Seasonally changing preen wax composition: red knots’ flexible defense against feather-degrading bacteria?

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Submitted

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 has been positively identified in the plumage of breeding and non-breeding red knots) on the feathers of red knots with natural amounts of mono- or diester preen waxes. The removal of preen waxes from feathers resulted in faster degradation of the feathers, confirming earlier studies that preen wax inhibits growth of feather-degrading bacteria. However, the rate of degradation of feathers with preen wax based on diesters did not differ from that of feathers with preen wax based on monoesters. We argue that preen waxes protect feathers by forming a physical barrier to microbes rather than by chemical properties of the waxes, such as acidity.
Introduction

Most birds apply waxes secreted by their preen gland onto their feathers as part of maintenance behavior (Jacob & Ziswiler 1982). The secreted preen waxes are species-specific, complex 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, UV 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 to branched fatty acids (Sweeney et al. 2004), and thus might occur more often in waterbirds. Furthermore, preen wax composition sometimes varies intra-specifically based on season (Jacob et al. 1979, Kolattukudy et al. 1987, Piersma et al. 1999, chapter 2). Such seasonal variation in preen wax composition may be the result of different selection pressures that birds encounter in the course of an annual cycle. For example, it is advantageous to secrete less volatile wax mixtures in periods when birds are exposed to a high risk of predation by mammals that search by olfaction (e.g., when incubating a clutch in an easily accessible nest; chapter 7).

Another selection pressure that may explain variation in preen wax composition is the occurrence and density of feather parasites (Sweeney et al. 2004). The plumages of birds harbor a variety of bacteria, many of which are able to degrade feathers (Burtt & Ichida 1999, 2004, but see Cristol et al. 2005). Degradation of feathers could increase thermoregulatory costs as a result of reduced insulation, increased heat loss and, as a consequence, reduced 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). 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 & Ichida 1999). In warm, moist environments vegetative cells of feather-degrading bacteria become metabolically active and degrade feathers rapidly (Burtt & Ichida 2004). Because relatively warm, moist conditions are created in the nest scrapes of incubating shorebirds (Ar & 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 test 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 has been shown to inhibit the
growth of feather-degrading and skin bacteria (Bandyopadyay & Bhattacharyya 1996, Shawkey et al. 2003). Just before the breeding period, sandpipers (Scolopacidae) show an abrupt shift in preen wax composition from preen waxes based on short-chained monoesters to more viscous secretions based on longer-chained diesters (Sinninghe Damsté et al. 2000, chapter 2). Secretion of diester preen waxes by sandpipers occurs only during the weeks when the eggs are laid and incubated and only in individuals (or sexes) that incubate (chapter 2 and 3). 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 & 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 (74º 28’ N, 20º 34’ W), Northeast Greenland and of 28 red knots staging in the Dutch part of the Wadden Sea, on a high tide roost on the sandbank Richel (53º 17’ N, 05º 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 re-sealed 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 B. licheniformis and other potential feather-degrading bacteria, media were chosen to accommodate different growth preferences and select for bacteria known to degrade feathers (B. licheniformis, in particular). These selective media included Tryptic Soy Agar (TSA), Glycerine Asparine Agar (GAA), Tomato Paste Oatmeal Agar (TPO), Yeast Maltose Agar (YMA) and Nutrient Broth Alkaline Salt solution (NBas). TSA and NBas were used as a selective medium for Bacillus sp.; GAA, TPO and YMA were used to isolate Actinomycetes. In the lab, the bacterial swabs were streaked across plates of TSA, GAA and TPO Media and then placed in test tubes of NBas. Plates were incubated at 37°C (TSA), or 28°C (GAA, YMA and TPO plates) for 48 hrs. After 48 h agar plates were removed from incubation and colonies were counted. Plates that did not show evidence of bacterial growth were discarded. Sterile loops were used to streak single colonies onto fresh plates of TSA and YMA media. These plates were incubated at 37°C (TSA) or 28°C (YMA) for 48 hrs. A sterile loop was used to inoculate tubes of the same media with an isolated colony and these tubes were in-
cubated for 48 hrs, as described above. The resulting tubes contained isolates of bacteria and were stored at 4°C. Media-specific keys were used to classify bacteria based on colony morphology. For example, *B. licheniformis* colonies were identified by their wrinkled, mounded appearance. Additionally, we used Gram-staining and oil immersion light microscopy to classify the bacteria from each isolate based on a positive or negative Gram stain and basic morphological characteristics, such as the rod shape of bacilli (Singleton 1997).

NBas tubes were incubated at 50°C for seven days with constant oscillation. The modified nutrient broth and high temperature favor the growth of *B. licheniformis* and inhibit the growth of most other microorganisms (Burtt & Ichida 1999). After seven days, tubes were removed and bacterial growth was assessed. If the broth remained clear, the colony was not *B. licheniformis* and the broth culture was discarded. If the broth became cloudy, bacilli were cultured by cross-streaking a loopful of the media on a sterile TSA plate and incubating it at 37°C for 48 hrs. If colonies grew, we removed one with a sterile loop and inoculated a tube of TSA, which was incubated at 37°C for 48 hrs. The resulting culture, which was stored at 4°C, was a pure isolate of *B. licheniformis* from a known red knot.

A known strain of *Bacillus licheniformis* (OWU 1455) was cultured following the procedures described above and used for comparison when identifying bacterial isolates. We did not grow control cultures of bacteria other than *B. licheniformis*. Details on preparation and identification of (feather-degrading) bacteria in feathers of red knots are given in table 6.1.

### Collection of feathers and preen waxes

Feathers were collected from 16 adult (i.e. more than 2 years old) red knots that were held in outdoor aviaries exposed to the local light regime at Texel, The Netherlands. The birds were caught with mistnets at high tide roosts in the western part of the Wadden Sea and had been in captivity for 4 to 9 years at the time of feather sampling. The red knots showed annual cycles in mass, molt and preen wax composition (chapter 4) similar to free-living conspecifics. On 4 May 2005 preen wax and feathers were collected from 17 birds in full breeding plumage. On 17 June 2005 these birds were sampled again. The birds had not molted their breast feathers between sampling dates. On both dates at least 0.16 g of feathers were collected with a pair of forceps to avoid rubbing wax off the feathers. A few mg of preen gland secretions were collected by gently rubbing a cotton bud over the papilla of the uropygial gland.

**Table 6.1 (right)** Description of the bacteria identified in plumages of red knots. In some occasions more than one colony of bacteria was isolated from swabs of individual birds.
<table>
<thead>
<tr>
<th>Ring no.</th>
<th>Date; location</th>
<th>Preen wax</th>
<th>Preparationa</th>
<th>Gram stain; description</th>
<th>Description plate growth</th>
<th>Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>8882036</td>
<td>July 4; NE Greenland</td>
<td>Diesters</td>
<td>TSA-TSA</td>
<td>+ ; Cocci in random packets</td>
<td>Glistening, white, raised; cocci</td>
<td>Staphylococcus sp.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TSA-YMA</td>
<td>+ ; Cocci in random packets</td>
<td>Small, white, raised, glistening colonies</td>
<td>Staphylococcus sp.</td>
</tr>
<tr>
<td>8882059</td>
<td>July 29; NE Greenland</td>
<td>Monoesters</td>
<td>NBas-TSA</td>
<td>+ ; Scattered rods, some in clusters</td>
<td>Raised, wrinkled, cream-colored, matte; spore-forming rods</td>
<td>B. licheniformis</td>
</tr>
<tr>
<td>Z023407</td>
<td>Aug. 29; Wadden Sea</td>
<td>Monoesters</td>
<td>NBas-TSA</td>
<td>+ ; Rods in net-like pattern</td>
<td>Raised, wrinkled, cream-colored, matte; spore-forming rods</td>
<td>B. licheniformis</td>
</tr>
<tr>
<td>Z023408</td>
<td>Aug. 29; Wadden Sea</td>
<td>Monoesters</td>
<td>NBas-TSA</td>
<td>+ ; Rods arranged in net-like pattern</td>
<td>Raised, wrinkled, cream-colored, matte; spore-forming rods</td>
<td>B. licheniformis</td>
</tr>
<tr>
<td>Z023411</td>
<td>Aug. 29; Wadden Sea</td>
<td>Monoesters</td>
<td>TSA-TSA</td>
<td>+ ; Even-numbered packets of cocci</td>
<td>Whitish opaque circular colonies; cocci</td>
<td>Unknown cocci</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NBas-TSA</td>
<td>+ ; Rods arranged in net-like pattern</td>
<td>Raised, wrinkled, cream-colored, matte; spore-forming rods</td>
<td>B. licheniformis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TSA-YMA</td>
<td>+ ; Even-numbered packets of cocci</td>
<td>Small, white, raised, glistening colonies</td>
<td>Unknown cocci</td>
</tr>
<tr>
<td>Z023412</td>
<td>Aug. 29; Wadden Sea</td>
<td>Monoesters</td>
<td>TPO-TSA-TSA</td>
<td>+ ; Scattered rods</td>
<td>Raised, wrinkled, cream-colored, matte; spore-forming rods</td>
<td>B. licheniformis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TPO-TSA-TSA</td>
<td>+ ; Scattered rods</td>
<td>Raised, wrinkled, cream-colored, matte; spore-forming rods</td>
<td>B. licheniformis</td>
</tr>
<tr>
<td>Z023414</td>
<td>Aug. 30; Wadden Sea</td>
<td>Monoesters</td>
<td>TSA-TSA</td>
<td>+ ; Bacilli with spores</td>
<td>Widespread translucent feathery colonies; two large clumps of pinkish-brown filamentous cells</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TPO-YMA-YMA</td>
<td>+ ; Rods with some spore-looking structures</td>
<td>Actinomycetes</td>
<td>Streptomyces sp.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TSA-YMA</td>
<td>+ ; Short rods scattered throughout</td>
<td>Actinomycetes</td>
<td>Streptomyces sp.</td>
</tr>
<tr>
<td>Z023424</td>
<td>Sept. 2; Wadden Sea</td>
<td>Monoesters</td>
<td>TSA-TSA</td>
<td>+ ; Cocci</td>
<td>Yellow translucent, circular colonies; cocci</td>
<td>Unknown cocci</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TSA-YMA</td>
<td>+ ; Cocci</td>
<td>Large, raised, yellow, glistening colonies with irregular shape</td>
<td>Unknown cocci</td>
</tr>
<tr>
<td>Z023426</td>
<td>Sept. 2; Wadden Sea</td>
<td>Monoesters</td>
<td>NBas-TSA</td>
<td>+ ; Rods arranged in net-like pattern</td>
<td>Raised, wrinkled, cream-colored, matte; spore-forming rods</td>
<td>B. licheniformis</td>
</tr>
<tr>
<td>Z023433</td>
<td>Sept. 4; Wadden Sea</td>
<td>Monoesters</td>
<td>TSA-TSA</td>
<td>+ ; Cocci, many in even-numbered clusters</td>
<td>Glistening, white circular; cocci</td>
<td>Unknown cocci</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TSA-YMA</td>
<td>+ ; Cocci in even-numbered clusters</td>
<td>Small, white, raised, glistening colonies</td>
<td>Unknown cocci</td>
</tr>
<tr>
<td>Z023436</td>
<td>Sept. 4; Wadden Sea</td>
<td>Monoesters</td>
<td>TSA-TSA</td>
<td>+ ; Long rods, some in clusters</td>
<td>Raised, wrinkled, cream-colored, matte; spore-forming rods</td>
<td>B. licheniformis</td>
</tr>
</tbody>
</table>

* The order of which (sub)cultures were transferred to plates with different media.
Gas chromatography of preen waxes
Preen wax samples of all 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-ionisation detector. Helium was the carrier gas. Separation of the chemical components was achieved using a fused-silica capillary column (Varian, 25 m x 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 mono- or diesters are easy to distinguish and identify visually based on 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 mono- or diester preen waxes onto their plumage. All birds secreted pure monoester preen waxes on 4 May, whereas the same birds secreted pure diester waxes on 17 June. The birds had not molted their breast feathers between the two sampling dates.

Treatment groups
To compare bacterial degradation of feathers coated with different preen wax compositions, feathers were collected from the 16 adult red knots. 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 hrs 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 mono- or diester preen waxes of red knots. The ethyl acetate removed part or all of the preen waxes. We made thirty photographs with a Scanning Electron Microscope of four untreated feathers and four feathers of which preen waxes were removed with ethyl acetate. We coded the pictures to remove knowledge of whether the feathers were untreated or had the wax removed and 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) to measure bacterial degradation of feathers. Here, bacterial growth is indirectly measured by determining the concentration of oligopeptides in a medium of *B. licheniformis* with feathers. Oligopeptides are a by-product of bacterial degradation of β-keratin, the structural protein of feathers (Goldstein et al. 2004).

Replicates of 0.075 g feathers of each treatment group were put in 25 ml feather medium (9.34 mM NH₄Cl, 8.55 Mm NaCl, 1.72 mM K₂HPO₄, 2.92 mM KH₂PO₄, 0.49 mM MgCl₂·6H₂O and 0.01% yeast extract) in 100 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 down, 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 nutrient broth from the flask and placed them in 15 ml tubes. The tubes were centrifuged for 10 min at 4500 rpm to separate the nutrient broth from the bacteria. The nutrient broth was discarded and the bacteria were resuspended in 1 ml of feather medium and added to the 100 ml flasks described above.

Following inoculation the flasks were put in a 37°C incubator, rotating at 120 rpm. After 96 hrs 0.5 ml was removed from each flask and diluted with 0.5 ml of feather medium in order to obtain an adequate volume to measure the absorbance. The sample was centrifuged for 10 min at 4500 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 increase in oligopeptides leveled off after 96 hrs 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 hrs after inoculation as our measure of feather degradation.

The initial quantity of oligopeptides in the solution had to be known in order to measure feather degradation by *B. licheniformis*; therefore, a first measurement was taken after one hr of incubation without bacteria, when the medium was well mixed, but bacteria had produced few oligopeptides. We subtracted these initial light absorbance values from those measured after 96 hrs to correct
for oligopeptides (and possible other proteins) in the feather medium that are not due to feather-degradation by the inoculum.

The data were analyzed with a repeated measures 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
Six of the seven red knots captured on the breeding grounds in Greenland had preen wax that contained only diesters and one chick-guarding bird secreted monoester preen wax. 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. (chapter 2) who showed that only incubating sandpipers secrete diester preen waxes. Bacteria found in the samples included Streptomyces sp., Staphylococcus sp., B. licheniformis and unidentified cocci. The feather degrading B. licheniformis occurred only in red knots that secreted monoester preen wax (table 6.1). This included the single, chick-guarding, bird that secreted monoester preen wax at the breeding grounds and 6 of 28 red knots during migration in the Wadden Sea. In addition, we cultured Staphylococcus sp. from one diester-secreting individual, and Streptomyces sp. and some unspecified cocci in plumages of monoester-secreting individuals during migration in the Wadden Sea. Details about the identified bacteria are given in table 6.1. The sample sizes are too small for sufficient statistical power to draw conclusions from these frequencies.

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 $F_{44,1} = 0.699, P = 0.408$), but the removal of the wax coat from these feathers significantly increased the bacterial breakdown of the feathers (repeated measures ANOVA $F_{44,1} = 11.480, P = 0.001$; fig. 6.1). The interaction between ‘presence of wax’ and ‘preen wax composition’ was not significant (repeated measures ANOVA $F_{44,1} = 0.498, P = 0.484$). Feathers incubated in the absence of B. licheniformis did not degrade regardless of the presence or absence of preen wax.
Discussion

Here we show for the first time that red knots harbor feather-degrading bacilli in their plumage during incubation 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 & 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 non-breeding individuals that secreted monoesters, although it is striking that *B. licheniformis* only occurred in monoester secreting red knots. Future study of seasonal changes in presence of feather bacteria 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

![Figure 6.1](image_url)
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 under natural conditions on living birds. 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 due to preening, sunning (Saranathan & Burtt 2007), or other maintenance behavior of the birds. They also argued that the optimal growing conditions for *B. licheniformis* (temperatures around 45 ºC, humid conditions) do not often occur under natural circumstances (Cristol et al. 2005). Although the temperatures in clutches incubated by High Arctic breeding shorebirds are approximately 36 ºC (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, the microclimate in bird nests is relatively humid (Ar & Sidis 2002). However, diester preen waxes secreted during incubation, when the damp, warm environment of the nest scrape may favor bacterial growth, did not protect of the plumage from potential bacterial degradation better than the usually secreted monoesters.

If we want to understand inter- and intraspecific variation in preen wax composition in the light of co-evolution 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 need to be understood. How would 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 anti-microbial agents. Indeed, Jacob et al. (1997) showed that 3,7-dimethyloctan-1-ol, one of the products of hydrolysis of preen wax of 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 or alcohols rarely occur in preen wax secretions (Jacob 1976, Jacob & Ziswiler 1982, Dekker et al. 1999, Sweeney et al. 2004), and not even in the preen waxes of 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 anti-bacterial capacities. Preen gland secretions consist of complex mixtures of often more than one hundred different types of wax esters that vary in chain length and branching (Jacob & 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 as well as on 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. chapters 5, 6 and 7) and also include (seasonal) quantitative variation in preen wax secretion (Bhattacharyya & Roy Chowdhury 1995, Montalti & Salibián 2000).

Incubating red knots create a relatively warm and humid microclimate in the nestcup that is likely also favourable for the growth of Bacillus licheniformis.
**Acknowledgements**

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**Box D  The effect of preen wax on the abrasion-resistance of primary feathers: a field experiment on High Arctic breeding sandpipers**

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Most birds possess a preen, or uropygial, gland from which lipid secretions (preen waxes) are smeared with the bill onto the feathers during preening activities (Jacob & Ziswiler 1982). Despite the ubiquity of the preen gland among birds, it still is largely unclear which function(s) the secreted waxes serve. The available experimental tests indicate that presence of preen wax reduces growth of feather-degrading bacteria (Shawkey et al. 2003, chapter 6), repels feather lice (Moyer et al. 2003) and that seasonal changes in preen wax composition cause a temporal (relative) olfactory crypsis against mammalian predators that use smell to locate prey (chapter 7). In some bird species, the preen gland secretions enhance colouration of the plumage (Delhey et al. in press), but not in others (chapter 5). Many more functions of preen wax have been proposed in the past but, to our knowledge, experimental evidence for these functions are lacking.

In the old discussion about the possible functions of preen waxes, an often proposed function is the protection of feathers against tear and wear (e.g. Elder 1954, Jacob & Ziswiler 1982). Feathers may wear by ultraviolet radiation (Bergmann 1982) and by contact with hard objects such as vegetation and airborne particles (Burtt & Ichida 2006) and by degradation by ectoparasites (Clayton 1990). That feather wear can have substantial effects
that accumulate over time becomes obvious when we consider that in great snipes *Gallinago media* individuals in their first summer plumage, that renew their primary feathers only in the next summer, can be distinguished from adults in summer plumage, that have primary feathers that are created in the past autumn, by the wear of their primary wing tips (Saether *et al.* 1994). In coastal shorebirds, in the middle of the non-breeding season, juveniles (that have carried their primaries for longer than adults) generally can be distinguished on the basis of primary wear (Prater *et al.* 1977).

It has been shown that different kind of feather keratins differently affect abrasion resistance, in which melanised feathers are stronger and resist tear and wear and bacterial degradation better than unmelanised feathers (Burtt 1981, 1986, Goldstein *et al.* 2004). Although recent studies show that preen wax protects feathers against ectoparasites that may cause feather degradation (Bandyopadhyay & Bhattacharyya 1996, 1999, Moyer *et al.* 2003, Shawkey *et al.* 2003, chapter 6), the effects of preen wax against physical wear and tear have remained unexplored. Rutschke (1960) suggested that preen waxes penetrate into the medulla cells of the barbs and shafts of feathers and thereby increase the flexibility of feathers that thereby break less easily. This effect and the penetration of preen wax into feathers itself have, however, never been substantiated. Here we examine the role of preen waxes secreted by sandpipers during incubation (consisting of diesters only, chapter 2) on the abrasion resistance of feathers in a relevant field context.

**Methods**

During the arctic summer of 2003 we tested this hypothesis in Zackenberg, Northeast Greenland (74°30’N, 20°30’ W). From 17 June till 15 July 2003 we searched for nests of dunlins *Calidris alpina arctica*, ruddy turnstones *Arenaria interpres*, sanderlings *Calidris alba*, red knots *Calidris canutus islandica* in a tundra area of more than 4 km² in the vicinity of the research station (Piersma *et al.* 2006). During the incubation period we tried to catch both of the incubating adults from the nest by use of small clap-nets. A small smear from the preen gland for chemical analysis in the laboratory of the Royal NIOZ was collected following procedures described by Reneerkens *et al.* (chapter 2).
Field experiment

We cut off 1 cm long tips of both the left and right 8th primary (P8; wing feather) with sharp scissors of the caught birds and collected the feather tips into small glass vials. The vials containing the feather tips were stored at 5 °C and kept still to avoid any possible damage to the feathers by scratching against the sides of the vial. The feather tips of the P8's served as a control for possible \textit{a priori} differences in abrasion between the two wings that were not related to the experimental treatment. The treatment consisted of chemically removing preen wax from a randomly chosen (left or right) wing by dissolving the wax into ethyl acetate, a potent solvent of hydrophobic waxes. This was done by stirring around the wing tip in a glass vial filled with ethyl acetate for ca. 30 sec, after the P8 feather tips were collected. The ethyl acetate, which is volatile at normal ambient temperatures, was allowed to evaporate from the wing in the field after which the birds were released again. The individual birds were recaptured on their nests after 8 days on average (range 1–28 days). At recapture, feather tips of the ninth primary (P9) were collected following the same procedure as for the P8 earlier. The ninth primary tips were collected to look for a treatment effect of preen wax removal. Our null-hypothesis was that removing preen wax would not result in different abrasion of the wing feathers during the days between preen wax removal and recapture. The left and right wings could be compared within an individual as preen wax was removed from only one of the two wings. In total 28 birds were treated and recaptured; nine dunlins, one red knot, three ruddy turnstones, and fourteen sanderlings. An additional twenty-seven birds were treated but could not be recaptured for the collection of the P9's.

The feather tips were studied with a dissection microscope (magnification 20–40) and scored for abrasion. The first 15 barbs starting from the tip of the feather both from the inner and the outer vane were given an abrasion score between 0 and 5. A score of 0 was an intact non-abraded barb, 1: a tiny tip of the barb was worn, 2: a small tip of the barb was broken off, 3: a significant part of barb missing, 4: up to half of the barb missing, 5: more than half of the barb missing. The scores of the 15 barbs from the inner and outer vane were added up to a ‘total abrasion score’.

The total abrasion scores were not normally distributed. Hence, we used a Wilcoxon paired-sample test to test for \textit{a priori} differences in total abrasion scores of the P8 on the side to be treated and the side that was not going to be treated with ethyl acetate, and similarly for treatment effects on the P9's.
Results

Total abrasion scores ranged between 6 and 135 (fig. D1). The control feather tips (P8) of left and right were not different from each other (Wilcoxon Signed Rank test, P = 0.484, fig. D2). Abrasion scores of the P9 with or without temporary wax removal also did not differ (Wilcoxon Signed Rank test, P = 0.484). As expected, given its position more to the end of the wing, tips of P9 were abraded more than the tips of P8 (Wilcoxon Signed Rank test, P < 0.001).

![Figure D1](image)

*Figure D1* An example of two feather tips of sanderlings with the most extreme abrasion scores encountered. The feather tip in (A) had a total abrasion score of 6 and a score of 135 in (B). The used microscope magnification for both pictures is 40.

Discussion

The removal of preen waxes from the wing tip did not result in a significantly different abrasion within the 1-28 days that the experiment lasted. There are several possible explanations for the lack of an effect of chemical preen wax removal on primary wing feather abrasion. First of all, the occurrence of preen wax on feathers might not play a biological role in the protection of feathers against tear and wear. It can, however, not be excluded that the birds had preened wax onto their treated wing, soon after we had experimentally removed the wax. In any case, however, (part of) the preen wax will have been temporally absent from the wing feathers. It is also possible that the experiment lasted too shortly for significant abrasion in feathers to take place.
The different abrasion scores between P8 and P9 suggest that outer primaries are more subjected to wear than more inner wing feathers. This can possibly be explained by the fact that outer primaries are more exposed and cover larger distances with each wing movement. This aspect deserves further attention, as it may be interesting with respect to the evolution of moulting strategies and/or preening behaviour.

**Figure D2** Total abrasion scores of the eight (P8) and ninth primaries (P9) of 28 shorebirds. A distinction is made between the feather tips that were treated with ethyl acetate to remove preen waxes and those that were not treated. Note that the P8’s served as a control and both sides were never treated with ethyl acetate. The boxes enclose 50% and vertical lines 95% of the value. The small dots are outliers. Black dots represent the average values, the dividing lines the median.

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