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BATS, BACTERIA, AND BAT SMELL: SEX-SPECIFIC DIVERSITY OF MICROBES IN A SEXUALLY SELECTED SCENT ORGAN

CHRISTIAN C. VOIGT,* BARBARA CASPERS, AND STEPHANIE SPECK

Research Groups Evolutionary Ecology and Wildlife Diseases, Leibniz-Institute for Zoo and Wildlife Research, Alfred-Kowalke-Strasse 17, D-10315 Berlin, Germany

Microbes interfere with the olfactory communication of animals by degrading chemical signals or by adding volatile metabolites. We report on the composition and diversity of the microflora in a sexually selected scent organ, the wing sacs of *Saccopteryx bilineata*, which are used by males for courting females. Wing sacs lack any glandular tissues. Instead, males clean and refill their wing sacs each day with genital and gular secretions. Females have only a nonfunctional rudiment of this organ. We isolated a total of 40 microbial species with only a moderate overlap in species composition between the sexes. The estimated microbial diversity was significantly lower in males than in females, with a minimum of 52.5 microbial species ± 5.0 *SD* in wing sac rudiments of females and 40.3 ± 4.2 *SD* in wing sacs of males (jackknife estimates). Males carried on average only 2 out of 40 possible microbial species in their wing sacs. Thus, individual scent profiles of males could originate from individual microflora. The daily routine of wing sac cleaning and refilling has possibly evolved to control microbial scent degradation, to support an individual microflora involved via volatile metabolites in mate choice, or both. Microbes may play a more prominent role in the evolution of morphological structures and behavioral adaptations than previously envisaged.

Key words: bacteria, bats, mate choice, microbes, olfactory communication, scent

Olfaction is an important cue for mate-choice decisions in almost all mammalian species, but because of methodological difficulties, chemical signals have long been neglected in studies of sexual selection (Andersson 1994). Most olfactory signals are temporary in nature because they usually evaporate readily and because they are exposed to chemical or microbial degradation (Bradbury and Vehrencamp 1998). Microbes also may add volatile metabolites to an animal's scent profile and because the microflora presumably is influenced by an animal's major histocompatibility complex, microbial volatiles may play an important role during mate-choice recognition (see review by Wyatt [2003]). Although microbial scent production and processing is involved in the olfactory communication of animals, few studies have focused on the microflora of scent organs. Anal sacs of carnivores contain numerous bacteria that add to the overall scent profile of the animals, for example, anaerobic bacteria in the red fox, *Vulpes vulpes* (e.g., Gosden and Ware 1976; Ware and Gosden 1980). Fish-eating bats, *Noctilio leporinus*, have paired inguinal structures that lack any secreting epithelia, but contain gram-positive bacteria, especially *Staphylococcus aureus*, creating the bats' typical strong odor (Dabson et al. 1977; Studier and Lavoie 1984). In humans, skin microflora, that is, coryneform bacteria, are responsible for scent production in the axillary region (Rennie et al. 1990, 1991). In particular, *Corynebacterium xerosis* converts odorless testosterone into several odoriferous metabolites (Rennie et al. 1990, 1991).

In the greater sac-winged bat, *Saccopteryx bilineata* (Emballonuridae), males have a pouchlike scent organ in the leading wing membrane (antebrachium) that contains an odoriferous liquid used for courtship (Bradbury and Emmons 1974). Microbial degradation of scents in the wing pouches may influence a male's scent and consequently also his mating success. As a 1st step toward an understanding of microbial interference with the chemical signals of a mammal, we studied the microbial flora of the wing pouches in *S. bilineata*.

The greater sac-winged bat is a common insect-feeding species in the neotropical lowlands. Daytime roosts are located in cavities of hollow trees or between buttress roots of trees (Bradbury and Emmons 1974). Occasionally, greater sac-winged bats also roost in or at the outside of buildings. In Central America, female *S. bilineata* give birth to a single young in June. The mating season is restricted to December and January (Bradbury and Emmons 1974). Because males

* *Correspondent: voigt@izw-berlin.de*

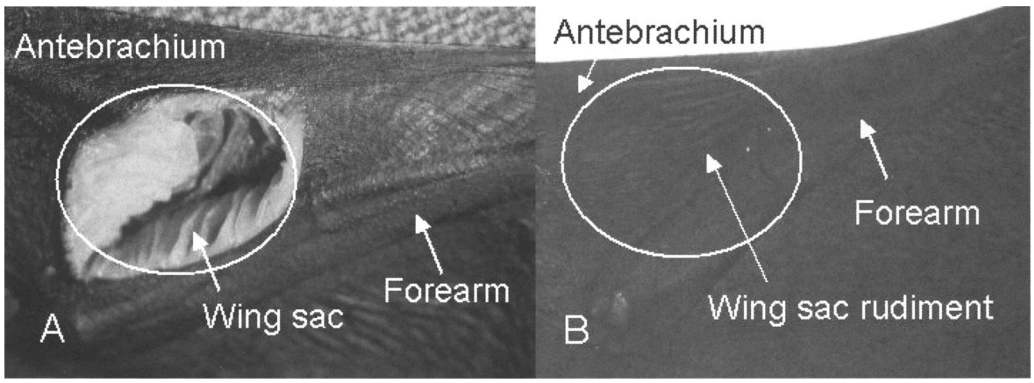

FIG. 1.—Dorsal view of the right antebrachium in *Saccopteryx bilineata*. A) Wing sac of a male and B) wing sac rudiment of a female. The areas from which microbial samples were taken are indicated by a circle.

defend year-round stable harems, the mating system of *S. bilineata* has been described as harem polygynous (Bradbury and Emmons 1974; Voigt et al. 2001). Recent paternity studies using microsatellite markers have confirmed that harem males sire, on average, more offspring than do nonharem males (Heckel and von Helversen 2002). However, harem males sire a mean of only 30% of the young within their territory. Probably because of energetic advantages during courting displays on the wing, small-sized males have a fitness benefit over large males (Voigt et al. 2005). Females are larger than males and are therefore superior over males during agonistic encounters (Bradbury and Emmons 1974). Thus, the potential for extraharem paternities and consequently female choice is high in this species. Males court females with hovering flights during which they fan scents from their wing pouches toward females. Histological studies have demonstrated that wing pouches do not include glandular tissue (Scully et al. 2000; Starck 1958). Instead, males transfer liquids from the genital and gular region into the wing sacs (Voigt and von Helversen 1999). This behavior, interpreted as cleaning and refilling of wing sacs, is repeated each day at almost the same time in the afternoon (Voigt 2002). During the 1st phase of this highly stereotypic behavior, males take up urine orally and afterward lick both wing sacs. During the 2nd phase, males press their gular region onto the penis and transfer a sperm-free droplet from their penis into their wing sacs (Voigt 2002). Both phases last for approximately 10–20 min each.

We suggest that males of *S. bilineata* repeat the time-consuming behavior of perfume-blending each day to control the microflora in their wing sacs, thus minimizing microbial fermentation, creating a microbial scent profile that is specific to the carrier, or both. Here, we compare the microbial composition of wing sacs of male sac-winged bats and the corresponding rudiments in females that lack any liquid and that are not used in any behavioral context. We predict that microbial species richness is lower in the wing pouches of males than in the wing pouch rudiments of females of *S. bilineata*.

MATERIALS AND METHODS

We collected samples from 11 males and 13 females in Costa Rica in December 2002 (La Selva Biological Station, 10°25′N, 84°00′W) and from 11 males and 13 females in Panama in December 2003 (the Biological Station at Barro Colorado National Monument, 9°9′N, 79°51′W). The Costa Rican study colony was located in an abandoned cottage and the colony in Panama roosted on outer walls of used buildings. At dusk, we captured the bats with mist nets (6 m long, 2 m high) at a distance of 2–10 m from the roost when they dispersed to their foraging habitat. For individual identification, we placed colored or numbered plastic bands (AC Hughes Ltd., Hampton Hill, United Kingdom) on the right forearm of males and on the left forearm of females. Treatment of animals followed guidelines of the American Society of Mammalogists (Animal Care and Use Committee 1998) and were approved by an institutional animal care and use committee.

Isolation and identification of bacteria.—Samples were collected from the wing sacs of males and wing sac rudiments of females by using sterile 0.9-mm cotton swabs (Hain Diagnostika, Nehren, Germany). Samples were taken from the antebrachium (Fig. 1). Swabs were stored in trypticase soy broth and kept at room temperature up to 1 h until plated onto Columbia (5% sheep blood) and McConkey II agar (Beckton Dickinson, Sparks, Maryland). Agar plates were incubated aerobically at 37°C in 5% CO_2 (AnaeroJar 2.5l, Oxoid, Wesel, Germany) for 24–72 h. Additionally, swabs were incubated in enrichment trypticase soy broth. Any microbial colony with notable morphological differences was subcultured on Columbia and McConkey II agar. Primary identification of microbial isolates was based on Gram staining, cellular morphology, and catalase and oxidase reaction. We used the commercially available analytical profile index (API) from bioMérieux (Nürtingen, Germany) for the identification of staphylococci and micrococci (API Staph), streptococci (API 20 Strep), coryneform bacteria (API Coryne), gram-positive bacilli (API 50 CH), *Enterobacteriaceae* (API 20 E), gram-negative nonfermenting bacteria (API 20 NE), and yeasts (API 20 C Aux). In addition, conventional biochemical tests (Bisping and Amtsberg 1988; Quinn et al. 2000) were used. For the identification of species that were unidentified by the biochemical tests, partial 16S rDNA sequences were amplified by polymerase chain reaction (PCR) according to the methods of Wyss et al. (1996). Amplified PCR products were sequenced bidirectionally without cloning by using an Applied Biosystems Genetic Analyser ABI 3100 (Weiterstadt, Germany). Sequence PCR was performed in a total volume of 10 µl by using the BigDye cycle sequencing kit (Applied Biosystems). The primers used for PCR amplification were likewise used for sequencing. Sequences obtained were compared to microbial sequences at NCBI BLAST, GenBank.

Estimation of species richness and sex-specific composition of microbial flora.—The species richness of microbial flora was

estimated on the basis of presence or absence data in female and male *S. bilineata* based on the jackknife 1 method (Burnham and Overton 1978, 1979; Heltshe and Forrester 1983) by using the software EstimateS (Colwell 2005; equation 1):

$$S_{\text{Jack1}} = S_{\text{obs}} + Q_1[(m-1)/m] \quad (1)$$

where S_{Jack1} is estimated species number, S_{obs} is number of observed species, Q_1 is number of species that were identified only once, and m is number of samples. We estimated the degree of species overlap between males and females with the Morisita index C_λ (Morisita 1959; equation 2):

$$C_\lambda = \frac{2\sum n_{1i}n_{2i}}{(\lambda_1+\lambda_2)N_1N_2} \quad (2)$$

where n_{1i} is frequency of microbes i in males, n_{2i} is frequency of microbes i in females, N_1 is number of males, and N_2 is number of females. Values for λ_1 and λ_2 were calculated as follows:

$$\lambda_1 = \frac{\sum[n_{1i}(n_{1i}-1)]}{N_1(N_1-1)} \quad (3)$$

$$\lambda_2 = \frac{\sum[n_{2i}(n_{2i}-1)]}{N_2(N_2-1)} \quad (4)$$

Mean values are reported ± 1 *SD*. Statistical tests were performed as 2-tailed by using the statistical software Instat version 3.06 (GraphPad Software, Inc., San Diego, California) and SPSS version 8.0 (SPSS Inc., Chicago, Illinois).

Results

In total, we found 40 microbial species in wing sacs or wing sac rudiments of *S. bilineata*, including 25 species in wing sacs of males and 35 in wing sac rudiments of females (Table 1). Most of the microbial species isolated from males belonged to the group of gram-positive cocci (36%; i.e., *Enterococcus*, *Micrococcus*, and *Staphylococcus*) as well as coryneform bacteria (28%; i.e., *Aureobacterium*, *Brevibacterium*, *Corynebacterium*, *Rhodococcus*, and *Rothia*). Sixteen percent of the species were gram-positive aerobic spore-forming rods (i.e., *Bacillus*). The remaining gram-negative rods (i.e., *Alcaligenes*, *Serratia*, and *Stenotrophomonas*), aerobic actinomycetes (*Nocardia*), and yeasts (*Candida*) contributed to the microflora to a lesser degree. Twenty-nine percent of the bacteria from females belonged to gram-positive cocci (i.e., *Micrococcus* and *Staphylococcus*) and gram-negative bacteria (i.e., *Acinetobacter*, *Alcaligenes*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Pseudomonas*, *Serratia*, and *Stenotrophomonas*). Twenty-six percent of the isolates revealed gram-positive coryneform rods (i.e., *Arthrobacter*, *Aureobacterium*, *Brevibacterium*, *Corynebacterium*, and *Rhodococcus*) and 14% of identified species comprised gram-positive aerobic spore-forming rods. The predominant genus found in both sexes was *Staphylococcus*.

Sex-specific microbial composition and diversity.—On average, we found 2.1 ± 1.3 *SD* microbial species in wing sacs of individual males and 2.7 ± 1.6 microbial species in wing sac rudiments of individual females, which was not significantly different (Student's *t*-test: $t = 1.5$, $d.f. = 46$, $P = 0.14$). The Morisita index equaled 0.62, indicating a moderate overlap in species composition between the sexes (0 = no overlap, 1.0 = complete overlap). The calculated asymptotic curve of species richness did not reach saturation either in males or in females (Fig. 2). Thus, reported numbers of species should be considered as conservative. Based on presence–absence data of microbes, we estimated a total number of 40.3 ± 4.2 microbial species in males ($n = 22$; Fig. 2). For 26 samples of females, we estimated a diversity of 57.2 ± 5.1 microbial species and for the same sample size of males ($n = 22$) 52.5 ± 5.0 microbial species (Fig. 2). The estimated number of microbial species was significantly higher in wing sac rudiments of females than in wing sacs of males based on equal sample sizes (Student's *t*-test: $t = 8.8$, $d.f. = 42$, $P < 0.001$).

Table 1.—List of 40 microbes identified in wing sacs of male and wing sac rudiments of female *Saccopteryx bilineata* (+ = present, − = absent).

Microbe	Females	Males
Acinetobacter	+	−
Acinetobacter baumannii/calcoaceticus	+	−
Acinetobacter junii/johnsonii	+	−
Aerobic spore-forming rods	+	+
Alcaligenes	+	+
Arthrobacter	+	−
Aureobacterium/Corynebacterium aquaticum	+	+
Bacillus	+	+
Bacillus cereus	+	+
Bacillus megaterium II	+	−
Bacillus mycoides	−	+
Bacillus sphaericus	+	−
Brevibacterium	+	+
Brevibacterium casei	+	+
Brevibacterium epidermidis	+	+
Candida parapsilosis	+	+
Citrobacter freundii	+	−
Corynebacterium bovis	+	+
Corynebacterium pseudodiphteriticum	+	−
Corynebacterium urealyticum	+	−
Enterobacter	+	−
Enterobacter cloacae	+	−
Enterococcus faecalis	−	+
Escherichia coli	+	−
Micrococcus	+	+
Nocardia	−	+
Pseudomonas aeruginosa	+	−
Rhodococcus	+	+
Rothia dentocariosa	−	+
Serratia marcescens	+	+
Staphylococcus	+	+
Staphylococcus aureus	+	+
Staphylococcus capitis	−	+
Staphylococcus hominis	+	−
Staphylococcus lentus	+	−
Staphylococcus saprophyticus	+	+
Staphylococcus sciuri	+	+
Staphylococcus warneri	+	+
Staphylococcus xylosus	+	+
Stenotrophomonas maltophilia	+	+

Discussion

Microbial skin flora of S. bilineata.—We identified a skin flora in *S. bilineata* that also is commonly found in other

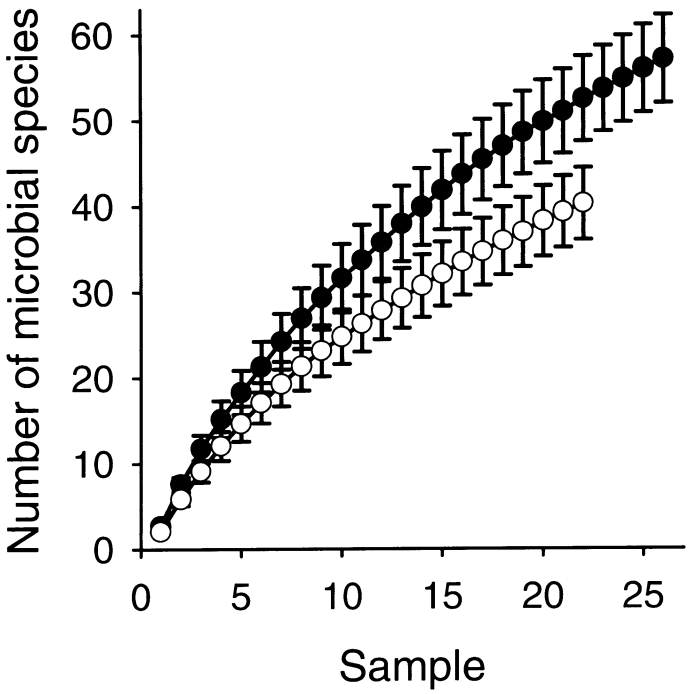

FIG. 2.—Estimates of microbial species richness (± *SD*) based on the jackknife 1 method for male (open circles) and female (closed circles) *Saccopteryx bilineata*.

animals. Gram-positive cocci are widespread in nature and commonly colonize skin, external glands, and mucous membranes of birds and mammals including humans. *Staphylococci* and *Enterococci* cause opportunistic infections in mammals although they are usually regarded as nonpathogenic. Gram-positive coryneform rods are part of the normal skin flora and mucous membranes of mammals. Most corynebacteria are able to cause suppurative infections. Gram-negative bacteria of the genera *Citrobacter*, *Enterobacter*, *Escherichia*, and *Serratia* (all *Enterobacteriaceae*) are widespread in the environment as well as in the intestine of animals. Most of the aerobic spore-forming gram-positive rods (e.g., *Bacillus*) are widely distributed saprophytes with little or no pathogenic potential. *Acinetobacter* species are common in soil and water and, so far, little is known about their pathogenicity. *Actinomycetes* species are present on mucous membranes and often in the oral cavity or nasopharynx of animals and are rarely pathogenic (Quinn et al. 2000).

Sexually dimorphic scent organs as habitats for bacteria.—The composition of the microflora in wing sacs and wing sac rudiments differed between the sexes. Specifically, we found a large portion of gram-positive cocci in males. This difference may be attributable to sex-specific morphological differences of the antebrachial wing membrane (Scully et al. 2000; Starck 1958) and also to the male-specific behavior of wing sac cleaning and refilling (Voigt 2002; Voigt and von Helversen 1999). In agreement with our prediction, microbial diversity was lower in males than in females. In contrast to our study, Gasset et al. (2000) showed a higher species richness on the glandular tuft organs of male white-tailed deer, *Odocoileus virginianus*, than in females. White-tailed deer do not remove old odoriferous liquids from their hair tufts, which could diminish microbial growth and species richness. Male *S. bilineata*, on the other hand, possibly control the microflora of their wing sacs by grooming. A controlled growth of microbes could help males of *S. bilineata* to produce a distinct, individual scent profile. This is supported by the observation that individual males on average carried only 2 microbial strains in their wing sacs, although a minimum microbial richness of 40 was estimated for the whole population.

Coevolution of scent organs and bacteria.—Morphological structures that host scent-producing microbes are common in many mammals (e.g., Dabson et al. 1977; Scully et al. 2000; Starck 1958). In some species, these structures are sexually dimorphic and possibly under sexual selection. Because microbes are involved in both scent production and degeneration, they most likely have coevolved during the evolution of these structures. A prominent example of microbial involvement in scent production is the modification of odorless androstene steroids by microbes of the human armpit regions to smelly compounds (Gower et al. 1994; Rennie et al. 1990, 1991). In *S. bilineata*, preliminary analysis of male scent profiles have revealed several volatile compounds that are likely of microbial origin, for example, indole derivatives and amino-acetophenon (F. Schröder, B. Caspers, C.C. Voigt, J. Meinwald, in litt.). However, it is not known whether these compounds are involved in mate recognition and female choice in *S. bilineata*, as has been shown and suggested for other animals including humans (see Vollrath and Milinski 1995). Microbes may be more involved in olfactory communication, especially female choice, and the evolution of morphological and behavioral adaptations than previously envisaged.

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