

Chapter 8

Bacterial Communities Associated with Junco Preen Glands: Preliminary Ramifications for Chemical Signaling

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8.1 Introduction

Renewed interest in the role of symbiotic bacteria in animal behavior—and particularly in producing chemical signals—has led to new insights and questions about the evolution of animal communication and host-symbiont coevolution (Archie and Theis 2011). The fermentation hypothesis for chemical recognition, first proposed in the 1970s (Albone et al. 1974; Gorman et al. 1974), suggests that symbiotic bacteria in mammalian scent glands produce volatile odorants that are used as recognition cues by the host animals and that variation in these bacterial communities contributes to variation in the animal scents. Support for this hypothesis has been demonstrated in several mammalian species, including spotted hyenas (Theis et al. 2012, 2013), meerkats (Leclaire et al. 2014), European badgers (Sin et al. 2012), North American porcupines (Roze et al. 2010), and greater sac-winged bats (Voigt et al. 2005). This hypothesis has rarely been applied outside of mammals and insects, although studies in birds and other taxa suggest that bacteria are present in glands related to chemical signaling (Ezenwa and Williams 2014). In this chapter, we explore whether symbiotic bacteria may play a role in manufacturing chemical signals in a songbird, the dark-eyed junco.

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Chemical communication among birds, especially songbirds, is a long-neglected area of study due primarily to the persistent belief that most birds have little to no sense of smell. However, recent studies have revealed a potentially significant role for intraspecific chemical communication in avian behavior (Caro and Balthazart 2010). The best-studied source of volatile odors in birds is preen oil secreted by the uropygial or “preen” gland, the largest exocrine gland in most birds (Jacob and Ziswiler 1982). While preening, birds spread preen oil on their feathers, which protects them in a number of ways, including enhancing their insulative capacity, maintaining feather condition, and defense against pathogenic bacteria and fungi (Jacob and Ziswiler 1982; Moyer et al. 2003; Giraudeau et al. 2010). Symbiotic bacteria associated with the gland appear to provide some of these services, by producing antimicrobial compounds that help protect against feather-degrading bacteria and other pathogenic microbes (Shawkey et al. 2003; Martín-Vivaldi et al. 2009, 2010; Soler et al. 2010). Preen oil also emits volatile and semivolatile compounds that contribute to a bird’s odor profile and thereby potentially function in communication (Mardon et al. 2010; Whittaker et al. 2010). These compounds vary among bird species (Soini et al. 2013), and within species, they can vary with individual identity (Mardon et al. 2010; Whittaker et al. 2010), sex (Soini et al. 2007; Whittaker et al. 2010), age (Shaw et al. 2011), and breeding condition (Whittaker et al. 2011b). In some bird species, they also reliably predict genetic and social reproductive success (Whittaker et al. 2013) and provide information about the relative quality of male rivals (Amo et al. 2012). Therefore, preen oil compounds may figure prominently in avian mate competition and choice. Importantly, many of these compounds, which include linear alcohols, methyl ketones, and carboxylic acids, are known to be end products of bacterial metabolism in other environments (Madigan et al. 2010; Agler et al. 2011), suggesting that the fermentation hypothesis for chemical recognition could explain the presence of these compounds in preen oil.

To our knowledge, no studies have yet tested whether bacteria play a role in avian chemical communication. However, several researchers have described relationships between preen glands and bacteria, including the effects of preen oil on feather-degrading bacteria, for example, in house finches (Shawkey et al. 2003), and beneficial bacteria found in breeding European hoopoe (Martín-Vivaldi et al. 2010) and wood hoopoe (Law-Brown and Meyers 2003) preen glands. Experimental work demonstrated that symbiotic bacteria in hoopoe uropygial glands produce preen oil volatile compounds with potent antimicrobial properties, though that study did not identify the symbiotic bacteria (Martín-Vivaldi et al. 2010). Shawkey and colleagues (2006) characterized the bacterial assemblages found on the plumage of several bird species and suggested that bacteria unique to the crested auklet could be a potential source for that species’ distinctive tangerine odor, but did not explore potential links between auklet bacteria and odors (Hagelin et al. 2003; Shawkey et al. 2006). In this chapter, we bring together data on bacteria and preen oil volatile compounds in the context of chemical communication in a songbird.

One of the best characterized songbird chemical communication systems is that of the dark-eyed junco (*Junco hyemalis*), a widespread North American sparrow whose behavior, ecology, and physiology are well understood (Nolan et al. 2002).

Thirty-nine volatile and semivolatile compounds have been identified in junco preen oil, 17 of which varied seasonally or differed in relative concentration between the sexes and were selected for further research in subsequent studies: linear alcohols 1-decanol through 1-octadecanol, methyl ketones 2-undecanone through 2-pentadecanone, and carboxylic acids dodecanoic acid, tetradecanoic acid, and hexadecanoic acid (Soini et al. 2007; Whittaker et al. 2010). These 17 compounds vary with many aspects of junco biology, including population of origin, sex, hormone levels, and reproductive success (Soini et al. 2007; Whittaker et al. 2010, 2011b, 2013). Furthermore, juncos are able to detect and differentiate among preen oil odors from different individuals, sexes, and species (Whittaker et al. 2009, 2011a). These qualities make preen oil volatiles reliable candidate cues for mate assessment and choice, yet their mechanism of production remains unknown.

To begin evaluating whether the fermentation hypothesis for chemical recognition can account for variation in junco odor profiles, here we characterize the bacterial communities associated with the preen glands of breeding adult dark-eyed juncos and determine whether the taxa present would logically contribute to junco chemical signals. We compare diversity in bacterial community composition and structure among nesting pairs and between sexes. Finally, we consider the ramifications of our findings for future studies of chemical communication, mate choice, and kin recognition in birds.

8.2 Methods

We sampled the preen gland bacterial communities of wild adult Carolina dark-eyed juncos (*J. h. carolinensis*) at Mountain Lake Biological Station in Pembroke, VA. In this region of the Appalachian Mountains, juncos are primarily altitudinal migrants, moving down into the valleys during the winter and returning to higher elevations to breed. Males typically arrive on the breeding grounds in March, with females arriving about 2 weeks later (Nolan et al. 2002). Juncos are socially monogamous, typically forming pair bonds that are maintained throughout the breeding season (May to August). Juncos also demonstrate appreciable levels of extra-pair fertilization, with about 28 % of all offspring being sired by a male other than their mother's social mate (Ketterson et al. 1997; Gerlach et al. 2012). Juncos display biparental care, with females incubating the eggs and brooding the hatchlings, and both pair mates provisioning the young (Nolan et al. 2002).

Throughout May 2012, we identified junco nests during egg laying or incubation and monitored them through the nestling phase to fledging (day 11 or 12 post-hatching). On the morning of fledging day, we captured the adult female and male at the nest using mist nets. For this study, we captured 25 juncos from 13 nests (one nest did not have an attendant male). From each junco, we sampled the bacterial communities associated with the preen gland by rubbing the tip of the gland using a sterile cotton swab. This rubbing motion mimics the birds' own preen oil collection behavior when preening and is similar to that used to stimulate preen oil secretion

for collection in capillary tubes (Whittaker et al. 2010). This collection method ensured that our samples included a small amount of preen oil and microbes from inside and outside the gland, which represent the mixture that birds collect on their bills in preparation for preening. We stored the samples at -80°C until analysis.

We extracted DNA from the bacteria on swabs using MO BIO PowerSoil[®] DNA isolation kits (MO BIO Laboratories, Inc., Carlsbad, CA). We followed the manufacturer's recommended protocol, except we added an initial 10 min saturation step during which the swab bathed in bead solution within the bead tube, and we subsequently vigorously vortexed the bead tube for 1 min before removing the swab and proceeding to the step in which solution 1 is added. Each DNA extraction yielded a discernible band, consistent with 16S rDNA, on an agarose gel following PCR amplification of the 16S rRNA gene. Aliquots of the original DNA extractions were provided to the Michigan State University Research Technology Support Facility's Genomics Core, where the V4 region of the 16S gene was targeted for sequencing on the Illumina MiSeq platform. Sample preparation, sequencing, and preliminary quality filtering were completed using previously published protocols (Caporaso et al. 2011, 2012).

We processed the MiSeq run files using mothur software, v. 1.31.2 (Schloss et al. 2009; Kozich et al. 2013). Specifically, we removed all sequences that (1) contained any ambiguous base calls, (2) had homopolymer runs longer than eight bases, (3) did not start and end at our specific V4 primer positions when aligned to the Silva bacterial database, (4) were deemed chimeric by mothur's uchime tool, or (5) were classified as originating from mitochondria, chloroplasts, archaea, eukaryotes, or other nonbacterial sources using the Ribosomal Database Project's trainset9_032012 (Wang et al. 2007; Claesson et al. 2009). This process revealed that the preen sample of one male junco was not successfully sequenced, so data from this sample were discarded. Each of the 24 remaining samples were subsampled to a depth of 6000 sequences, and these sequences were binned into operational taxonomic units (OTUs) using mothur's average neighbor split-clustering algorithm and a 97 % sequence similarity cutoff. We then removed all singleton and doubleton OTUs from the data set and derived a consensus taxonomy for each of the remaining OTUs using a conservative 80 % confidence threshold (Claesson et al. 2009).

We generated a Clearcut cladogram, v 1.0.9, in mothur to illustrate the phylogenetic and taxonomic relationships among the prominent (i.e., widespread) OTUs in samples (Sheneman et al. 2006). We then used this information in conjunction with data on the typical production of volatiles by bacterial taxa, available through the mVOC database (Lemfack et al. 2014), to determine whether the prominent OTUs associated with junco preen glands are likely to manufacture any of the 17 volatile compounds of interest. Variation in the OTU profiles of samples among nests and between sexes was visualized via two-dimensional, principle coordinates analyses (PCoA) and statistically evaluated using nonparametric MANOVA with 10,000 permutations (Anderson 2001). We conducted these analyses using Dice and Bray-Curtis similarity indices, reflecting similarities in bacterial community membership and structure, respectively (Hammer 2011). Community membership addresses the shared presence or absence of OTUs, while community structure further considers

similarities in their relative abundances. Prior to conducting community structure analyses, OTU abundance data were $\log_{10}(x+1)$ transformed (Ramette 2007). All analyses were completed using PAST software, v 2.17 (Hammer et al. 2001; Hammer 2011).

8.3 Results and Discussion

Our preliminary bacterial survey suggests that junco preen glands, like mammalian scent glands, harbor diverse communities of symbiotic odor-producing bacteria. Figure 8.1 shows a cladogram of the most common OTUs found in our samples. Sixteen OTUs were unclassified, while 18 were assigned to 16 genera in the phyla Actinobacteria, Firmicutes, and Proteobacteria (classes Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria). Several of these genera were previously found on the plumage of seabirds, chickens, and songbirds, including *Staphylococcus*, *Enterococcus*, *Pseudomonas*, and *Acinetobacter*; *Burkholderia* was found on the plumage of chickens and auklets (Shawkey et al. 2005, 2006). *Enterococcus* was also found in the preen glands and secretions of hoopoes and wood hoopoes (Martín-Vivaldi et al. 2009; Law-Brown and Meyers 2003). These findings suggest that these genera may be commonly found on birds, as they are on mammals.

Most of the bacterial genera associated with junco preen glands contain species that are known odor producers (Balkwill et al. 2006; Ezaki et al. 2006; Towner 2006; López del Castillo-Lozano et al. 2008; Blom et al. 2011; Latorre-Moratalla et al. 2011; Filipiak et al. 2012; Spraker et al. 2014). Most notably, *Burkholderia* and *Pseudomonas*, which can survive in a wide variety of habitats and utilize diverse nutrients, including oils (Haas and Défago 2005; Mahenthiralingam et al. 2005), can produce 9 of the 17 volatile compounds of interest (53 %) in juncos. Most of these nine volatile compounds are known to be produced by multiple species within each genus (e.g., nine species of *Burkholderia* and five species of *Pseudomonas* produce 2-tridecanone, Table 8.1). Thus, even without species-level identification in this study, the available data suggest that *Burkholderia* and *Pseudomonas* are strong candidates for the production of volatile compounds involved in junco chemical communication. We have begun efforts to successfully cultivate and metabolically characterize these bacteria from junco preen oil to directly test this hypothesis.

Not all of the identified genera contain odor producers, or at least they are not known to produce volatile compounds described in junco preen oil. However, while mammalian scent glands are specialized for a single purpose, secretions from avian preen glands perform diverse functions in addition to chemical signal production, including parasite defense, feather protection, and thermoregulation. Several genera associated with junco preen glands have documented antifungal (*Arthrobacter*, *Burkholderia*, *Pseudomonas*) or antibacterial (*Methylobacterium*, *Enterococcus*, *Pseudomonas*) effects (Fernando et al. 2005; Haas and Défago 2005; Green 2006; Jones and Keddie 2006; Soler et al. 2008; Groenhagen et al. 2013),

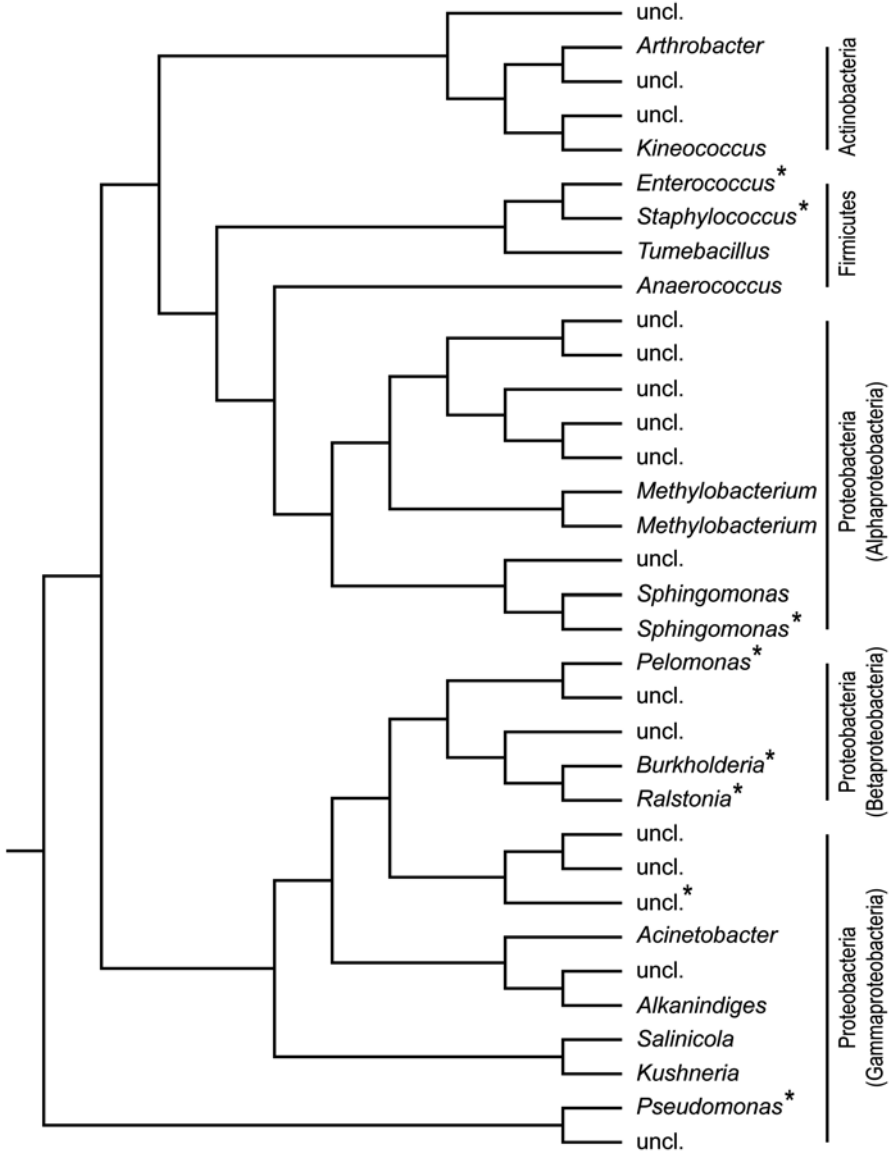


Fig. 8.1 Clearcut, relaxed neighbor-joining cladogram of the most common operational taxonomic units (OTUs) associated with junco preen glands. All OTUs listed were found in at least 50 % of birds sampled; those marked with *asterisks* were found in at least 75 % of the birds sampled. Taxonomic assignments were made using the Ribosomal Database Project’s classifier tool in mothur, with an 80 % confidence threshold

Table 8.1 A count of how many species of *Burkholderia* and *Pseudomonas* have been found to emit junco volatile compounds

Volatile compound	# of species producing compound	
	<i>Burkholderia</i>	<i>Pseudomonas</i>
1-Decanol	0	4
1-Dodecanol	0	1
1-Heptadecanol	0	4
2-Undecanone	21	6
2-Dodecanone	1	0
2-Tridecanone	9	5
2-Pentadecanone	1	0
Dodecanoic acid	7	1
Tetradecanoic acid	5	3

and *Burkholderia* and *Pseudomonas* species are also used for biocontrol in agriculture and in bioremediation to clean up pollutants (Haas and Défago 2005; Mahenthiralingam et al. 2005). Therefore, these bacteria may be providing other valuable services to their junco hosts.

Although male and female juncos have significantly different volatile profiles (Whittaker et al. 2010), we did not find a consistent effect of sex on bacterial community composition or structure (NPMANOVA, $N=22$, $df=1$, Dice: $F=0.858$, $p=0.7713$, Bray-Curtis: $F=0.8024$, $p=0.8511$). Instead, paired males and females clustered together (Fig. 8.2). Similarly, in a study of captive zebra finches (*Taeniopygia guttata*), bacteria applied to the feathers of one zebra finch were found in the cloaca of its pair mate 24 h later, most likely transmitted via copulation (Kulkarni and Heeb 2007). Humans—and their pet dogs—living in the same household have significantly more similar skin microbiomes than those living in different households (Song et al. 2013), and individual human microbiomes quickly colonize the individual’s surroundings (Lax et al. 2014). Since we collected our samples in May, near the beginning of the breeding season, our data suggest that adult birds that spend time in close physical proximity or have frequent contact may develop similar microbial communities in a fairly short period of time.

Previous studies have suggested that preen oil volatile compounds may be important for mate recognition and assessment, as they vary with species, sex, and individual identity (Soini et al. 2007; Mardon et al. 2010; Whittaker et al. 2010), and they predict reproductive success in juncos (Whittaker et al. 2013). These volatiles may also be important for recognizing kin (Leclaire et al. 2012) and one’s home nest or burrow (Bonadonna and Bretagnolle 2002; Caspers and Krause 2010). Cross infection of symbiotic microbes between mates and between parents and offspring may contribute to the development of a recognizable, “signature” home scent (Archie and Theis 2011).

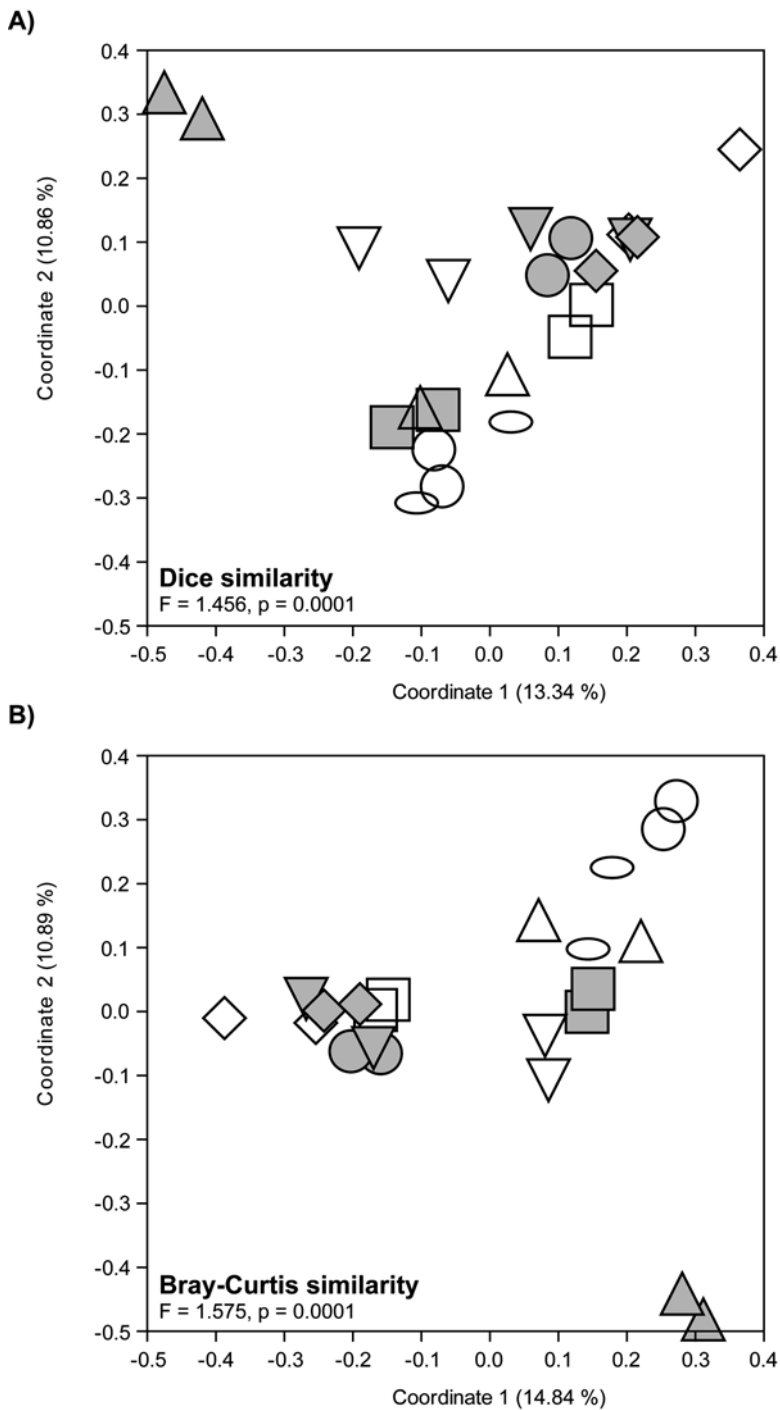


Fig. 8.2 Principal coordinates analyses (PCoA) illustrating nest-specific variation in the (a) composition and (b) structure of bacterial communities associated with junco preen glands. *Symbol shape and color* are indicative of nest identity. Statistical reports are for one-way NPMANOVA ($N=22$, $df=10$)

8.4 Conclusion

To our knowledge, this is the first study to examine avian microbiomes in the context of chemical signaling. Previous studies of symbiotic bacteria on birds' skin, feathers, or glands have focused on microbes that produce beneficial, antibacterial substances (e.g., Martín-Vivaldi et al. 2010) or on pathogenic microbes, especially feather-degrading bacteria (e.g., Shawkey et al. 2003; Saranathan and Burt 2007; Saag et al. 2011). Our study suggests that the fermentation hypothesis for chemical recognition, originally formulated for mammals, may apply to a much broader range of taxa and opens new pathways for research. Future studies should evaluate covariance between preen gland bacterial communities and odors, the effect of manipulating bacterial communities on odor profiles, and the extent to which subsequent changes in odor profiles influence birds' behavioral responses to preen oil. They should also further elucidate the effect of social behavior on the transmission of preen gland bacterial communities and determine the extent to which preen gland bacterial communities differ from those associated with other avian organs.

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