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SEASONALLY CHANGING PREEN-WAX COMPOSITION: RED KNOTS' (*CALIDRIS CANUTUS*) FLEXIBLE DEFENSE AGAINST FEATHER-DEGRADING BACTERIA?

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ABSTRACT.—**During incubation, ground-breeding sandpipers such as Red Knots (***Calidris canutus***)** create a warm, humid microclimate in the nest, conditions that favor the growth of feather-degrading bacteria present in their plumage. Just before incubation, the composition of waxes secreted by the uropygial gland of Red Knots and other sandpipers changes quickly and completely from a mixture of only monoesters to a mixture of only diesters. We hypothesized that the change in composition of the preen wax helps protect the plumage against feather-degrading bacteria. We tested the hypothesis by studying growth of the feather-degrading bacterium *Bacillus licheniformis*, which we found in the plumage of Red Knots. The removal of preen waxes from feathers resulted in faster bacterial degradation, confirming earlier findings that preen wax inhibits growth of feather-degrading bacteria. However, the degradation rate of feathers with preen wax based on diesters did not differ from that of feathers with preen wax based on monoesters. We suggest that preen waxes protect feathers by forming a physical barrier to microbes rather than through chemical properties of the waxes.
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Key words: *Bacillus licheniformis*, *Calidris canutus*, **feather degradation, preen wax, Red Knot, sandpipers, uropygial gland.**

Cambio Estacional en la Composición de la Cera de Acicalamiento: ¿Una Defensa Flexible ante Bacterias Degradadoras de Plumas en *Calidris canutus*?

RESUMEN.—**Durante la incubación, las aves playeras que anidan en el suelo como** *Calidris canutus* crean un microclima cálido y húmedo en el nido, condiciones que favorecen el crecimiento de bacterias que degradan las plumas presentes en sus plumajes. Justo antes de la incubación, la composición de las ceras secretadas por la glándula uropigial de *C. canutus* y otras aves playeras, cambia de composición completa y rápidamente de una mezcla compuesta solamente por monoésteres a una mezcla compuesta por diésteres. Planteamos la hipótesis de que el cambio en la composición de la cera de acicalamiento ayuda a proteger el plumaje ante bacterias degradadoras de plumas. Probamos esta hipótesis a través del estudio del crecimiento de la bacteria degradadora de plumas *Bacillus licheniformis,* que se encuentra en el plumaje de *C. canutus*. La remoción de las ceras de acicalamiento de las plumas condujo a una degradación bacteriana más rápida, confirmando estudios anteriores que documentaron que la cera inhibe el crecimiento de las bacterias degradadoras de plumas. Sin embargo, la tasa de degradación de plumas con ceras compuestas por diésteres no difirió de la de plumas con ceras compuestas por monoésteres. Sugerimos que las ceras de acicalamiento protegen a las plumas formando una barrera física ante los microbios, y no mediante sus propiedades químicas.

AS PART OF maintenance behavior, most birds spread waxes secreted by the preen gland onto their feathers (Jacob and Ziswiler 1982). **The secreted preen waxes are complex, species-specific** mixtures usually consisting of wax esters (i.e., fatty acids condensed with alcohols; Jacob 1976, Sweeney et al. 2004). The species-specificity of these waxes suggests that different habitats subject birds to different selective forces (e.g., humidity, predation pressure, ultraviolet radiation) and may have led to the evolution of varied preen-wax compositions to accommodate specific needs (Sweeney et al. 2004). Preen-wax esters consisting of alcohols esterified to unbranched fatty acids, for example, are more water-repellent than preen-wax esters consisting of alcohols esterified

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to branched fatty acids (Sweeney et al. 2004); thus, the former may occur more often in waterbirds. Furthermore, preen-wax composition sometimes varies intraspecifically with season (Jacob et al. 1979; Kolattukudy et al. 1987; Piersma et al. 1999; Reneerkens et al. 2002, 2007b). Such seasonal variation may result from different selection pressures that birds encounter in the course of an annual cycle. For example, it is advantageous to secrete less-volatile wax mixtures when incubating eggs in a nest accessible to predation by mammals that search by olfaction (Reneerkens et al. 2005).

Another selection pressure that may explain variation in preen-wax composition is the occurrence and density of feather parasites (Sweeney et al. 2004, Haribal et al. 2005). The plumage of birds harbors a variety of bacteria, many of which are able to degrade feathers (Burtt and Ichida 1999, 2004; but see Cristol et al. 2005). Degradation of feathers could increase thermoregulatory costs as a result of reduced insulation and increased heat loss, which could reduce body mass and survival (Booth et al. 1993, Clayton 1999). In addition, degradation of the flight feathers could reduce aerodynamic efficiency of the bird (Barbosa et al. 2002). Many feather-degraders are soil bacteria (Wood 1995, Lucas et al. 2003, Shawkey et al. 2005). Consequently, birds foraging on the ground have a higher incidence of feather-degrading bacteria than birds that forage in foliage, on bark, or in the air (Burtt and Ichida 1999). In warm, moist environments, vegetative cells of feather-degrading bacteria become metabolically active and degrade feathers rapidly (Burtt and Ichida 2004). Because relatively warm, moist conditions are created in the nest scrapes of incubating shorebirds (Ar and Sidis 2002), these birds are more likely to encounter metabolically active feather-degrading bacteria than non-incubating conspecifics or bird species that nest above the ground.

We tested whether a seasonal change in preen-wax composition might offer specific protection against feather-degrading bacteria in a ground-nesting sandpiper, the Red Knot (*Calidris canutus*). Preen wax inhibits the growth of feather-degrading and skin bacteria (Bandyopadhyay and Bhattacharyya 1996, Shawkey et al. 2003). Just before the breeding period, sandpipers (Scolopacidae) abruptly shift preen-wax composition from a mixture of short-chain monoesters to a more viscous mix of longer-chained diesters (Sinninghe Damsté et al. 2000, Reneerkens et al. 2002). Secretion of diester preen waxes by sandpipers occurs only during the weeks when the eggs are laid and incubated and only in individuals that incubate (Reneerkens et al. 2002, 2007b). These two facts suggest that the chemical shift is related to some demand of incubation. We quantified the effect of preen-wax composition on the growth of *Bacillus licheniformis*, a common feather-degrading bacterium found in many species of wild birds (Burtt and Ichida 1999).

Methods

Occurrence of feather-degrading bacteria in Red Knots.—In the summer of 2003, bacterial samples were collected from the plumage of seven Red Knots on the breeding grounds near Zackenberg Research Station on Wollaston Forland (74°28′N, 20°34′W), northeast Greenland, and from 28 Red Knots staging in the Dutch part of the Wadden Sea, on a high-tide roost on the sandbank Richel (53°17′N, 05°07′E). Samples were taken by wetting a sterile Dacron swab with sterile saline and rubbing it over the plumage of the birds. The swabs were resealed in their sterile packaging to prevent contamination and refrigerated at 5°C until processed.

Because the types of bacteria in the plumage were unknown and our goal was to identify feather-degrading bacilli (e.g., *B. licheniformis*) from among all those present, media were chosen to accommodate a broad spectrum of plumage bacteria from which we could isolate bacilli that degraded feathers. We used tryptic soy agar (TSA), a rich, nonselective medium, for the initial broad-spectrum collection of microorganisms. In the lab, the Dacron swabs from the field were streaked across plates of TSA media and then placed in test tubes of nutrient broth alkaline salt (NBas) solution. The TSA plates were incubated at 37°C. After 48 h, we removed the plates from incubation and counted the colonies. Plates with no bacterial growth were discarded. Colonies that showed the wrinkled, mounded morphology of *B. licheniformis* were transferred to tubes of NBas. All NBas tubes were incubated at 50°C for seven days with constant oscillation (120 rpm). The slightly alkaline (pH 7.7), slightly salty (7% NaCl) nutrient broth and prolonged high temperature favor the growth of *B. licheniformis* and inhibit the growth of most other microorganisms, including most other bacilli (Burtt and Ichida 1999). After seven days, bacterial growth was assessed. If the broth remained clear, the colony was not *B. licheniformis* and the broth culture was discarded. If the broth was cloudy, bacteria were cultured by cross-streaking the media on a sterile TSA plate and incubating it at 37°C for 48 h. We again checked the isolated colonies for the wrinkled, mounded appearance characteristic of *B. licheniformis*. Additionally, we used Gram-stain and oil-immersion light microscopy to identify each isolate. As seen under the microscope, *B. licheniformis* is positive for Gram stain, has a cylindrical, rod-like shape, and forms internal, centrally placed spores that do not cause a central swelling of the cell (Singleton 1997). If colonies on the plate were confirmed as *B. licheniformis*, we removed one with a sterile loop and inoculated a tube of TSA, which was incubated at 37°C for 48 h. The resulting culture, which was stored at 4°C, was a pure isolate of *B. licheniformis* from a known Red Knot. A sample of each isolate was later placed in media that contained a white secondary covert from a Domestic Goose (*Anser domesticus*) and incubated at 37°C. All isolates degraded feathers. A known strain of *Bacillus licheniformis* (OWU 1455), identified by its cellular fatty-acid profile, was cultured following the procedures described above and used for comparison when identifying bacterial isolates.

Collection of feathers and preen waxes.—Feathers were collected from 16 adult (i.e., >2 years old) Red Knots in full breeding plumage on 4 May and 17 June 2005, for use in an experiment on feather degradation. The birds were held in outdoor aviaries exposed to the local light regime at Texel, The Netherlands. The birds were mist-netted at high-tide roosts in the western part of the Wadden Sea and had been in captivity for four to nine years at the time of feather-sampling. The Red Knots showed annual cycles in mass, molt, and preen-wax composition similar to those of free-living conspecifics, even though they do not migrate or lay eggs (Reneerkens et al. 2007a). The birds had not molted their breast feathers between sampling dates. On both dates ≥0.16 g of feathers were collected with forceps to avoid rubbing wax off the feathers. A few milligrams of preen-gland secretions were collected by gently rubbing a cotton bud over the papilla of the uropygial gland.

These were the feathers and wax samples used throughout the procedures described below.

Gas-chromatography of preen waxes.—Preen-wax samples of all free-living and experimental birds were obtained immediately after a feather sample or bacterial swab was taken. The wax samples were dissolved in ethyl acetate to a concentration of 1 mg ml^{-1} and injected into a gas chromatograph (Shimadzu UV-1601) using an on-column injector. Detection was accomplished using a flame-ionization detector. Helium was the carrier gas. Separation of the chemical components was achieved using a fused-silica capillary column (Varian, 25 m × 0.32 mm i.d.) coated with CP-Sil 5CB (film thickness 0.12 μm). The samples were injected at 70°C, and the oven was subsequently heated to 130°C at 20°C min^{-1} followed by 4°C min^{-1} to 320°C, and held at this temperature for 35 min. Gas chromatograms of pure monoesters and diesters are easy to distinguish and identify visually on the basis of previous molecular analysis of the intact monoester and diester preen waxes (Dekker et al. 2000, Sinninghe Damsté et al. 2000). This enabled us to determine whether individual birds had preened either monoester or diester waxes onto their plumage. All captive birds secreted pure monoester preen waxes on 4 May, whereas the same birds secreted pure diester waxes on 17 June.

Treatment groups.—The feathers collected from the 16 adult captive Red Knots were used to compare bacterial degradation of feathers coated with different preen-wax mixtures. Half of the samples from each collection date were placed in ethyl acetate, a solvent of hydrophobic waxes, and gently shaken in an automatic shaker. After 8 h, the feathers were taken out of the ethyl acetate and air-dried. Gas chromatograms of the ethyl acetate that had been used to wash the feathers showed the peak pattern typical for monoester or diester preen waxes of Red Knots. The ethyl acetate removed part or all of the preen waxes. With a scanning electron microscope, we made 30 photographs of four untreated feathers and four feathers from which preen waxes were removed with ethyl acetate. We had no prior knowledge of whether the feathers were untreated or had the wax removed when we examined them for any signs of damage (holes, broken barbules), paying special attention to where barbules connect to barbs. The ethyl acetate did not affect the feathers in any way that we could see. The washed feathers were used to measure the growth of *B. licheniformis* on feathers without waxes. In addition to looking for photographic evidence of damage, we incubated two uninoculated samples of washed feathers and one uninoculated sample of unwashed feathers to serve as controls for the effect of shaking on washed and unwashed feathers in the absence of bacteria.

Feather-degrading experiment.—We followed the procedure of Goldstein et al. (2004), in which bacterial degradation of feathers was measured by determining the concentration of oligopeptides in feather medium inoculated with *B. licheniformis*. Oligopeptides are a breakdown product of bacterial degradation of β-keratin, the structural protein of feathers (Goldstein et al. 2004).

We placed 0.075 g of feathers per treatment group in 25 mL of feather medium (9.34 mM NH_4Cl, 8.55 Mm NaCl, 1.72 mM K_2HPO_4, 2.92 mM KH_2PO_4, 0.49 mM $MgCl_2$-$6H_2O$, and 0.01% yeast extract; Williams et al. 1990) in 125-mL Erlenmeyer flasks with lids. The flasks were sterilized in an autoclave for 20 min at 15 psi and 120°C. Gas chromatography of heated and unheated preen wax showed no difference in the structure of the different preen waxes.

After the flasks had cooled, they were inoculated with *B. licheniformis* strain OWU 138B (available from the American Type Culture Collection as strain ATCC 55768). To prepare the inoculum, we transferred a small sample of strain 138B from an isolation tube to a 250-mL flask containing 100 mL of Luria broth and incubated the flask at 37°C and 120 rpm. After 24 h, we removed 2.5 mL of bacteria and broth from the flask and placed them in 15-mL tubes. The tubes were centrifuged for 10 min at 4,500 rpm to separate the broth from the bacteria. We discarded the broth, resuspended the bacteria in 1 mL of feather medium, and added 0.1 mL to each 125-mL flask described above.

Following inoculation, the flasks were put into a 37°C incubator, rotating at 120 rpm. After 96 h, 0.5 mL was removed from each flask and diluted with 0.5 mL of feather medium to obtain an adequate volume for measuring the absorbance. The sample was centrifuged for 10 min at 4,500 rpm to sediment the feather fragments and bacteria. The absorbance of the supernatant was measured at a wavelength of 230 nm with a Beckman DU UV/VIS spectrophotometer. At this wavelength, light is maximally absorbed by the oligopeptides (Goldstein et al. 2004). The samples were discarded after measurement. The concentration of oligopeptides stabilized after 96 h for some feather samples. For that reason, and because an earlier pilot study showed that the oligopeptide concentration increased linearly during the first four days, we decided to use the oligopeptide concentration 96 h after inoculation as our measure of feather degradation.

We had to know the initial concentration of oligopeptides in the solution to measure feather degradation by *B. licheniformis*; therefore, a first measurement was taken after 1 h of incubation without bacteria, when the medium was well mixed. Following measurement, the flasks were inoculated with *B. licheniformis*. We subtracted these initial light-absorbance values from those measured after 96 h to correct for oligopeptides (and other possible contaminants) in the feather medium that are not attributable to feather degradation by the inoculum.

The data were analyzed with a repeated-measures analysis of variance (ANOVA) with two within-subjects ("presence of wax" and "wax composition"). One of the four measurements was missing from three birds, and these individuals were excluded from the analysis. Another individual was excluded because absorbance values were clear but unexplained outliers for all treatments (maximal absorbance of 0.0614).

Results

Occurrence of feather-degrading bacteria.—The six incubating Red Knots captured on the breeding grounds in Greenland had preen wax that contained only diesters. The one chick-guarding bird secreted preen wax that contained only monoesters. All 28 migrating Red Knots captured in the Wadden Sea secreted preen wax that contained only monoesters. This follows closely the pattern described by Reneerkens et al. (2002, 2007b), who showed that only incubating sandpipers secrete diester preen waxes. Feather-degrading *B. licheniformis* occurred only in Red Knots that secreted monoester preen wax. This included the single chick-guarding bird that secreted monoester preen wax at the breeding

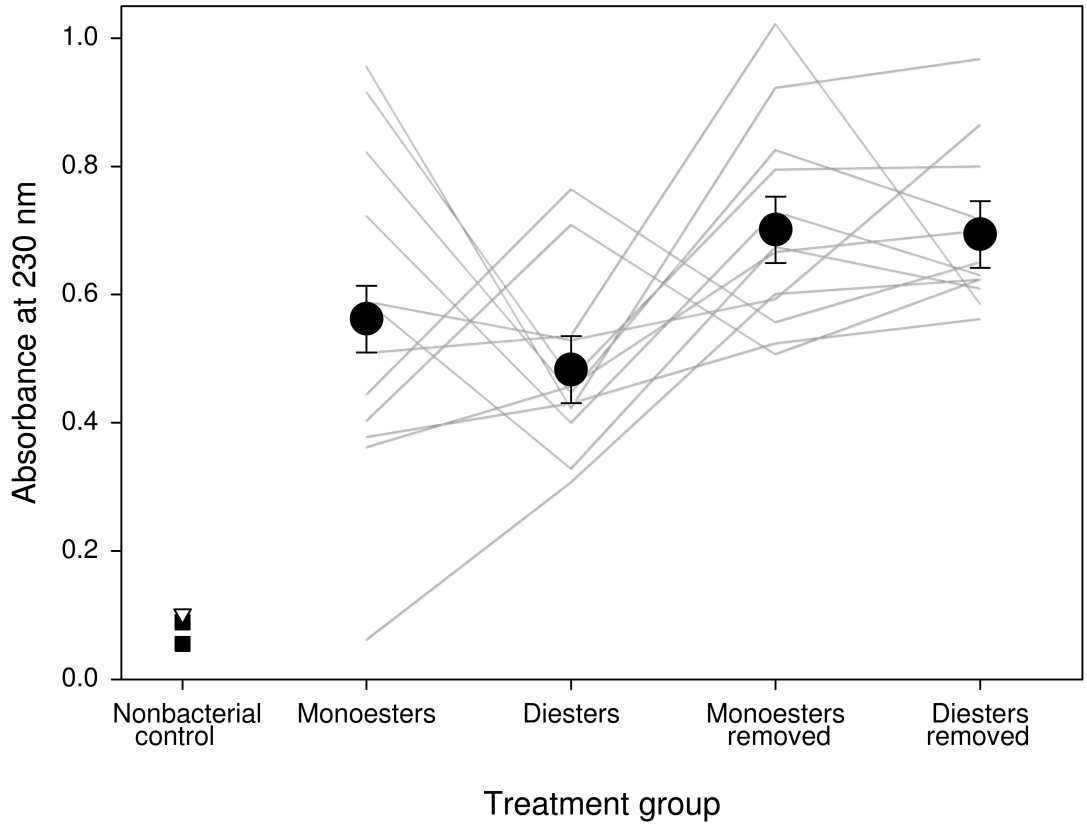

Fig. 1. Absorption of radiation at 230 nm by media containing dissolved oligopeptides of β-keratin released after 96 h of degradation by *B. licheniformis* from Red Knot feathers preened with monoesters, with diesters, or without monoester or diester waxes, as well as the three nonbacterial controls (black squares: feathers in medium treated with ethyl acetate; white triangle: feather medium only). Symbols represent the means in accordance with the repeated-measures ANOVA we used (i.e., least-square means, which are the means after correction for inter-individual variation). Error bars represent standard errors of these means. Gray lines connect the absorbance measurements of each replicate.

grounds and 6 of 28 Red Knots captured on migration in the Wadden Sea. The sample sizes are too small for statistical analysis.

Effects of preen waxes.—The rate of degradation by *B. licheniformis* of feathers with a coat of monoester waxes did not differ from that of feathers with a coat of diester waxes (repeated-measures ANOVA, $F = 0.699$, df = 1 and 44, $P = 0.408$), but removal of the wax from these feathers significantly increased bacterial breakdown of the feathers (repeated-measures ANOVA, $F = 11.480$, df = 1 and 44, $P = 0.001$; Fig. 1). The interaction between "presence of wax" and "preen-wax composition" was not significant (repeated-measures ANOVA, $F = 0.498$, df = 1 and 44, $P = 0.484$). Feathers incubated in the absence of *B. licheniformis* did not degrade, regardless of the presence or absence of preen wax.

Discussion

Red Knots harbor feather-degrading bacilli in their plumage on the High Arctic breeding grounds and at intertidal migration stopover sites in temperate climates. This is the first evidence that *B. licheniformis* occurs in sandpipers (Scolopacidae). Its occurrence supports the conclusion of Burtt and Ichida (1999), based on the pattern of occurrence in passerines, that *B. licheniformis* would be found in the plumage of all avian taxa. Sample sizes were too small to draw definite conclusions about the differential occurrence of *B. licheniformis* in plumages of breeding Red Knots that secrete diester preen waxes and nonbreeding individuals that secrete monoesters, though the trend is for *B. licheniformis* to occur only in Red Knots secreting monoesters. Future study of this trend is needed.

This is the first time that growth inhibition of feather-degrading bacteria has been tested with feathers to which preen waxes were applied by the birds themselves. We show that preen waxes, in the amounts preened onto the feathers by Red Knots, effectively diminish feather degradation. These results are consistent with those of disc-diffusion experiments (Shawkey et al. 2003) that showed that preen wax of House Finches (*Carpodacus mexicanus*) delayed the growth of *B. licheniformis*.

It remains to be investigated whether *B. licheniformis* is able to degrade feathers on living birds under natural conditions. Cristol et al. (2005) could not detect feather damage caused by experimentally applied bacteria on plumages of captive songbirds. However, they could not exclude the possibility that no feather damage was found because of preening, sunning (Saranathan and Burtt 2007), or other maintenance behavior of the birds. Cristol

et al. (2005) also argued that the optimal growing conditions for *B. licheniformis* (temperatures around 45°C, humid conditions) do not often occur under natural circumstances. Although the temperatures in clutches incubated by High Arctic breeding shorebirds are ~36°C (Cresswell et al. 2004), the temperature of the plumage in these conditions is probably higher and may approach the optimal temperature for *B. licheniformis*. Additionally, *B. licheniformis* can grow at temperatures below its optimum, and the humid microclimate in bird nests (Ar and Sidis 2002) would favor such growth. However, diester preen waxes secreted during incubation, when the damp, warm environment of the nest scrape may favor bacterial growth, did not protect the plumage from potential bacterial degradation better than monoesters, which are secreted at all other times and by nonincubating individuals.

If we want to understand inter- and intraspecific variation in preen-wax composition in the light of coevolution with microbes on birds' plumage (Shawkey et al. 2003, Sweeney et al. 2004), the mechanisms responsible for the inhibition or enhancement of microbial growth by preen waxes needs to be understood. How do preen waxes inhibit bacterial growth on feathers? Shawkey et al. (2003) suggested that preen waxes act as a chemical repellent in which alkyl-substituted fatty acids and alcohols are antimicrobial agents. Indeed, Jacob et al. (1997) showed that 3,7-dimethyloctan-1-ol, one of the products of hydrolysis of preen wax of Northern Gannets (*Morus bassanus*), negatively affects growth of Gram-positive bacteria and dermatophytes. However, preen waxes of most bird species consist of esters, which are fatty acids condensed to alcohols, but free fatty acids and alcohols rarely occur in preen-wax secretions (Jacob 1976, Jacob and Ziswiler 1982, Dekker et al. 2000, Sweeney et al. 2004), even in the preen waxes of Northern Gannets (Jacob et al. 1997). It remains to be seen whether hydrolysis of preen waxes takes place under natural conditions (e.g., under the influence of ultraviolet light or by bacteria that use waxes as a substrate).

Our study suggests that the chemical composition of the wax esters does not affect their antibacterial properties, at least not against *B. licheniformis*. Preen-gland secretions consist of complex mixtures, often of >100 different types of wax esters that vary in chain length and branching (Jacob and Ziswiler 1982, Haribal et al. 2005). The chemical composition of the preen-wax mixtures affects their physical characteristics (e.g., melting temperatures; Patel et al. 2001). However, all avian preen waxes consist of chemically stable esters. Therefore, we propose that preen waxes do not chemically combat microbes but form a physical barrier between microbes and feathers.

More knowledge of the physical aspects of preen-wax esters, and of the (micro)distribution of preen waxes on the plumage, will be required to test this idea. Although diesters are larger molecules than monoesters (Sinninghe Damsté et al. 2000), which should affect mechanical properties, the different preen-wax mixtures found in Red Knots did not differ in their ability to inhibit growth of feather-degrading bacteria. Future descriptive and experimental studies of the function of inter- and intraspecific variation in preen waxes in an ecological context need to consider the chemical and physical aspects of the secretions. Such studies should not only focus on the interaction between preen-wax secretions and microbial flora (Shawkey et al. 2003) or ectoparasites (Moyer et al. 2003), but should also consider other selective factors, such as mate choice and predation (cf. Reneerkens and Korsten 2004, Reneerkens et al. 2005), and include (seasonal) quantitative variation in preen-wax secretion (Bhattacharyya and Roy Chowdhury 1995, Montalti and Salibián 2000).

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