Cambridge birds, with Cambridge birds having higher allelic diversity (more MHC class II alleles) than London birds. It should be noted that a prior study of song sparrows from the London and Newboro populations did not find evidence of population differences in allele frequencies at MHC class II (Slade et al. 2016b). However, that study did not address population differences in allelic diversity. Based on my exploratory analysis of MHC allelic differences between populations, investigating whether and how genetic differences within and between populations explain differences in host microbiota should be a promising area for future research. I also found significant differences in the mean number of preen oil chemical peaks between London and Cambridge birds, with Cambridge birds having higher preen oil chemical diversity (more preen oil peaks). Population differences in preen gland microbiota could potentially be explained by differences in preen oil chemical diversity if gland-associated microbes feed on preen oil substrates that differ among populations. This could be explored by culturing preen gland microbes from different populations on ‘home’ and ‘away’ preen oil and looking for differential growth and abundance of microbes based on preen oil origin.

The relative abundances of *Methylobacterium*, *Sphingomonas*, *Xylophilus*, *Pantoea*, and *Lachnoclostridium* were higher in London birds, while Newboro birds had greater relative abundances of *Pseudomonas*, *Bacillus*, and *Staphylococcus*, and Cambridge birds tended to have more *Bacillus*, *Enterococcus*, *Clostridium*, and *Anaeropshaera*. As discussed previously, *Pseudomonas*, *Bacillus*, and *Staphylococcus* spp. are known odour producers involved in microbially-mediated chemical communication in closely related dark-eyed juncos (Whittaker and Theis 2016; Whittaker et al. 2019). *Pseudomonas* and *Enterococcus* may also have antimicrobial properties (Fernando et al. 2005; Haas and Défago 2005; Soler et al. 2008), whereas at least some *Bacillus* species are feather-degrading bacteria (Reneerkens et al. 2008; Soler et al. 2008). *Staphylococcus* and *Clostridium* contain pathogenic strains (Dworkin 2006), and these gut pathogens can affect both domesticated (Lowder and Fitzgerald 2010; Hafez 2011) and wild (Harry 1967; Brittingham et al. 1988; Mitscherlich and Marth 2012) birds. *Pantoea* are primarily plant pathogens (Falkow et al. 2006), and thus may have been acquired by song sparrows from their environment; however, they are also found as
gut bacteria (Davidson et al. 2019), as are many of the other bacterial genera that have been found in the preen gland (Waite and Taylor 2014; Hird et al. 2015; Waite and Taylor 2015; Davidson et al. 2019). However, among the avian host species that have been studied to date, cloacal (i.e., partially gut-derived) and preen gland-associated bacterial communities differ overall (Whittaker et al. 2016; Leclaire et al. 2019).

Although I found no evidence for sex differences in the mean number of MHC class II alleles or preen oil chemical peaks, I found subtle, albeit statistically significant, sex differences in song sparrows’ preen gland microbiota. However, I was unable to identify specific sequence variants to which these sex differences are primarily attributable. Sex differences in host microbiota have been documented in both mammals (Theis et al. 2013; Leclaire et al. 2014a) and birds (Pearce et al. 2017; Leclaire et al. 2019, but see Whittaker et al. 2016), suggesting that sex differences in microbiota are common across taxa. Sex differences in preen gland microbiota may be due to physiological differences between males and females, particularly during the breeding season. For example, seasonal fluctuations in reproductive hormones (e.g., estradiol and testosterone) can alter bacterial communities (discussed in Pearce et al. 2017; reviewed in Maraci et al. 2018).

Sex differences in behaviour may also affect host bacterial communities. For example, sex differences in microbiota have been attributed to differences in time spent at the nest in several bird species (Møller et al. 2009; Saag et al. 2011; Goodenough et al. 2017), given that bacteria at the nest likely differ from bacteria in the surrounding environment. Thus, we might predict that sex differences in host microbiota should be more likely in species with greater role division, particularly with respect to parental care duties, and less likely in species that invest similarly in parental care. However, in socially monogamous Leach’s storm petrels and blue petrels, breeding pairs share a nest burrow and parental care duties, and yet sex differences in preen gland-associated microbiota have been found in both species (Pearce et al. 2017; Leclaire et al. 2019).

Frequent physical contact and close proximity between members of mated pairs could lead to the social transfer of microbiota, leading to a lack of sex differences. Shared
microbiota between social mates has been found in both captive (zebra finches, *Taeniopygia guttata*, Kulkarni and Heeb 2007) and free-living (barn swallows, *Hirundo rustica*, Kreisinger et al. 2015; dark-eyed juncos, Whittaker and Theis 2016) birds. In Leach’s storm petrels, the sexes have limited physical contact during the nesting period, which may limit the potential for shared microbial communities to develop between mates (Pearce et al. 2017). In fact, individual Leach’s storm petrels shared the same amount of microbiota with their social mates as with randomly chosen non-mates, suggesting that in this species, sex-specific differences in microbiota outweigh potential contributions from interactions with mates (Pearce et al. 2017).

Like the evidence for sex differences in preen gland-associated microbes, evidence for sex differences in the chemical composition of preen oil is also mixed. Results of a literature review and meta-analysis I conducted suggest that sex differences in preen oil composition are related to both time of year and incubation type, with sex differences being more likely in breeding than nonbreeding birds and in species with uniparental rather than biparental incubation (Chapter 1, Section 1.5). As with sex differences in microbes, seasonal fluctuations in reproductive hormones have also been associated with sex differences in preen oil composition (Bohnet et al. 1991; Whittaker et al. 2011b). Leach’s storm petrels, blue petrels and song sparrows all exhibit sex differences in preen gland-associated microbiota (Pearce et al. 2017; Leclaire et al. 2019; this study) and, while no data are available for Leach’s storm petrels, preen oil composition differs between the sexes in breeding blue petrels (Mardon et al. 2010), and song sparrows (Grieves et al. 2019a, Chapter 4). Interestingly, both petrel species have biparental incubation, while song sparrows have uniparental (female only) incubation.

Disentangling the influences of reproductive hormones and behaviour (e.g., parental role division) on sex differences in both preen gland microbes and preen oil composition may provide further insight into the relationship between microbes and body odour, particularly with respect to intraspecific chemical cues. Experimentally manipulating estradiol and testosterone levels and testing for changes in preen oil composition (as in Whittaker et al. 2011b) and preen gland microbiota before and after hormonal manipulation would help to infer whether circulating hormone levels are
directly related to differences in preen oil chemical composition and host microbial community composition.

7.4.3 Preen gland microbiota, MHC genotype, and preen oil chemical composition

MHC-based mating preferences have been demonstrated in all major vertebrate groups (mammals, Setchell et al. 2010; birds, Bonneaud et al. 2006; Strandh et al. 2012; reptiles, Olsson et al. 2003; amphibians, Bos et al. 2009; and fish, Landry et al. 2001). In birds, olfactory-based discrimination of the MHC genotype of potential mates using preen oil odour cues has recently been reported in song sparrows (Grieves et al. 2019b, Chapter 6) and blue petrels (Leclaire et al. 2017b). However, it is unclear why preen oil composition reflects MHC class II genotype, and the mechanisms underlying this link are poorly understood.

I hypothesized that variation at MHC underlies some of the variation in preen gland microbiota, and that this in turn contributes to variation in the chemical composition of preen oil. Consistent with my hypothesis, song sparrows that were more similar at MHC class II had more similar preen gland microbiota. Similarly, MHC class II genotype covaries positively with the microbiota of feathers surrounding the preen gland in blue petrels (Leclaire et al. 2019). Counter to my hypothesis, I did not detect a significant relationship between preen gland microbiota and the wax ester composition of preen oil in song sparrows. Similarly, a recent study on closely related dark-eyed juncos found no significant relationship between preen gland microbiota and the volatile chemical composition of preen oil (Whittaker et al. 2016). Finally, song sparrows that were more similar at MHC class II were also more similar in their preen oil composition. Positive covariation between MHC class II genotype and preen oil composition has also been previously reported in song sparrows (Slade et al. 2016a; Grieves et al. 2019b, Chapter 6) and black-legged kittiwakes (*Rissa tridactyla*; Leclaire et al. 2014b).

The effect sizes I observed do not appear to be consistent with my hypothesis that MHC genotype has an indirect effect on preen oil chemical composition mediated solely
through preen gland microbiota. This hypothesis would predict a relatively strong relationship between MHC and preen gland microbiota (reflecting direct effects of MHC), a relatively strong relationship between preen gland microbiota and preen oil chemical composition (reflecting direct effects of preen gland microbiota), and a relatively weak relationship between MHC genotype and preen oil composition (reflecting indirect effects of MHC mediated through preen gland microbiota). My effect sizes (MHC versus microbes, $r = 0.23$; microbes versus preen oil, $r = 0.08$; MHC versus preen oil, $r = 0.38$) are consistent with a direct effect of MHC on preen gland microbiota (as predicted). Because MHC class II molecules are involved in immune defense against extracellular pathogens such as bacteria, MHC genes may indeed shape host microbiota (Penn 2002). However, the fact that preen oil composition was more strongly related to MHC genotype than to preen gland microbiota suggests that, counter to my prediction, the effects of MHC on preen oil composition are not mediated exclusively through preen gland bacteria.

Host microbiota, shaped by MHC genotype, might contribute to host odour by metabolizing compounds in preen oil, consistent with the fermentation hypothesis of chemical recognition, and/or by metabolizing MHC-derived peptides secreted in bodily fluids such as preen oil. However, these hypotheses are not consistent with the relatively weak relationship I found between preen gland microbiota and preen oil chemical composition. Alternatives to the fermentation hypothesis, not mutually exclusive, have been proposed to explain how MHC might influence odour (reviewed in Penn 2002). MHC peptides bound to MHC proteins directly reflect the structure of the polymorphic peptide binding regions of MHC proteins. These MHC peptides can be secreted in bodily fluids and, accordingly, MHC peptides may act as chemical cues that convey information about individual MHC genotype (Penn 2002; Boehm and Zufall 2006; Hinz et al. 2013). MHC molecules and/or the metabolites of MHC-bound peptides secreted in preen oil may themselves be odorous, and MHC genotype may thus shape host odour more directly (Penn 2002). Although this speculation is consistent with my findings of a relatively large effect of MHC genotype on preen oil chemical composition, I note that I analyzed the whole wax ester composition of preen oil. I did not measure volatile compounds or identify potentially MHC-derived peptides or metabolites.
The main prediction of the fermentation hypothesis of chemical recognition is that, if symbiotic microbes contribute to host odour, bacteria inhabiting scent-producing glands should covary with the volatile profiles of those glands (Albone et al. 1974; Gorman et al. 1974; Archie and Theis 2011). Although the relationship between preen gland microbiota and preen oil chemical composition approached significance, it was not significant at alpha = 0.05. Thus, my findings are not strictly consistent with the fermentation hypothesis. However, there are alternative explanations for why I did not detect a significant relationship between preen gland microbes and preen oil chemicals.

First, I measured the whole wax ester composition of preen oil rather than the volatile fraction. Preen oil is a complex mixture made up of hundreds of compounds (Dekker et al. 2000; Campagna et al. 2012), and preen oil secretions have multiple non-mutually exclusive functions (Moreno-Rueda 2017). Second, and relatedly, multiple and diverse functions for bacteria inhabiting the preen gland have been proposed (Jacob and Ziswiler 1982; Shawkey et al. 2003; Martín-Vivaldi et al. 2010; Soler et al. 2010; Whittaker et al. 2019). If symbiotic preen gland bacteria contribute to chemical cues involved in avian social communication, it is likely that only a subset of preen oil chemicals and preen gland bacteria contribute to these processes. My analysis, using whole preen oil and whole preen gland bacterial communities, may have masked covariance that exists between specific subsets of preen oil compounds and microbes. Relatedly, bacterial community function (e.g., their metabolic capabilities) cannot be adequately inferred from bacterial community composition (Moya and Ferrer 2016). Determining the subset of volatile compounds that most contribute to chemical cues, as has been done for other species (e.g., dark-eyed juncos; Whittaker et al. 2010), combined with metagenomics and metabolomics approaches (Turnbaugh and Gordon 2008; Tang 2011), will provide meaningful insights into the functional diversity and metabolic capacity of preen gland microbes and elucidate their role in avian chemical communication.

Finally, I note that the effect size I report for MHC genetic similarity and preen oil similarity ($r = 0.38$ for all pairwise dyads) is larger than previously reported in song sparrows ($r = 0.11$ for male-female dyads; Slade et al. 2016a; $r = 0.13$ for male-female
dyads; Grieves et al. 2019c) and in black-legged kittiwakes (r = 0.22 for male-male dyads, r = 0.13 for male-female dyads; Leclaire et al. 2014b). Previous studies focused on a single population, while I screened two populations separated by approximately 100 km. The variation in genetic and chemical diversity I detected between these two populations, with Cambridge birds having higher MHC allelic diversity and preen oil chemical diversity compared to London birds, may explain the larger effect sizes reported here.

7.4.4 Conclusion
The preen gland of song sparrows harbors diverse bacterial communities that differ among populations, between the sexes, and with MHC genotype. Overall, my results are consistent with general findings that the symbiotic bacterial communities of vertebrate hosts are shaped by the physical and social environment, host physiology and behaviour, and host genotype (Archie and Theis 2011).

Song sparrows with more similar MHC genotypes have more similar preen gland microbiota and more similar preen oil chemical composition, suggesting that variation at MHC contributes to variation in both preen gland bacterial communities and preen oil chemical composition. Antibiotic treatments alter preen gland microbiota (Martín-Vivaldi et al. 2010; Whittaker et al. 2019) and preen oil chemical composition (Martín-Vivaldi et al. 2010; Jacob et al. 2014; Whittaker et al. 2019), but to my knowledge no behavioural trials have been performed to test host responses to odour cues of birds with altered microbiota. A crucial next step is to experimentally manipulate preen gland microbiota (e.g., through the administration of antibiotics) to test whether this impairs or abolishes the ability of birds to discriminate the MHC genotype of potential mates.
7.5 References


Davidson G, Wiley N, Cooke AC, Johnson CN, Fouhy F, Reichert MS, de la Hera I, Crane JM, Kulahci IG, Ross RP. 2019. Diet-induced changes to host gut microbiota are linked to foraging innovation in a wild bird. bioRxiv. :827741.


Gloor GB, Macklaim JM, Pawlowsky-Glahn V, Egozcue JJ. 2017. Microbiome datasets are compositional: and this is not optional. Front Microbiol. 8:2224.


Pearce DS, Hoover BA, Jennings S, Nevitt GA, Docherty KM. 2017. Morphological and genetic factors shape the microbiome of a seabird species (Oceanodroma leucorhoa) more than environmental and social factors. Microbiome. 5:146.


Chapter 8

8 General discussion

Avian chemical ecology is an emerging field with fertile ground for discovery. Chemical communication involves sensory modalities that are evolutionarily ancient, and this type of communication is thus widespread across taxa (Bradbury and Vehrencamp 1998). Indeed, all species, from single celled bacteria and prokaryotes to multicellular animals, are sensitive to chemical information (Wyatt 2014). In birds, chemical communication has historically been understudied due to the misconception that smell is unimportant in these taxa (Stager 1967; Hagelin and Jones 2007; Caro et al. 2010). However, it is now well established that birds use olfaction in many contexts (Chapter 1, Sections 1.2, 1.3).

Despite the rapidly growing body of research on chemical communication in birds, many knowledge gaps remain. Of particular interest is the role of chemical communication in avian mate choice and other social contexts. Preen oil, a waxy substance secreted from the uropygial gland, is the main source of avian body odour involved in social communication via chemical cues (Jacob 1978; Caro et al. 2015). Thus, throughout my thesis, I used preen oil secretions to test for chemical differences among groups of interest and to test birds’ ability to discriminate among such groups using preen oil odour cues. My goal in these chapters was to assess what potential information is available in preen oil and whether birds are capable of using this information. Finally, as a first step towards understanding the role of symbiotic microbes in avian chemical communication, I characterized the preen gland microbiota of song sparrows from different populations and sexes and evaluated their relationship to MHC genotype and the chemical composition of preen oil.
8.1 Summary of findings

8.1.1 Odour cues of malarial infection

Parasitic infection can alter body odour in mammals, a phenomenon that has allowed the evolution of olfactory mechanisms to identify and avoid parasitized conspecifics (Kavaliers and Colwell 1995; Penn and Potts 1998a; Kavaliers et al. 2005; Shirasu and Touhara 2011; Olsson et al. 2014). Relatedly, avian influenza alters fecal odour in birds, but whether birds can detect such cues of infection is unknown (Kimball et al. 2013). Avian malaria parasites (genus *Plasmodium*) are transmitted by insect vectors (biting flies), and are thus not directly contagious through social contact between infected and uninfected birds. However, proximity to infected birds may increase the likelihood of becoming infected as a result of increased exposure to infected insects (Aron and May 1982). Given the negative effects of malaria infection on fitness (Valkiunas 2005; Asghar et al. 2015), selection should favour the ability of birds to detect and avoid parasitized individuals, as has been shown in mammals.

I hypothesized that infection with avian malaria alters the chemical composition of preen oil, providing an olfactory cue of infection status that may be used by birds to detect and avoid infected conspecifics. To test this, I experimentally inoculated song sparrows with malaria parasites (*Plasmodium* sp.) and compared their preen oil chemical composition prior to inoculation and at peak infection. In Chapter 2, I showed that the pre- and post-inoculation preen oil profiles differed for both *Plasmodium*-infected birds and birds that were inoculated with infected blood but that resisted infection. In contrast, there was no difference in the pre- and post-inoculation preen oil profiles of sham-inoculated birds (i.e., birds inoculated with unparasitized blood from an uninfected individual). Thus, I found support for my hypothesis that infection with avian malaria alters preen oil composition. Unexpectedly, I also found that simply being exposed to malaria parasites alters preen oil composition. Mounting an immune response, regardless of infection outcomes, has been shown to alter body odor in other species (e.g., mice; Kimball et al. 2014).
Building on these findings, in Chapter 3 I tested the hypothesis that birds use olfactory cues to avoid infected conspecifics. I tested this using a two-choice design in which song sparrows could associate with preen oil from uninfected or *Plasmodium*-infected conspecifics. There was no difference in the amount of time birds spent with either stimulus type; thus, I did not find support for my prediction that song sparrows would avoid the preen oil odour of infected conspecifics. The preen oil samples I used were collected during the acute-stage of infection (Sarquis-Adamson and MacDougall-Shackleton 2016; Grieves et al. 2018, Chapter 2). Gametocytes, which are capable of infecting mosquito vectors, enter the red blood cells of the vertebrate host during the chronic, not the acute, phase of infection (Valkiunas 2005; Rivero and Gandon 2018). Although I detected significant changes in the preen oil chemical profiles of acutely-infected song sparrows compared to sham-inoculated controls (Grieves et al. 2018, Chapter 2), it is possible that chronic-stage infection is more biologically relevant to both hosts and vectors, given that this is the time during which the disease can be spread. It is also possible that birds can detect cues of *Plasmodium* infection but do not behaviourally discriminate in their response to infected and uninfected conspecifics. Because *Plasmodium* parasites are not transmitted directly from bird to bird or by environmental contamination but are instead transmitted through vectors, the risks of proximity to infected conspecifics may not be very high.

My work in Chapters 2 and 3 is the first to demonstrate that preen oil chemistry is altered by both exposure to malaria and malaria infection, and the first to test whether birds can use olfactory cues to discriminate among infected and uninfected conspecifics. Together, these chapters make a novel contribution to the study of olfactory cues of disease in birds. Future work should test whether olfactory cues in preen oil differ between uninfected birds and chronically-infected birds, and whether birds can discriminate between these odours.
8.1.2 Preen oil as a reproductive chemical cue

While much of our current theory on mate choice and communication in birds has involved examining visual and acoustic signals such as plumage and song (Hamilton and Zuk 1982; Searcy and Nowicki 2005; Andersson and Simmons 2006; Gill 2007), recent advances in chemical ecology have begun to shift our understanding of the role of chemical cues in avian mate choice and communication. In Chapter 4, I used gas chromatography-mass spectrometry (GC-MS) to characterise the wax ester composition of song sparrow preen oil and I explored the evidence for preen oil as a reproductive chemical cue by using GC with flame ionization detection (GC-FID) to test whether the chemical composition of preen oil differs between breeding and nonbreeding seasons and between sexes, ages, and populations.

Song sparrow preen oil was comprised of at least two homologous series of fatty alcohols and fatty acids esterified in different combinations to form monoesters of 30 – 38 carbons. I identified 53 unique monoesters and detected a characteristic pattern of doublet peaks having the same total carbon number and molecular weight. For a given carbon number and molecular weight, doublet peaks were comprised of isomeric monoester mixtures that varied in the proportions of each component found in each peak. These results are generally consistent with findings from closely related white-throated sparrows (Thomas et al. 2010) and other bird species (Dekker et al. 2000). Species differences in preen oil, particularly among passerines (e.g., Soini et al. 2013) are thus likely due primarily to variations in the proportions of common preen oil compounds, rather than differences in the compounds themselves.

As predicted, the chemical composition of preen oil was significantly different between breeding (April – May) and nonbreeding (July – August) seasons, between breeding condition males and females, between adults and juveniles (i.e., recently fledged birds), and between breeding populations. Interestingly, the sex differences that were detectable in the breeding season were not detectable during the nonbreeding season. This study established preen oil as a candidate reproductive cue and provided the foundation for behavioural experiments I completed in Chapters 5 and 6 to test whether song sparrows respond to preen oil odour cues.
8.1.3 Discriminating odour cues of sex and species

Previous work has shown that preen oil odour cues mediate chemical communication in seabirds (Bonadonna et al. 2007; Mardon et al. 2010) and in gregarious passerine species (Whittaker et al. 2011; Amo et al. 2012a,b; Krause et al. 2012; Caspers et al. 2017). However, prior to my thesis research, little was known about how nonsocial passerines respond to social odour cues (but see Krause et al. 2014). To address this, and building on my findings from Chapter 4, in Chapter 5 I tested the responses of song sparrows, a relatively asocial species, to preen oil odour cues of sex and species. Specifically, I used a two-choice design to test the responses of breeding condition adult male and female song sparrows to same-sex conspecific preen oil versus no odour, same-sex versus opposite-sex preen oil, and heterospecific female cowbird preen oil versus no odour. I also used GC-FID and multivariate statistics to test for differences in the chemical composition of male and female song sparrow preen oil and between song sparrows and female brown-headed cowbirds.

My overarching hypothesis was that song sparrows can detect preen oil odour cues. Accordingly, I predicted that they would be attracted to same-sex conspecific odour, consistent with findings from seabirds (Bonadonna and Nevitt 2004; Coffin et al. 2011) and gregarious passerines (Krause et al. 2014). Next, I predicted that breeding condition adults would prefer opposite-sex odour over same-sex odour, as has been found in budgerigars (Melopsittacus undulatus; Zhang et al. 2010). Finally, I tested whether song sparrows respond to heterospecific odour cues. I used preen oil odour from brown-headed cowbirds, song sparrows’ major brood parasite (Arcese et al. 2002), reasoning that this would be an ecologically relevant odour that song sparrows should be under strong selection pressure to detect, given the high costs associated with brood parasitism (Rothstein 1975).

I found significant species differences in preen oil composition (i.e., between song sparrows and female brown-headed cowbirds), consistent with prior work in passerines (Soini et al. 2013), as well as replicating my previous finding of significant sex differences in breeding condition song sparrows. Although in contrast to my first prediction, song sparrows did not discriminate between the presence and absence of
(same-sex) odour stimuli, both male and female song sparrows spent more time with opposite-sex than with same-sex preen oil odour, a pattern consistent with my second prediction. Finally, I found a sex by stimulus-type interaction: males spent more time and females spent less time with female cowbird odour. Thus, I established that song sparrows, a relatively nonsocial species with small olfactory bulbs, can use olfactory stimuli for chemical communication both within and between species.

The lack of discrimination between same-sex odour cues and no odour, coupled with the discrimination of same-sex versus opposite-sex odour, suggests that song sparrows can detect conspecific odour cues but do not always respond to them. This finding highlights the importance of considering carefully whether it is appropriate to interpret an absence of evidence for discrimination as an inability to detect a stimulus. Few studies have compared time spent with ecologically relevant odours to time spent with neutral control odours such as solvent or water (but see Amo et al. 2008). Future studies should incorporate trials using stimulus and neutral control odours to facilitate clearer interpretation of results. Relatedly, my same-sex odour cues were collected from birds housed in the same room (albeit in different individual cages). Thus, these stimuli were likely familiar. Future work testing responses to familiar versus novel stimuli could help disentangle how familiarity and individual recognition may affect behavioural responses to odour cues of sex and species.

As I established in Chapters 4 and 5, chemical cues of sex are present in song sparrow preen oil, and as I established in Chapter 5, both sexes appear to use this information. Whereas many studies focus on female responses to male signals or cues, I found that preference for opposite-sex odour was actually more pronounced in males (based on effect size) than females. Odour cues of sex may be particularly useful for species without sex differences in plumage, because visual cues of sex are limited. The relative importance of chemical cues in mate choice compared to other signal modalities has not yet been explored in birds. Experimental studies could be designed to test the relative importance of visual, acoustic, and chemical cues of sex and reproductive status in birds by taking a hierarchical approach (Anderson et al. 2013; Searcy et al. 2014). For example, in a traditional choosy-female songbird model, one might predict that breeding
condition females would first assess acoustic signals of male quality through song (a long-range signal), next assess visual signals of quality through plumage (a medium-range signal), and finally assess short-range chemical cues. Whether or not ‘unattractive’ or inappropriate chemical cues paired with attractive visual and acoustic signals of male quality would alter female choice remains to be determined, although some progress has been made, particularly in crested auklets (Hagelin et al. 2003; Hagelin 2007).

Song sparrows responded in behaviourally appropriate ways to heterospecific odour cues of female brown-headed cowbirds. Song sparrows are commonly parasitized by cowbirds (Lowther 1993) and generally accept parasitic eggs (Rothstein 1975). However, song sparrows do respond aggressively to adult cowbirds in the wild (Smith et al. 1984; Arcese et al. 2002): this behavior may have reduced the value of egg rejection mechanisms (Robertson and Norman 1976). It is unknown whether song sparrows respond to odour cues of cowbird eggs; however, magpies (Pica pica) recognize novel egg odours and use these odour cues to reject brood parasitic eggs (Soler et al. 2014). In dark-eyed juncos (Junco hyemalis), females significantly reduced their incubation bouts after heterospecific (northern mockingbird, Mimus polyglottos), but not conspecific, preen oil secretions were applied to their eggs, suggesting that passerines may have similar capabilities (Whittaker et al. 2009). Chemical analyses of the volatile odours given off by host versus parasitic eggs would address whether or not there are species differences in egg odours. Then, studies could be designed to test host responses to odour-free eggs experimentally coated with host and parasite odours (Soler et al. 2014).

8.1.4 Discriminating odour cues of genotype

Prior work has shown that preen oil chemical composition is positively correlated with MHC class II genotype in song sparrows (Slade et al. 2016). Building on this, in Chapter 6 I confirmed this finding in captive birds and then used a two-choice design to test song sparrows’ responses to preen oil odour from MHC-similar versus MHC-dissimilar and less MHC-diverse versus more MHC-diverse potential mates (i.e., opposite sex conspecifics). MHC-based mate choice, particularly preferences for MHC-dissimilar or
MHC-diverse partners, appears to be widespread among vertebrates (Milinski et al. 2005; Bonneaud et al. 2006). Consequently, I predicted that song sparrows would spend more time with preen oil odour from MHC-dissimilar and MHC-diverse potential mates.

In this chapter, I provided the first evidence that passerines may use odour cues to mate disassortatively at the MHC. Consistent with prior work on wild song sparrows (Slade et al. 2016), the preen oil chemical similarity of captive song sparrows was positively correlated with MHC class II similarity. Consistent with my predictions, both sexes spent more time with preen oil from MHC-dissimilar than MHC-similar opposite-sex conspecifics, and more time with MHC-diverse than less-diverse opposite-sex conspecifics. These preferences are consistent with predictions of both compatible genes models of mate choice (preferences for dissimilar mates should maximize genetic diversity of offspring, allowing them to benefit from heterozygote advantage) and direct benefit models of mate choice (preferences for MHC-diverse mates should result in pairing with mates who themselves experience heterozygote advantage, and are thus capable of providing higher quality care to offspring) (Zelano and Edwards 2002; Neff and Pitcher 2004).

In song sparrows, like most passerines, both sexes invest in parental care (Arcese et al. 2002). Mutual mate choice is probably widespread among socially monogamous species like song sparrows, but most experiments in these systems focus on female choice for male ornaments (Fitzpatrick and Servedio 2018). Unlike many visual and acoustic ornaments, preen oil is produced by both sexes, and my findings suggest that both sexes attend to the odour cues it conveys. In another monogamous (but non-passerine) bird (blue petrels, *Halobaena caerulea*), males preferred the odour of MHC-dissimilar females (consistent with my findings in song sparrows) but incubating females preferred the odour of MHC-similar males (Leclaire et al. 2017a). In humans, female preferences for the body odour of MHC-dissimilar males are reversed when females use oral contraceptives, which are hormonally comparable to pregnancy (Wedekind et al. 1995; though see Roberts et al. 2008). Relatedly, female house mice (*Mus musculus*) prefer to mate with MHC-dissimilar males (Penn and Potts 1998b), but prefer to nest and nurse
communally with MHC-similar females, presumably because MHC-similar females are more likely to be kin (Manning et al. 1992).

More experimental work is needed to test whether odour-based discrimination of MHC genotype is widespread among birds and if reproductive status (i.e., breeding versus incubating versus nonbreeding) and breeding system (i.e., socially monogamous versus promiscuous species) influences preferences for MHC-similar versus MHC-dissimilar potential mates. Field studies are also required to investigate whether lab-based preferences lead to direct mate choice in the wild. For example, in blue petrels, mated pairs are significantly more dissimilar at MHC compared to random mating (Strandh et al. 2012), consistent with my lab findings. In contrast, the exact opposite pattern was found in a recent study on wild song sparrows: mated pairs are significantly more similar at MHC compared to randomly generated pairings (Slade et al. 2019).

8.1.5 Microbially-mediated chemical communication

MHC genes may influence host body odour. MHC molecules and/or the antigens that bind to them may be odorous (Hinz et al. 2013; Milinski et al. 2013). Further, an individual’s MHC class II genotype may influence host bacterial communities, shaping host odour indirectly (Penn 2002; Kubinak et al. 2015). In birds, MHC class II diversity has been implicated in shaping the community composition of symbiotic microbes inhabiting feathers and skin (Pearce et al. 2017; Leclaire et al. 2019). In dark-eyed juncos, symbiotic preen gland bacteria produce volatile compounds that are known chemical cues involved in conspecific social interactions, and these preen oil volatiles are positively associated with the relative abundances of specific preen gland bacteria (Whittaker et al. 2019). Moreover, olfactory-based discrimination of the MHC genotype of potential mates using preen oil odour cues has recently been reported in song sparrows (Grieves et al. 2019a, Chapter 6) and blue petrels (Leclaire et al. 2017a).

In Chapter 7, I sequenced a portion of the 16S rRNA gene to identify the microbes inhabiting the uropygial (preen) glands of adult male and female song sparrows
sampled from three populations. I tested for population and sex differences in song sparrows’ preen gland microbiota. Then, hypothesizing that variation at MHC class II underlies variation in preen gland microbes which in turn contributes to variation in preen oil composition, I tested for correlations between MHC class II genotype and preen gland microbiota; preen gland microbiota and preen oil chemical composition; and MHC genotype and preen oil chemical composition. The identification of such relationships could provide a potential mechanism to explain how and why avian preen oil conveys information about MHC genotype in birds.

I found significant population and sex differences in the preen gland microbiota of adult song sparrows, consistent with my predictions and with the results of prior studies on both mammals (Theis et al. 2013; Leclaire et al. 2017b) and birds (Pearce et al. 2017; Leclaire et al. 2019; Whittaker et al. 2019). Contrary to my prediction, pairwise similarity in preen gland microbiota was not significantly correlated with similarity in preen oil chemical composition. However, birds with more similar preen gland microbiota had more similar MHC class II genotypes, consistent with findings in blue petrels (Leclaire et al. 2019).

My findings are consistent with previous work demonstrating that the symbiotic bacterial communities of vertebrate hosts are shaped by the environment (population differences) as well as host physiology (sex differences) and genotype (MHC differences) (Archie and Theis 2011). This latter pattern provides some evidence of a link between host genotype and microbiota, but more experimental work is needed to determine if and how this relationship is involved in microbially-mediated chemical communication. My finding that song sparrows with more similar MHC genotypes have more similar preen gland microbiota supports the hypothesis that variation at MHC underlies variation in preen gland bacterial communities. Birds with more similar MHC genotypes also had more similar preen oil composition. The fact that preen oil composition was more strongly related to MHC genotype than to preen gland microbiota suggests that the effects of MHC on preen oil are not mediated exclusively through preen gland bacteria. MHC molecules and/or the metabolites of MHC-bound peptides secreted in preen oil may themselves be odorous, and MHC genotype may thus shape host odour more directly
(Penn 2002). Although this is consistent with my findings, it should be noted that I analyzed whole preen oil, and not the volatile components.

Antibiotics can alter preen gland microbiota (Martín-Vivaldi et al. 2010; Whittaker et al. 2019) and preen oil chemical composition (Martín-Vivaldi et al. 2010; Jacob et al. 2014; Whittaker et al. 2019), but no behavioural trials have been performed to test host responses to odour cues of birds with altered microbiota. A crucial next step is to experimentally manipulate preen gland microbes using antibiotics to test whether this disrupts the ability of birds to discriminate the MHC genotype of potential mates.

8.2 Future directions

In the previous sections (8.1.1 – 8.1.5) I suggested next steps associated with each of my data chapters. However, there are many other future directions for research in avian chemical ecology. For example, ongoing work is bringing exciting new insights into olfactory-based kin recognition mechanisms, focusing on maternal and embryonic odour cues (Caspers and Krause 2013; Caspers et al. 2013, 2015; Webster et al. 2015; Costanzo et al. 2016; Caspers et al. 2017), while other research groups are focusing on the avian microbiome (Soler et al. 2008; Jacob et al. 2014; Martín-Vivaldi et al. 2014; Rodríguez-Ruano et al. 2015; Soler et al. 2016; Veelen et al. 2018; Escallón et al. 2019; Lora et al. 2019) and the antimicrobial properties of preen oil (Law-Brown 2001; Martín-Platero et al. 2006; Soler et al. 2008; Martín-Vivaldi et al. 2010; Magallanes et al. 2016; Braun et al. 2018). In this section, I highlight some research topics in avian chemical ecology that have yet to be explored.

8.2.1 Bill-wiping in chemical communication

Preen oil is a proxy for avian body odour (Caro et al. 2015) and, similar to mammalian scent-marking behaviour, preen oil may persist in the environment through its frequent reapplication during preening. Bill-wiping typically refers to rubbing the bill side to side,
from base to tip, on a surface such as a perch or other foreign object (Clark Jr 1970). The main proposed function of bill-wiping has been to clean the bill. However, observations of bill-wiping outside of a feeding or cleaning context have led to the suggestion that it is also a displacement activity. Whether this is truly the case, or whether there are other functions of bill-wiping behaviour remains uncertain (Clark Jr 1970). Birds spend a lot of time preening, which frequently involves the application of preen oil to the body and feathers by rubbing the bill on the uropygial gland to stimulate preen oil secretions and then rubbing or combing the bill on other body surfaces (Delius 1988). Because birds preen so frequently, bill-wiping likely releases preen oil odour into the environment, and it may thus be an olfactory display used in social interactions (Whittaker et al. 2015). However, this has never been tested.

Many birds are territorial during the breeding season, and I hypothesize that bill-wiping, in addition to other possible functions, is a territorial behaviour. My hypothesis could be tested by applying preen oil secretions and control odours to preferred perches or other objects in birds’ territories and comparing bird responses to the application of preen oil from ‘intruders’ (i.e., unfamiliar rivals) versus ‘nonintruders’ (i.e., self-odour or mate-odour) and water controls. If bill-wiping is a territorial behaviour, I predict that birds would bill-wipe overtop of ‘intruder’ odour significantly more than they would bill-wipe overtop of ‘nonintruder’ odours or controls.

8.2.2 Chemical ecology in group living birds

Social animals must navigate a suite of benefits and challenges associated with group living. Accordingly, highly social animals tend to have more complex communication (Freeberg et al. 2012). For example, group-living birds tend to have larger vocal repertoires than nonsocial birds (Grieves et al. 2015). Cooperative breeders are highly social group living species in which offspring receive care from both their parents and less-related (e.g., siblings from a prior year) or unrelated adult group members. In some cooperatively breeding species, multiple unrelated females lay eggs in a single nest. Within these joint-laying groups, adults cooperate by provisioning young but they also...
compete by tossing eggs from their shared nest and burying eggs under a new nest floor (Quinn and Startek-Foote 2000; Koenig and Dickson 2004). Importantly, rates of egg loss are higher and reproductive success is lower in joint-laying groups that take longer to synchronize egg laying (Schmaltz et al. 2008). Thus, mechanisms and signals enhancing reproductive synchrony are critical to ensuring group stability and success.

Given that the composition of avian body odour changes over time and with breeding condition (Grieves et al. 2018, Chapter 2; Grieves et al. 2019b, Chapter 4), olfactory cues are a compelling candidate mechanism by which individuals might assess both group membership and reproductive status. Thus, odour cues may facilitate egg-laying synchrony and enhance reproductive success. The highly social nature of cooperatively breeding birds suggests that, as with their complex vocal communication, they may also engage in complex chemical communication. However, this has never been investigated.

Future studies could explore these ideas in cooperatively breeding birds by testing for differences in the preen oil composition between group members and non-group members. If preen oil odour cues indicate group membership, I predict that the odours of group members would be more similar to that of non-group members, as has been found in social mammals (Burgener et al. 2008; Theis et al. 2013; Leclaire et al. 2017b). If odour cues facilitate egg-laying synchrony, I predict that more synchronous breeding groups would have more similar preen oil profiles than less synchronous breeding groups.

8.2.3 Disentangling environmental and genetic effects on symbiotic microbes

Animals’ chemical profiles correlate with symbiotic microbial communities on skin and in scent glands (Theis et al. 2013; Jacob et al. 2014), and bacteria are important sources of host animal’s odour signals (Ezenwa et al. 2012; Ezenwa and Williams 2014). Shared microbial communities among individuals can correlate with proximity, which may be related to a shared environment. For example, dark-eyed junco parents have more similar
preen gland microbial communities to each other than to their same-sex counterparts (Whittaker and Theis 2016). Genetic factors may also play a role in shaping microbial communities. For example, the microbial communities of three closely related finch species differed significantly, despite these captive birds experiencing the same environmental conditions and diet (Engel et al. 2018). In captive zebra finches (Taeniopygia guttata), feather and preen gland microbial communities are most similar between full siblings, intermediate between parents and offspring, and least similar between parents (i.e., unrelated adults; Engel and Caspers 2019).

Currently, our understanding of the mechanisms by which microbes are transferred across generations is limited (Maraci et al. 2018). Joint-laying species are an ideal system in which to explore the role of symbiotic microbes in animal communication and the mechanisms by which these microbes are transferred across generations, as they provide a natural experiment in which to disentangle the effects of environment (shared nests) and genetics (unrelated adults and nestlings of varying relatedness to each other) on the composition of symbiotic microbial communities in nature. Future studies could test the prediction that, within joint-nests, there should be greater similarity in the microbiota of full siblings compared to non-kin nest mates, between parents and offspring compared to parents and non-kin nestlings (genetic effects), and between social mates compared to other adult group members (environmental effects).

8.3 Conclusions

My thesis has established that there is a wealth of potential information available in avian preen oil and that birds are capable of using preen oil odour cues in ecologically appropriate ways. My thesis provides some of the first evidence that exposure to parasites alters chemical cues emitted by birds (Chapter 2), that birds use odour cues to discriminate the MHC genotype (diversity as well as dissimilarity) of potential mates (Chapter 6), and that MHC genotype is positively correlated with both preen gland microbes and preen oil chemical composition (Chapter 7).
I showed that preen oil differs between birds exposed and not exposed to avian malaria, and differs between populations, age classes, the sexes, and breeding versus nonbreeding seasons. I also replicated findings that the chemical composition of preen oil is positively correlated with genotype at the major histocompatibility complex. While I found no evidence that birds discriminate between odour cues of healthy versus infected conspecifics or between same-sex conspecific odour versus no odour, birds spent significantly more time with preen oil odour cues from opposite-sex than same-sex conspecifics and with MHC-dissimilar over MHC-similar and more MHC-diverse over less MHC-diverse potential mates. I also demonstrated a sex-specific response to heterospecific brood parasite odour cues, where males spent more time and females spent less time with preen oil from female cowbirds. Finally, I demonstrated that, like preen oil chemical composition, preen gland microbial communities differ among populations and sexes. Furthermore, MHC genotype is positively correlated with both preen gland microbiota and the chemical composition of preen oil. These results suggest a role for microbially-mediated chemical communication in birds, similar to findings in mammals.

Collectively, my results show that even relatively nonsocial passerine birds—long thought to possess little or no sense of smell—are capable of using odour cues in social and mate choice contexts. My thesis broadens our understanding of the rapidly growing body of literature on avian chemical ecology, which suggests that chemical communication is widespread across avian taxa. Birds are extremely well-studied in terms of their visual and acoustic communication (Searcy and Nowicki 2005; Gill 2007). They can see into the ultraviolet spectrum (Cuthill et al. 2000) and use infrasound (Kreithen and Quine 1979; Freeman and Hare 2015). At least some bird species respond to vibrational cues (Dorward and McIntyre 1971; Shen 1983; Hill 2008) and many species can detect the earth’s magnetic field (Leask 1977; Mouritsen et al. 2004). Establishing birds’ sophisticated capacity for chemical communication as well suggests that birds possess among the greatest sensory capabilities of any extant taxon. It is an exciting time to be an ornithologist!
8.4 References


Engel K, Caspbers BA. 2019. Family Matters: Bacterial communities on the skin of birds. Behaviour Meeting, University of Chicago, Chicago, IL, USA.


Soler JJ, Pérez-Contreras T, De Neve L, Macías-Sánchez E, Möller AP, Soler M. 2014. Recognizing odd smells and ejection of brood parasitic eggs. An experimental test


### Appendix A

**Table A1** Studies and species included in meta-analysis testing for an effect of time of year (breeding *versus* nonbreeding stage) and incubation type (uniparental *versus* biparental) on the probability of detecting sex differences in preen oil chemical composition. Effect size calculations are based on reported sample sizes and test statistics (e.g. F, t, U, and Z), where possible. NA indicates that effect size calculations were not possible due to missing or unclear data.

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<th>Incubation</th>
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<td>Breeding</td>
<td>Biparental</td>
<td>Volatile compounds: 0.8</td>
<td>NA</td>
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- **Charadriiformes**: Order of seabirds
- **Scolopacidae**: Family of sandpipers and plovers
- **Laridae**: Family of gulls

- **Breeding Status**: Yes for breeding, No for non-breeding
- **Breeding Type**: Uniparental or Biparental
- **PC1** and **PC2**: Principal Component Analysis values
- **References**: Names of studies for the data

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<th>Parental Care</th>
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<th>Reference</th>
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<td>NA</td>
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<td>Uniparental</td>
<td>NA</td>
<td>Zhang et al. 2013</td>
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Nonvolatile compounds: 0.9
<p>| Passeriformes | Paridae | Great Tit, <em>Parus major</em> | Yes | Breeding | Uniparental | PC1: 1.28 | Jacob et al. 2014 |
|               |               |                                           |    |            |            | PC2: 0.47 |               |
|               |               |                                           |    |            |            | PC3: 1.29 |               |
| Passeriformes | Paridae | Black-capped Chickadee, <em>Poecile atricapillus</em> | No | Nonbreeding | Uniparental | NA | Van Huynh and Rice 2019 |
| Passeriformes | Paridae | Carolina Chickadee, <em>Poecile carolinensis</em> | No | Nonbreeding | Uniparental | NA | Van Huynh and Rice 2019 |</p>
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* This study examined colour changes in preen oil between the sexes, not the chemical composition of preen oil
References


Appendix B

Table B1 Chemical composition of song sparrow (*Melospiza melodia*) preen oil wax esters as determined by gas chromatography-mass spectrometry (GC-MS; N = 21; all sexes, seasons, age classes, and populations combined). Carbon numbers refer to the total number of carbons in the monoester. Percent of ester is an average for all birds measured. Percent of ester at each carbon number represents the total amount of isomeric monoesters by combining the contributions from the individual isomers (i.e., peaks A and B combined). Monoester peak A and B refers to two peaks resolved by GC-MS that had the same carbon number but different retention times (see Chapter 4 text for details).

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<th>% of Ester</th>
<th>Peak A</th>
<th>% of Ester</th>
<th>Peak B</th>
<th>Carbon # Alcohol:Acid</th>
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Elevated in post-breeding adults, juveniles (Newboro)

Elevated in Newboro males

≥ 0.1% in post-breeding Newboro males

≥ 0.1% in post-breeding males & juveniles (Newboro), breeding females (Cambridge only); ≤ 0.1% in Cambridge males
| 35 | 522 | 1.44 | x | 21:14 | 229 |
| 35 | 522 | 4.85 | x | 20:15 | 243 |
| 35 | 522 | 4.46 | x | 19:16 | 257 |

Elevated in post-breeding adults, juveniles (Newboro)

| 35 | 522 | 3.40 | x | 18:17 | 271 |

Elevated in breeding males (Newboro only)

| 36 | 536 | 0.08 | x | 23:13 | 215 |

≥ 0.1% in breeding males (Newboro only), breeding females (Cambridge only)

| 36 | 536 | 0.60 | x | 22:14 | 229 |
| 36 | 536 | 2.46 | x | 21:15 | 243 |
| 36 | 536 | 3.98 | x | 20:16 | 257 |

Elevated in post-breeding adults, juveniles (Newboro)

| 36 | 536 | 3.90 | x | 19:17 | 271 |
| 36 | 536 | 1.24 | x | 18:18 | 285 |
249

36  536  x  18:18  285
36  536  0.22 x  17:19  299  ≥ 0.1% in breeding males, juveniles (Newboro)
36  536  x  17:19  299
37  550  0.10 x  23:14  229
37  550  0.89 x  22:15  243  ≤ 0.1% in Cambridge males
37  550  x  22:15  243
37  550  1.89 x  21:16  257
37  550  x  21:16  257
37  550  2.77 x  20:17  271
37  550  x  20:17  271
37  550  1.37 x  19:18  285  Elevated in post-breeding adults, juveniles (Newboro)
37  550  x  19:18  285
37  550  0.31 x  18:19  299
37  550  x  18:19  299
38  564  0.34 x  24:14  229
38  564  0.13 x  23:15  243
38  564  0.55 x  22:16  257
38  564  1.08 x  21:17  271
38  564  0.57 x  20:18  285
38  564  0.17 x  19:19  299
Figure B1 Complete wax ester composition of breeding stage adult song sparrow preen oil at Newboro (N_{females} = 3, N_{males} = 3, mean ± SD). Peaks that were at least 0.1% of the total chromatogram area were retained for analysis, while peaks that were < 0.1% were counted as zero (see Chapter 4 text for details).
Figure B2 Selected wax ester composition of breeding stage adult song sparrow preen oil at Newboro ($N_{\text{females}} = 3$, $N_{\text{males}} = 3$, mean ± SD). For complete wax ester composition see Appendix B, Fig. B1.
Figure B3. Wax ester composition of song sparrow preen oil from post-breeding stage adults and juveniles (sexes pooled for juveniles; sampled at Newboro). Peaks that were at least 0.1% of the total chromatogram area were retained for analysis, while peaks that were < 0.1% were counted as zero (see Chapter 4 text for details).
Figure B4. Complete wax ester composition of breeding-stage song sparrow preen oil at Newboro and Cambridge (N_{Newboro males} = 3, N_{Newboro females} = 3, N_{Cambridge males} = 3, N_{Cambridge females} = 3, mean ± SD). Peaks that were at least 0.1% of the total chromatogram area were retained for analysis, while peaks that were < 0.1% were counted as zero (see Chapter 4 text for details).
**Figure B5.** Selected wax ester composition of breeding-stage song sparrow preen oil at Newboro and Cambridge (N\textsubscript{Newboro males} = 3, N\textsubscript{Newboro females} = 3, N\textsubscript{Cambridge males} = 3, N\textsubscript{Cambridge females} = 3, mean ± SD). For complete wax ester composition see Appendix B, Fig. B4.
Figure B6. Complete wax ester composition of adult song sparrow preen oil sampled during breeding and post-breeding stages at Newboro (N_{early-season females} = 3, N_{late-season females} = 2, N_{early-season males} = 3, N_{late-season males} = 3, mean ± SD). Peaks that were at least 0.1% of the total chromatogram area were retained for analysis, while peaks that were < 0.1% were counted as zero (see Chapter 4 text for details).
Figure B7. Selected wax ester composition of adult song sparrow preen oil sampled during breeding and post-breeding stages at Newboro (N_{early-season females} = 3, N_{late-season females} = 2, N_{early-season males} = 3, N_{late-season males} = 3, mean ± SD). For complete wax ester composition see Appendix B, Fig. B5.
Appendix C

Table C1 MHC class II exon 2 alleles* and associated GenBank accession numbers (Acc. No.).

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Each allele has the prefix Sosp-Dab*# (e.g., Allele 1 is equivalent to Sosp-Dab*1).

Data accessibility

Appendix D

Detailed bacterial DNA extraction protocol

I extracted bacterial DNA from swabs using Qiagen DNeasy PowerSoil DNA isolation kits, with some modifications to the manufacturer’s recommended protocol (available at https://www.qiagen.com/ca/products/discovery-and-translational-research/dna-rna-purification/dna-purification/microbial-dna/dneasy-powersoil-kit/?clear=true#resources).

Before starting the protocol, I added an initial saturation step in which I placed the swabs in the PowerBead tubes then bathed the swabs in the bead solution for 10 min before vortexing for 1 min, following Whittaker and Theis (2016). I then aseptically removed the swab and proceeded with the protocol instructions by adding solution C1 (sodium dodecyl sulfate, SDS), following Whittaker and Theis (2016). After adding solution C1, I inverted the sample tubes to mix them. Next, I vortexed the samples at maximum speed for 10 min, followed by centrifugation at 10 000 × g for 30 s. I then transferred the supernatant to a clean 2 mL collection tube.

Next, I modified the manufacturer protocol by combining steps 7 and 10, skipping steps 8 and 9. Specifically, I added solutions C2 and C3 (proprietary mixtures that contain inhibitor removal reagents that precipitate non-DNA organic and inorganic materials out of solution) at the same time rather than separately. After adding both solutions C2 and C3 to the collection tubes, I vortexed the tubes briefly, incubated them at 4 °C for 5 min, then centrifuged at 10 000 × g for 3 min. Avoiding the pellet, I then transferred up to 750 µL of the supernatant to a clean 2 mL collection tube.

After shaking thoroughly to mix Solution C4 (a proprietary mixture that is a high concentration salt solution containing guanidine hydrochloride and 2-propanol that precipitates DNA), I added 1200 µL to the supernatant and vortexed for 5 sec. I loaded 675 µL of the solution onto an MB Spin Column, centrifuged at 10 000 × g for 1 min, and discarded the flow through. This step was repeated twice, so that all of the sample was processed in this way.
Then, I added 500 µL of Solution C5 (an ethanol-based wash), centrifuged at 10 000 × g for 30 sec, and discarded the flow through before centrifuging again at 10 000 × g for 1 min. Following this, I placed the MB Spin Column into a clean 2 mL collection tube in a heat block held at 60 °C. Then, I modified the protocol again by adding 60 µL of 1X TE + 0.1 M EDTA (instead of Solution C6, an EDTA-free sterile 10 mM Tris elution buffer) to the centre of the filter membrane, and incubated the samples at 60 °C for 5 min before centrifuging at 10 000 × g for 1 min. Finally, I discarded the MB Spin Column and stored the DNA at -20 °C pending PCR amplification.

All centrifugation steps were carried out at room temperature (20 – 22 °C).

References

**Table D1** Bayesian Ribosomal Database Project taxonomic assignment of sequence variants (SVs) collected from the uropygial gland of adult song sparrows that were removed from further analysis as putative contaminants (see text for details).

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Table D2 Bayesian Ribosomal Database Project taxonomic assignment of sequence variants (SVs) collected from the uropygial gland of adult song sparrows. Superscripts indicate taxa previously reported from the uropygial gland, feathers surrounding the gland, feathers on rump (near the gland), or from body and wing feathers of other bird species.

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</tr>
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</table>

*a* Pearce et al. 2017. Microbiome 5:146


*d* Braun et al. 2018. Syst Appl Microbiol 41:564–569


Figure D1 The mean number of A) MHC class II alleles and B) preen oil peaks per individual differs between free-living song sparrows sampled from London and Cambridge breeding populations separated by approximately 100 kms. Open circles show individual values, lines show mean ± SE. Sample sizes are reported in parentheses.
Appendix E

Ethics Statement

All birds were captured under permission from the Canadian Wildlife Service and Environment and Climate Change Canada (Scientific Collection Permit CA 0244; banding subpermits 10691B,E,F). All animal procedures were approved by The University of Western Ontario Animal Use Subcommittee (protocols 2015-047 and 2016-017 to EAM-S.).
Curriculum Vitae

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Post-secondary Education and Degrees:
The University of Winnipeg
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2005–2011 B.Sc. (Hons)

McMaster University
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Honours and Awards:
Vanier Canada Graduate Scholarship
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Western University Malcolm Ferguson Award in Life Sciences
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Western University Biology Graduate Student Travel Award
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Bird Studies Canada/Society of Canadian Ornithologists Graduate Student Travel Award
2018

Bird Studies Canada/Society of Canadian Ornithologists Fred Cooke Student Award
2017

Western University Biology Graduate Student Travel Award
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Western University Ruth H. Arnold Fellowship in Biology 2016

**Related Work Experience**

- Teaching Assistant
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  2016-2017

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  2015

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  The University of Winnipeg
  2014–2019

- Teaching Assistant
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  2007–2011

**Publications:**

**Grieves LA,** MacDougall-Shackleton EA. No evidence that songbirds use odour cues to avoid malaria-infected conspecifics. Journal of Parasitology In Prep.


**Grieves LA†,** Bottini CLJ†, Branfireun BA, Bernards MA, MacDougall-Shackleton SA, MacDougall-Shackleton EA. 2020. Food stress, but not experimental exposure to mercury, affects songbird preen oil composition. Ecoxoticology 1–11. †Co-first authors.


