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Interactions between Feather-Degrading Bacteria and Feather Coloration

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Interactions between Feather-degrading Bacteria and Feather Coloration

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Bacteria that can damage feathers have been isolated from the plumage of several avian species. These feather-degrading bacteria (FDB) may contribute to feather wear and could thus influence avian fitness and evolution, as do feather-feeding lice. Relatively little research has addressed how birds interact with FDB, but recent work suggests that avian traits such as feather pigmentation and/or preen oil composition may, in part, be adaptive defenses against FDB. FDB have also been theorized to positively influence some birds by enhancing color characteristics. My research has attempted to answer two questions: 1) Does feather melanization make feathers resistant to bacterial degradation relative to unpigmented feathers?, and 2) Does the action of FDB cause feather coloration of individual birds to change over time? I addressed the first question using in vitro experiments. Using three separate metrics of bacterial activity, I conclude that melanized feathers are more resistant to FDB than unpigmented feathers. This suggests that the evolution of avian color patterns may be in part influenced by the need to protect feathers from damaging bacteria. I addressed the second question by studying a wild population of eastern bluebirds (Sialia sialis). I found that feather color of individual bluebirds does change over time, and that this change is correlated with an individual’s load of FDB for males, but not for females. The correlation between FDB load and color change in males is the first evidence consistent with FDB degrading the feathers of live birds. Furthermore, I found that plumage bacteria load correlates positively with male body condition, but negatively with female body condition. The results of this study suggest that plumage bacteria can influence the coloration and health of birds, but that plumage bacteria appear to affect males and females in fundamentally different ways. These two studies combined suggest that plumage bacteria may be an important and understudied facet of avian biology, and that they could be an important selective forces shaping avian evolution.
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Chapter 1. Feather-Degrading Bacteria: A new frontier in avian and host-parasite research?
Introduction

Birds are important models for the study of host-parasite interactions and coevolution (Loye and Zuk 1991, Clayton and Moore 1997). Much of this research has focused on arthropod ectoparasites that feed on feathers (Clayton et al. 2003, Proctor 2003) because feathers are so important to avian life history traits. For example, feathers function in thermoregulation (Stettenheim 2000), communication (Andersson 1994, Shuster and Wade 2003), and flight (Rayner 1988). Damaged feathers have reduced abilities to perform these functions (Booth et al. 1993, Swaddle and Witter 1997, Ferns and Lang 2003, Williams and Swaddle 2003) and so there are likely fitness consequences for individuals possessing damaged feathers. A subset of plumage bacteria that can degrade feathers have recently garnered interest since they may impose significant evolutionary selection pressures on birds similar to arthropod ectoparasites (Burtt and Ichida 1999, Clayton 1999). Aspects of avian morphology, behavior, and life history may be influenced by a coevolutionary battle between birds and feather-degrading bacteria that damage their plumage.

Research on feather-degrading bacteria and birds is in its nascent stages; however, there is a substantial body of literature that has attempted to understand how they interact. In this review I will synthesize what we currently know, focusing on these fundamental questions: What are feather-degrading bacteria and how do they degrade feathers? How prevalent are feather-degrading bacteria on birds? How do feather-degrading bacteria and birds influence one another? At each step, I will attempt to elucidate some of the important gaps in our knowledge and make suggestions as to how they can be addressed. I will also highlight some of the most exciting prospects of this field of study. For
instance, there is evidence that feather-degrading bacteria could influence sexually selected feather-color signals. I will end by summarizing the most important questions that need to be addressed in future research.

**What are feather-degrading bacteria?**

Feather-degrading bacteria (hereafter FDB) are a polyphyletic group related only in that they can decompose feathers (Onifade et al. 1998). They are phylogenetically and physiologically diverse (see Table 1), and appear to be cosmopolitan. The ability to decompose feathers is uncommon among bacteria, as feathers contain over 90% \( \beta \)-keratin by mass (Onifade et al. 1998, Ramnani et al. 2005). \( \beta \)-keratins are extensively cross-linked within and between polypeptides through hydrogen and disulfide bonds, making them compact and resistant to degradation by most proteolytic enzymes (Gupta and Ramnani 2006). How FDB decompose feathers is not fully understood, but the process likely involves two steps. First, the disulfide bonds of \( \beta \)-keratin are reduced, possibly by the production of disulfide reductases (Yamamura et al. 2002b) or sulfite (Ramnani et al. 2005). Then proteolytic keratinases (Onifade et al. 1998, Ramnani et al. 2005) that are specialized in hydrolyzing keratins break the remaining bonds (Gupta and Ramnani 2006).

Most biochemical studies of bacterial feather degradation are carried out in a biotechnological context, as keratinolytic enzymes have a number of industrial applications (Gupta and Ramnani 2006). Unfortunately, research has not focused explicitly on characterizing FDB found on birds (with some exceptions, e.g. Ichida et al. 2001), or under conditions that could realistically be found within avian plumage.
Biochemical data for FDB isolated from plumage would be useful to make predictions about the conditions in which bacterial feather degradation should be most intense.

**How prevalent are feather-degrading bacteria on birds?**

FDB are common within avian plumage. Burtt and Ichida opportunistically sampled temperate birds and found FDB of the genus *Bacillus* on 32 of 83 species (Burtt and Ichida 1999). The number of individuals sampled per species were generally low, but FDB occurred on 16 of 18 (89%) of the species studied at reasonable sample sizes (N>20). Within species, the prevalence (% of individuals contaminated) ranged from 0-29% (mean ± SD, 8.4% ± 0.2) (Burtt and Ichida 1999). The authors found that ground foraging and water birds have a higher prevalence of *Bacillus* than aerial or bark probing species, suggesting that FDB are acquired through contact with environmental substrates rather than conspecifics (Burtt and Ichida 1999); however, their analyses did not control for sample sizes and are thus only cursory. Furthermore, a recent study of the total bacteria communities of plumage found little evidence that birds acquire bacteria from their immediate environment (Bisson et al. 2007). Whitaker et al. surveyed eight temperate birds and found FDB on all species sampled (Whitaker et al. 2005). FDB prevalence ranged from 21-59% among the different species, with a mean prevalence of 38%. This is in sharp contrast to the 8.4% mean prevalence reported by Burtt and Ichida. When considering only the five avian species sampled in both studies, a similar result is found (7% vs. 40% mean prevalence). The cause of this discrepancy is not clear, but could be due to differences in sampling and cultivation protocols (Whitaker et al. 2005) or geographic variation in microbial communities between study sites. In any case, these
studies indicate that FDB are pervasive among birds, and suggests considerable among-
species and among-population variation in FDB prevalence.

These reports provide valuable information about the relationship between FDB
and birds, but they likely underestimate the prevalence of FDB (Clayton 1999, Shawkey
et al. 2007). Both studies used highly selective cultivation protocols to isolate FDB of the
genus *Bacillus* (Burtt and Ichida 1999, Whitaker et al. 2005), which are mildly
thermotolerant, halotolerant, and gram positive. Isolating bacteria with these
characteristics narrows the range of bacteria that can be detected. More inclusive
cultivation methods detected FDB on 88% of male Eastern Bluebirds (*Sialia sialis*)
(Shawkey et al. 2007) and found a phylogenetically diverse assemblage of FDB on House
Finches (*Carpodacus mexicans*) (Shawkey et al. 2003). Similar methods isolated 13
strains of putative FDB from soil, suggesting a high diversity of FDB that birds could
encounter in their environment (Lucas et al. 2003). FDB are physiologically diverse and
this diversity must be accommodated in culture-based surveys to determine the exposure
of birds to FDB as a group.

Culture-independent methods could also be useful in detecting FDB on birds.
Approximately 99% of bacterial species are unculturable due to their ability to enter non-
culturable states or a lack of established culture methods (Amann et al. 1995). Thus, a
significant portion of FDB species could go undetected in culture-based surveys.
Identifying plumage bacteria without cultivation can circumvent this problem. A number
of techniques can accomplish this, and typically involve sequencing rRNA genes
extracted directly from cells in a microbial community sample (Head et al. 1998).
However, this would not identify non-culturable FDB specifically. A more direct method
would be to amplify the keratinase genes present in a sample of the plumage microbial community. The amplification of keratinase genes could indicate the presence of FDB that cannot be grown in culture, if they are present. In order for this method to work, primers specific to keratinases must be used. Most keratinases characterized to date are subtilisin proteases that contain conserved regions (Gupta and Ramnani 2006). Unfortunately, the keratinase genes of known sequence are mostly from bacteria of the genus *Bacillus* (Gupta and Ramnani 2006), and thus may not reflect keratinase sequence variation among phylogenetically diverse FDB. In order to construct the most effective primers for the amplification of keratinase genes in bacterial community samples, direct DNA sequencing of keratinase genes from a diverse assemblage of FDB is needed. Ultimately, for surveys of FDB on birds in the future, culture-dependent and independent methods should be combined since particular bacteria may be detectable using only one method or the other (Shawkey et al. 2005).

Surveys of FDB on birds to date have generally not addressed variation in FDB intensity (the number of parasite individuals associated with a host individual) among individual birds in a population (but see Shawkey et al. 2007). Among-individual variation in parasite intensity within a population is indicative of the distribution of the parasites within that population (Goater and Holmes 1997). High among-individual variation in FDB intensity, coupled with a correlation between FDB intensity and fitness, is expected if FDB are mediating selection in a population of birds. Surveys that collect quantitative, rather than simply presence/absence, FDB data from sampled birds would be useful in beginning to answer the question of whether or not FDB could currently be a selective force.
FDB are likely a ubiquitous feature of avian plumage (Burtt and Ichida 1999). Surveys of the prevalence, diversity, and quantity of FDB on birds will help determine broad geographic, ecological, and phylogenetic patterns of avian contamination with FDB. Importantly, one or several model systems for the study of birds and FDB could emerge. Large scale, multi-species sampling of birds using standardized sampling techniques would be beneficial. At the very least, researchers working with their own avian model systems should begin to characterize the bacteria that live on their birds.

**Have birds evolved defenses against feather-degrading bacteria?**

Clearly, FDB commonly inhabit avian plumage. Have birds evolved mechanisms to combat these bacteria? If FDB have placed selective pressure on birds over time, one would expect birds to mount adaptive responses. Several lines of evidence suggest that this may have occurred.

*Feather structure and color*

Feather microstructure is a bird’s first line of defense against bacterial feather degradation. As mentioned previously, the tightly folded keratins of feathers cannot be cleaved by most proteolytic enzymes. Selection exerted by FDB is probably not responsible for the utilization of keratin in feathers; however, the action of FDB may favor the evolution and maintenance of microstructural characteristics that inhibit the action of FDB. As a corollary to this selection, the deposition of particular feather pigments may be selected for because of their protective value against FDB.
Melanin pigments are responsible for the majority of black, brown, and earth-toned colors of birds' feathers (McGraw 2006), and are important for signaling (Griffith et al. 2006) and crypsis. Feathers colored by melanins may also be more resistant to FDB than unpigmented feathers. Evidence suggests that *B. licheniformis* degrade black feathers more slowly than white feathers *in vitro* (Goldstein et al. 2004); however, conflicting results have been reported (Grande et al. 2004). Both of these studies used the same species (but different strains) of FDB, but inoculated feathers from different species of bird. There are also aspects of both studies that complicate the interpretation of their results. Goldstein et al. report no sample sizes, measures of error, or statistical tests. Furthermore, Goldstein et al. had a control only for unpigmented and not melanized feathers, and did not presented or discuss the data for this control. Grande et al. similarly did not have controls for all feather types, and scored feather damage subjectively using criteria that they created after the experiment was over (Grande et al. 2004). Grande et al. also combined degradation results of feathers taken from different bird species for statistical analyses. These studies have received a considerable amount of attention (Shawkey and Hill 2004, Bortolotti 2006, McGraw 2006), but at present the data on whether or not melanized feathers resist FDB are equivocal. It is also important to consider that the results using one species or strain of FDB cannot be generalized to all FDB. While some FDB could be inhibited by feather melanization, others could be unaffected or adapted to feeding on melanized feathers. The two types of feather melanin, eumelanin and phaeomelanin, may also differ in their influence on FDB.

Burtt and Ichida (2004) looked for an association between feather pigmentation and FDB activity in a field study. They compared the prevalence and feather-degradation
rates of *B. licheniformis* isolated from a dark subspecies of Song Sparrow (*Melospiza melodia morpha*) in Washington to those isolated from a lighter subspecies of Song Sparrow (*Melospiza melodia fallax*) in Arizona. It was assumed that the darker subspecies has a higher concentration of melanin in its feathers. The prevalence of *B. licheniformis* between the two subspecies did not significantly differ; however, using a subjective feather degradation measure, they do report that *B. licheniformis* from the darker-colored sparrows degrade unpigmented chicken feathers more rapidly than *B. licheniformis* from the lighter-colored sparrows. More effective bacteria on birds with higher feather melanin concentrations suggest that an evolutionary arms race may be occurring, with increases in bacterial efficiency selecting for birds with increased melanin deposition, and vice-versa (Burtt and Ichida 2004). However, how bacterial degradation of unpigmented chicken feathers relates to the degradation of melanized sparrow feathers is unclear.

At present there is no definitive answer as to whether or not melanized feathers are more resistant to FDB than unpigmented feathers, but it is still a possibility. Melanized feathers are harder and more resistant to physical abrasion than unmelanized feathers (Bonser 1995), and melanins can bind to proteolytic enzymes (Kuo and Alexander 1967). One or both of these mechanisms could protect melanized feathers from FDB. Future studies that compare feather types for resistant to FDB should use objective measures of feather degradation and bacterial activity, such as protein released from feathers (Lucas et al. 2003, Goldstein et al. 2004), change in feather mass (Lucas et al. 2003), bacterial growth (Lucas et al. 2003), and keratinase production (Lucas et al. 2003) to confidently assess the degradability of feathers. This needs to be carried out with
multiple species of FDB and with feathers from several different species of birds to
determine the generality of any trend that may arise.

Preen oil and preening

Birds could manipulate the bacterial composition of their plumage by the selective use of
preen oil on feathers. Preen oil is a holocrine secretion of the external avian uropygial
gland (Jacob and Zisweiler 1982) that birds apply to feathers while preening. Plumage
condition deteriorates with surgical removal of the gland (Moyer et al. 2003) and it is
assumed that preen oil maintains feather condition by waterproofing and/or maintaining
feather flexibility (Jacob and Zisweiler 1982). However, to my knowledge there is no
direct experimental evidence to support either of these assumptions. Preen oil could
maintain feather condition by inhibiting FDB. Removal of the preen gland from domestic
fowl shifted the structure and composition of microbial communities on the birds skin
(Bandyopadhyay and Bhattacharyya 1996). Notably, Bacillus became the second most
prevalent genus of bacteria on glandless birds but was never found on birds with
uropygial glands (Bandyopadhyay and Bhattacharyya 1996). House Finch preen oil
inhibits the growth of several FDB in vitro, including those with high rates of keratinase
production (Shawkey et al. 2003). Red-billed Hoopoe (Phoeniculus purpureus) preen oil
inhibits the growth Bacillus licheniformis (Burger et al. 2004).

There are two modes by which preen oil could influence FDB. First, the lipids
composing preen oil could be antibiotic. The wax 3,7-dimethyloctan-1-ol isolated from
the preen oil of the Northern Gannet (Sula bassana) inhibits the growth of several
bacteria in vitro (Jacob et al. 1997). Second, antibiotic-producing bacteria could be
cultivated within the uropygial gland and subsequently applied throughout the plumage within preen oil. *Enterococcus feacalis* isolated from Red-billed Hoopoe preen oil produces antibiotic bacteriocins that are effective against *B. licheniformis* and several other bacteria (Martin-Platero et al. 2006). It is not known if the antibiotics produced by *E. feacalis* affect plumage (or egg and nest) bacterial communities, but the possibility is intriguing.

Preen oil can clearly affect FDB. However, it is not known if this effect benefits birds and could, therefore, be under selection. It is possible that the anti-bacterial properties of preen oil are a by-product of preen oil composition that have no influence on avian fitness. It is also worth mentioning that some feather mites feed on preen oil (and possibly on feather microbes) (Proctor and Owens 2000, Proctor 2003) and could thus influence the relationship between birds and FDB. Longitudinal studies that monitor changes in communities of FDB, feather wear, and fitness metrics before and after preen gland removal would be powerful in determining if preen oil functions to inhibit or otherwise alter the influence of FDB on birds. Also, the act of preening, irrespective of preen oil, could physically dislodge bacteria (Clayton 1999).

*Anting, dustbathing, and sunbathing*

Dustbathing and sunbathing are avian behaviors that have eluded explanation, but could influence FDB (Burtt and Ichida 1999, Clayton 1999). Dustbathing dries the plumage, but would also expose birds to FDB which are common in soil (Lucas et al. 2003). This behavior could also expose plumage to microorganisms that displace or otherwise influence FDB. Sunbathing would most likely be detrimental to any plumage bacteria, as
it would dry the plumage and expose bacteria to damaging UV radiation. No experiments have addressed these behaviors in the context of FDB.

Anting describes the behavior in which birds either take ants between their bills and rub them throughout their plumage or stand on ant colonies and allow the ants to swarm over their bodies. The purpose of anting is unknown but has been proposed to serve an anti-microbial function (Ehrlich et al. 1986). Some passerines ant with formicine ants (Formicidae: Hymenoptera) that produce formic acid as a defense mechanism. Extracts from five species of Formicidae did not inhibit FDB growth in culture (Revis and Waller 2004). The ant extracts were also tested against several other species of pathenogenic bacteria and fungi, but no inhibition was reported. Birds also “ant” with other objects that contain anti-microbial compounds, including snails (VanderWerf 2005) and fruit (Clayton and Vernon 1993, VanderWerf 2005). Experimental tests of anting behavior in live birds, such as that conducted by Lunt et al. (2004), should be conducted in the context of FDB to determine if anting reduces the numbers of, or otherwise alters, communities of FDB on birds.

Choice of nest materials
Many birds line their nests with fresh green vegetation. The nest protection hypothesis proposes that birds place fresh plant material in their nests to protect against parasites (Clark 1991). In Corsican Blue Tits (Parus caeruleus ogliastre) and European Starlings (Sturnus vulgaris), preferred nest plants are high in volatile compounds that inhibit bacterial growth (Clark and Mason 1985, Petit et al. 2002). Corsican blue tits use olfactory cues to determine when to bring fresh plant material to the nest, suggesting that
birds use fresh plants for the volatile compounds that they contain (Petit et al. 2002). No study has addressed the topic of nest plant material in relation to FDB, yet it seems an area worth consideration.

*Feather molt*

Molt occurs once or twice a year in most temperate birds and many tropical birds molt continuously. It has been suggested that molt evolved to replace worn and damaged feathers, and there is some evidence to support this (Williams and Swaddle 2003). FDB could have selected for the evolution of molt by wearing down feathers (Burtt and Ichida 1999, Clayton 1999). Molt could also reduce plumage loads of FDB. There is evidence that loads of *B. licheniformis* are lower on temperate birds during the spring and fall, which corresponds with primary molting times (Burtt and Ichida 1999), although this has not yet been addressed systematically. Simple experiments that measure the intensity of FDB on individuals in a population before, during, and after molt could indicate whether or not molt reduces FDB load.

*Feather-degrading bacteria and feather color expression*

Variation in feather color signals can communicate information about the nutrition (Hill and Montgomerie 1994), immunocompetence (Saino et al. 1999), endoparasite load (Hamilton and Zuk 1982), age (Siefferman et al. 2005), and dominance (McGraw et al. 2003) of the signaler. However, these mechanisms typically influence feather color while the feather is being produced. FDB have the potential to alter feather color expression after the feather is fully formed and is dead tissue. The effects of FDB could be positive
or negative. Certainly, feather degradation could influence the reflective properties of feathers in such a way that a color signal is reduced. However, many birds acquire breeding plumage coloration after molt by the wearing of the ends of feathers (Veiga 1996, Willoughby et al. 2002). FDB could aid this process and thus influence signaling by weakening the ends of feathers through degradation.

The affect of FDB on feather color expression could also be more subtle. Blue rump feathers of Eastern Bluebirds degraded by FDB in vitro are significantly brighter and have greater spectral saturation than feathers not degraded by FDB (Shawkey et al. 2007). Furthermore, bacterial feather damage correlates negatively with UV-chroma (the percentage of total light reflected in the UV portion of the spectrum) (Shawkey et al. 2007). The blue in eastern bluebird feathers is structural, produced by the coherent refraction of light in the feathers spongy medullary layer (Prum 2006). FDB thin the outer cortex of feathers, which may allow more light to pass to and from the inner spongy medullary layer and thus effect color change (Shawkey et al. 2007). The blue color of male Eastern Bluebird rump feathers appear to be a sexually selected signal, as variation in rump coloration is indicative of reproductive success (Siefferman and Hill 2003). Specifically, males with brighter blue feathers fledge more, heavier offspring than dull males (Siefferman and Hill 2003). Thus, the action of FDB could positively influence a sexually selected trait, meaning FDB may not necessarily be parasitic.

The abundance of culturable FDB on individual bluebirds does not correlate with structural feather brightness in the wild (Shawkey et al. 2007). Assuming (cautiously) that the abundance of culturable FDB correlates with total FDB abundance, this lack of association suggests that bacteria do not currently affect feathers in this system. However,
the authors argue that certain FDB may be more effective at feather degradation than others, and thus bacterial damage may not correlate with bacterial abundance (Shawkey et al. 2007). Indeed, there is variation among FDB in their rates of keratinase production, keratinase activity, and rates of feather degradation (Kim et al. 2001, Lucas et al. 2003). A number of alternative (though not mutually exclusive) factors could also contribute to the lack of correlation. For instance, an important environmental covariate may not have been considered, or individual birds may be differentially susceptible to bacterial feather degradation. Bluebirds may also currently be winning an evolutionary arms race against FDB. If this is the case, FDB would exert little influence on feathers, which would be seen as a lack of correlation between abundance of FDB and feather wear. Importantly, feather color change on wild birds in relation to FDB abundance has not been addressed, which may be more important than color characteristics at any one moment. For example, an individual with dull feathers and the most FDB may get brighter, but will not necessarily be the brightest. Furthermore, degradation could likely be so great that brightness is eventually negatively affected.

Structural feather brightness of Eastern Bluebirds in the wild does correlate with overall abundance of culturable bacteria, inclusive of all bacteria, not just FDB (Shawkey et al. 2007). This correlation could result from reduced self-maintenance (i.e., preening) in the more dominant bright males (Shawkey et al. 2007). Dominant males spend more time defending territories and perhaps have to provision more offspring. Observations from populations of Eastern Bluebirds in Virginia, USA, confirm a negative correlation between the amount of time spent preening and the number of fledglings produced from a brood (Kight and Swaddle 2007). Consistent with this trend, European Starlings with
experimentally increased broods harbor more bacterial cells (Lucas et al. 2005). Brighter bluebirds may also harbor more bacteria because they can better promote the growth of beneficial bacteria, perhaps by way of preen oil composition (see above) (Shawkey et al. 2007). However, even if birds can promote the growth of certain bacteria, it does not necessitate an increase in total bacterial abundance. More beneficial bacteria would likely come at the expense of other species, particularly if the beneficial bacteria act by inhibiting the growth of detrimental bacteria. This would be seen as a shift in the relative abundance of species present, not an increase in total bacterial abundance.

Structural colors are important for a number of birds. Blue structural coloration is condition dependent in Blue Tits (Parus caeruleus) (Johnsen et al. 2003), Blue Grosbeaks (Guiraca caerulea) (Keyser and Hill 1999), and Blue-black Grassquits (Volatinia jacarina) (Doucet 2002). Variation in structural coloration influences mate preferences of European Starlings (Bennett et al. 1997) and Blue Tits (Andersson et al. 1998). Interestingly, the blue coloration of male and female Blue Tits in the wild increases in brightness but has reduced UV chroma after molt and throughout the breeding season (Ornborg et al. 2002), a pattern of structural color change remarkably similar to that inflicted by FDB in vitro (Shawkey et al. 2007). Structural color is also important in carotenoid color expression (Shawkey and Hill 2005), and some carotenoid-colored feathers increase in brightness over time (Blanco et al. 2005, Figuerola and Senar 2005). If FDB positively influence sexually selected color signals on birds, and these characteristics correlate with condition, it is possible that good condition is partially indicated by the ability to cultivate beneficial exogenous microorganisms (Shawkey et al. 2007).
Do feather-degrading bacteria degrade the feathers of live birds?

Do FDB affect the feathers of live birds? As obvious as this question may seem, it is rarely addressed in the literature. Most of the evidence I have reviewed is either correlational or experimental but from highly artificial culture conditions. Only one study to date has attempted to experimentally detect bacterial degradation of feathers on live birds (Cristol et al. 2005).

In two separate experiments, Cristol et al. inoculated flight feathers of captive birds with the FDB *B. licheniformis* and treated control feathers with an antibiotic (Cristol et al. 2005). One experiment was conducted on Northern Cardinals (*Cardinalis cardinalis*) during the winter, and the second on European Starlings during the summer under conditions of experimentally increased humidity. Feather damage was quantified by counting feather lesions using a scanning electron microscope. Feather damage did not differ between the two treatments in either experiment. However, aspects of the experiments may have compromised their ability to detect bacterial feather degradation (Cristol et al. 2005). The cold and dry winter conditions of the first experiment were likely too harsh for the mildly thermophilic *B. licheniformis* to be active (Cristol et al. 2005). The use of starlings in the second experiment, whose black feathers are melanized and could be resistant to *B. licheniformis*, may have negated a positive influence of increased temperature and humidity on bacterial activity. Perhaps most importantly, the birds in both experiments were inoculated with only one species of FDB. Given the complexity of the bacterial community of plumage (Shawkey et al. 2005), the addition of
a large number of one species FDB may not create realistic conditions conducive to FDB activity (see below) (Shawkey et al. 2007).

Despite the negative result, the conclusions of this study do not close the book on research into the effects of FDB on birds. Hopefully, this review convincingly describes several avenues of research that are promising and have not been explored. Below, I suggest some specific ways in which we can take this intriguing field of inquiry several steps forward.

**The next step should be the first step**

Feathers serve many functions, including thermoregulation (Stettenheim 2000), visual (and sometimes auditory) signaling (Andersson 1994, Shuster and Wade 2003), and flight (Rayner 1988). Feather alteration that affects any of these functions can directly affect avian survival and/or fitness. Published studies investigating FDB on birds, inclusive of this review, are replete with speculations as to the potential influence of FDB on avian life history and evolution. However, as indicated above, there are many gaps in this line of supposition and evidence is lacking of a direct link between FDB and changes in feather condition. Despite this, several authors have stated unequivocally that bacteria degrade feathers on live birds (see Gill 1995, Burtt and Ichida 2004, Figuerola and Senar 2005). Research on FDB and birds cannot move past speculation until bacterial feather degradation has been demonstrated on a live bird. To put previous research into an ecological and evolutionary context, experiments must address whether FDB alter feathers *in situ*, particularly in the wild (Clayton 1999). If FDB do negatively impact birds, they would be a unique group of avian ectoparasite. Two criteria must be met to
demonstrate that parasites mediate natural selection on a host (Little 2002). First, infection must reduce host reproduction or survival. Second, host genotypes must differ in their susceptibility. The criteria for parasite-mediated natural selection should be kept in mind when designing experiments addressing FDB. The potential for a positive influence of FDB on birds also exists via alteration of feather color expression and should also be considered, particularly in birds with structurally derived feather-color signals (Shawkey et al. 2007).

Microbial community ecology will be important in determining if FDB affect feathers, as microbially mediated biological processes are often a function of bacterial group composition (Balser et al. 2002). Most FDB studies to date have focused on the bacterial genus Bacillus, and more specifically on the species Bacillus licheniformis. Several other species of FDB can occur within plumage (Table 1) and thus significant feather degradation may result only from the concerted action of the group. Along these lines, FDB cannot be considered in isolation from other bacteria. Non-FDB could inhibit or promote the growth of FDB (Burtt and Ichida 1999, Clayton 1999, Shawkey et al. 2007) or otherwise contribute to feather degradation (Shawkey et al. 2007). Investigation of the function of FDB will necessitate multilevel selection analyses where group and individual bacterial selection is considered in concert with host bird selection. A number of techniques are available to assess microbial community structure and composition (reviewed in Head et al. 1998, Kirk et al. 2004, Dorigo et al. 2005) and should be employed in in situ studies of FDB.

Studies that look for correlations between FDB load and/or microbial community composition and feather damage would be useful. However, because feathers can incur
damage in a number of ways, a more direct demonstration of bacterial degradation may ultimately be needed. For instance, scanning electron microscopy could be used to determine if bacteria aggregate at areas of feather damage. Fluorescent in situ hybridization could possibly be used to specifically locate FDB on feathers, either targeting mRNA for keratinase or rRNA specific to certain species of FDB. Keratinases can also be probed with fluorescently tagged antibodies (Noronha et al. 2002). A number of other techniques, such as environmental functional gene arrays, are available to determine if a process is bacterially mediated (reviewed in Torsvik and Ovreas 2002, Tringe and Rubin 2005) and could have application in detecting bacterial feather degradation on live birds.

A further consideration

Along with bacteria, complex communities of fungi also exist within plumage and in nests (Apinis and Pugh 1967, Pugh and Evans 1970b, Pugh and Evans 1970a, Pugh 1972, Hubálek et al. 1973, Hubálek 1976, 1978, reviewed in Hubalek 2000). Many fungi produce anti-bacterial compounds and could thus directly influence the plumage bacterial community. Some plumage and nest fungi can also degrade feathers (referred to as keratinophilic fungi). In fact, research on these fungi has been ongoing since the 1960’s. Culture-based surveys of wild bird populations have isolated keratinophilic fungi from up to 67% of individuals (Deshmukh 2004). 14 species of feather-degrading fungi were isolated from the feathers of 100 live chickens (Kaul and Sumbali 1999). Chrysosporium georgiae, a fungus also isolated from chicken feathers, degrades feathers but not human
or bovine hair or wool (El-Naghy et al. 1998). This suggests *C. georgiae* specializes in degrading β-keratin, the form of keratin found in feathers.

To date, no experimental work has addressed the impact of plumage fungi on either plumage bacterial communities or feathers of live birds. However, a great deal of biochemical (reviewed in Kunert 2000, Gupta and Ramnani 2006) and ecological (see references above) studies of keratinophilic fungi have laid the foundation for such work. Experiments that test for impacts of FDB on birds could be easily adapted to test for impacts of keratinophilic fungi on birds. Fungi add another intriguing aspect to the ecology of plumage microbes. The interactions between feather fungi, feather bacteria, and birds are more or less completely unknown. This is an area of research wide open and ready to be explored.

**Conclusion**

Demonstrating unequivocally that bacteria (or fungi) are responsible for observed feather wear on live birds will be difficult, as ascribing function to microbes is a common problem in microbial ecology (Balser et al. 2002, Torsvik and Ovreas 2002). However, tackling this question opens the door for creative interdisciplinary research, potentially integrating methods of microbiology with field behavioral ecology. Rigorous experimental studies of FDB and birds that integrate host fitness metrics with bacterial activity are needed to shed light on this potentially novel system of host-parasite (or, more generally, host-symbiont) interaction.
Acknowledgements

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References


Table 1. Bacteria with keratinolytic activity. Unless noted, see references for specific strains identified. Note that this list is conservative as many bacteria have not been tested for keratinolytic activity and many cannot currently be tested because they are uncultureable. Keratinolytic bacteria unlikely to be found on birds, such as those from hot springs (Kim et al. 2004), are not included.

<table>
<thead>
<tr>
<th>Bacterium</th>
<th>Source</th>
<th>Bacterial Phylum</th>
<th>Reference</th>
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<tbody>
<tr>
<td><em>Bacillus licheniformis</em></td>
<td>wild bird</td>
<td>Firmicutes</td>
<td>(Burtt and Ichida 1999, Whitaker et al. 2005)</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>wild bird</td>
<td>Firmicutes</td>
<td>(Burtt and Ichida 1999, Whitaker et al. 2005)</td>
</tr>
<tr>
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<td>Firmicutes</td>
<td>(Burtt and Ichida 1999)</td>
</tr>
<tr>
<td><em>Bacillus pseudofirmus</em></td>
<td>poultry farm soil</td>
<td>Firmicutes</td>
<td>(Gessesse et al. 2003, Kojima et al. 2006)</td>
</tr>
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<td>poultry waste</td>
<td>Firmicutes</td>
<td>(Kim et al. 2001)</td>
</tr>
<tr>
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<td>wild bird</td>
<td>Firmicutes</td>
<td>(Shawkey et al. 2003)</td>
</tr>
<tr>
<td><em>Staphylococcus hemolyticus</em></td>
<td>wild bird</td>
<td>Firmicutes</td>
<td>(Shawkey et al. 2003)</td>
</tr>
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<td>(Shawkey et al. 2003)</td>
</tr>
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<td>(Shawkey et al. 2003)</td>
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<td><em>Kocuria rhizophila</em></td>
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<td>Actinobacteria</td>
<td>(Shawkey et al. 2003)</td>
</tr>
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<td>Actinobacteria</td>
<td>(Tiquia et al. 2005)</td>
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<tr>
<td><em>Psuedomonas fulva</em></td>
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<td>Proteobacteria</td>
<td>(Shawkey et al. 2003)</td>
</tr>
<tr>
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<td>(Yamamura et al. 2002a, Yamamura et al. 2002b)</td>
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<td>poultry industry</td>
<td>Cytophaga-Flavobacterium</td>
<td>(Riffel and Brandelli 2002)</td>
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Chapter 2. Resistance of melanized feathers to bacterial degradation: is it really so black and white?
Introduction

Melanins are the most common pigments of birds’ feathers, and are responsible for the majority of black, brown, and earth-toned colors of avian plumage (McGraw 2006). The putative functions of melanized feathers are many, and range from thermoregulation to crypsis and sexual/social signaling. In terms of their function in condition-dependent conspecific signaling, melanized feathers are typically thought to be of less importance than carotenoid colored feathers because variation in melanin coloration is assumed to be less affected by environmental variation that would enforce condition-dependent signal honesty. A recent review by Griffith et al (2006) refutes this generalization, however, and cites a myriad of physiological mechanisms that require melanins and their precursors which suggests the placement of melanins into feathers is costly and subject to environmental influences (Griffith et al. 2006, also reviewed in McGraw 2006). For example, the reproductive load of house sparrows (Passer domesticus) influences the melanization of their throat feathers in the subsequent molt (Griffith 2000). Variation in feather melanization may also influence feather condition, as melanized feathers are harder and more resistant to physical abrasion than unpigmented (white) feathers (Bonser 1995). Feather wear can influence flight performance (Swaddle and Witter 1997) and thermoregulatory ability (Booth et al. 1993) in birds, and thus melanization may help to protect birds from incurring these costs.

As melanized feathers are relatively more abrasion resistant, some have suggested that they are also more resistant to feather-feeding ectoparasites such as lice, and there is some evidence consistent with this (Kose et al. 1999, but see Bush et al. 2006). Melanized feathers may also resist feather-degrading bacteria (FDB). FDB are a
polyphyletic assemblage of microbes that can hydrolyze β-keratin, the primary constituent of feathers. FDB occur within the plumage of several avian species (Burtt and Ichida 1999, Whitaker et al. 2005), and, for some species at least, are a ubiquitous feature of plumage (e.g. FDB detected on 99% of eastern bluebirds (Sialia sialis) in southern Virginia, U.S.A.; Gunderson et al., unpubl. data). Despite the potential for FDB to be a potent selective force shaping avian evolution (Burtt and Ichida 1999, Clayton 1999), little is known about how these microbial symbionts function on live birds. FDB were first isolated from wild bird feathers less than 10 years ago (Burtt and Ichida 1999), and in the short time since this discovery only a handful of studies have addressed mechanisms by which FDB and birds may influence one another (e.g. Shawkey et al. 2003, Burtt and Ichida 2004, Cristol et al. 2005, Shawkey et al. 2007).

Two studies have tested the hypothesis that melanized feathers are more resistant to feather degrading bacteria than un-melanized (white) feathers; however, these studies draw opposite conclusions. Goldstein et al. (2004) put either white (presumably unpigmented) or black (melanized) secondary flight feathers of domestic chickens (Gallus gallus) into flasks in a buffer media (referred to as “feather solution”) and inoculated them with the feather-degrading bacterium Bacillus licheniformis. Feather degradation rates were determined by measuring the soluble protein content of the feather solution spectrophotometrically at intermittent times over the course of six days. This method assumes that proteins in solution are oligopeptides released from bacterial hydrolysis of feather β-keratins, a reasonable assumption, as soluble protein content correlates significantly with bacterial density, keratinase production, and feather decomposition (Lucas et al. 2003). Goldstein et al. (2004) found higher soluble protein
content in feather solution around white unpigmented feathers than around black melanized feathers, suggesting that feather melanization inhibits the activity of *B. licheniformis*. On the contrary, Grande et al. (2004) found that melanized feathers degrade more quickly than unpigmented feathers. In their study, melanized and unpigmented feathers from two and three bird species, respectively, were placed in feather solution and inoculated with *B. licheniformis*. Rates of feather degradation were scored subjectively by observing each flask and visually estimating the amount of feather degradation. Melanized feathers showed signs of degradation before unpigmented feathers, and melanized feathers were more damaged than unpigmented feathers at the conclusion of the experiment (Grande et al. 2004). This study also included feathers colored with carotenoids, which appeared more resistant to bacterial degradation than melanized feathers as well (Grande et al. 2004).

The results of Goldstein et al. (2004) and Grande et al. (2005) have received considerable attention (see Shawkey and Hill 2004, Bortolotti 2006, McGraw 2006) because the results of both suggest that feather pigmentation may play a role in the resistance of feathers to a ubiquitous feather-degrading bacterium. Thus, those studying avian coloration may have overlooked some important mechanisms that modulate color expression. The question remains, however; do melanins impart bacterial resistance to feathers or do they make feathers more susceptible to bacterial degradation? There is currently no unequivocal answer to this question, and we felt there were aspects of both the Goldstein et al (2004) and Grande et al (2005) studies which suggested that their results be regarded cautiously. Goldstein et al. (2004) do not present data on replicate flasks for each pigment type. Their results include no measures of error and were not...
subjected to statistical scrutiny. Furthermore, the authors included an uninoculated control flask for unpigmented feathers only, and not melanized feathers. This could confound their results, for, as the authors themselves state and as is discussed above, melanized feathers are physically stronger than unpigmented feathers. Thus, the discrepancy in degradation rates could have been the result of their initial feather sterilization method (autoclaving at 121 °C for 15 minutes) and/or their incubation conditions (agitation at 120 rpm at 37 °C) weakening unpigmented feathers more than melanized feathers, causing them to be less resistant to bacterial degradation. As feathers on live birds would rarely experience such conditions, it is difficult to interpret the results of Goldstein et al. (2004) in terms of evolutionary or ecological processes. Interpretation of their data is further complicated by the fact that they do not present or discuss the results for the unpigmented control that they prepared. Grande et al. (2004) did have replication in their study; however, they determined feather degradation rates subjectively on a scale of 1-5 based upon visual inspection of the feathers in the flasks, and determined the criterion for inclusion in a particular degradation category after the experiment had ended (Grande et al. 2004). Grande et al. (2004) similarly autoclaved their feathers but did not have controls for all feather pigment types, let alone species of bird, used in their study.

The goals for our study were two-fold. First, we sought to answer the question of whether or not melanized feathers are more resistant to bacterial degradation than unpigmented feathers, while addressing our concerns about the studies discussed above. Second, we wanted to begin to explore variation among species of FDB in their ability to degrade melanized and unpigmented feathers. Goldstein et al. (2004) and Grande et al.
(2004) looked at degradation rates with *B. licheniformis* only; however, well over a dozen phylogenetically diverse species of FDB have been reported and, thus, it is unlikely that pigmentation would affect all of them equally. We postulate that melanized feathers may inhibit some FDB and have no effect on others. Some FDB could even specialize in degrading melanized feathers and, thus, would degrade melanized feathers more quickly than unpigmented feathers. We ran feather degradation trials with two species of FDB; *B. licheniformis* (the same strain used in Goldstein et al. 2004) and *Kocuria rhizophila*, a bacterium that can degrade feathers and has been isolated from house finch (*Carpodacus mexicanus*) plumage (Shawkey et al. 2003). Because of the physical characteristics of melanized feathers, we hypothesized that melanized feathers would be more resistant to FDB than unpigmented feathers. We thus predicted that FDB would degrade melanized feathers more slowly than unpigmented feathers. We made no predictions about the direction of differences in feather degradation rates between the two species of FDB, as this aspect of our study was exploratory in nature. However, we did expect the two bacterial species to differ in their interaction with melanized feathers.

**Methods**

Unpigmented (white) and melanized (dark brown) tail feathers of domestic geese (*Anser anser domesticus*) were used in feather degradation trials. In each trial, 300.0 mg of feather (consisting of several small feather pieces from different feathers, and, probably, different individual birds) was placed in each of twelve 125 ml Erlenmeyer flasks; six flasks containing unpigmented feathers and six flasks containing melanized feathers. We used only the distal (approximately) 10 cm of feathers to ensure that most of the feather
mass was from barbs and barbules. The rachis, or central vein of a feather, contributes most of the mass to the proximal region and is not usually degraded by *B. licheniformis* (Ramnani et al. 2005). We conducted degradation trials with feathers sterilized with autoclaving or ethylene oxide gas. In trials in which feathers were sterilized by autoclaving, feathers were placed dry in flasks and autoclaved for 15 min at 121° C (melanized feathers in the *B. licheniformis* trial had to be autoclaved for 18 min, as 15 min of autoclaving repeatedly failed to sterilize them). After autoclaving, 50ml of sterile phosphate buffered saline (PBS), pH = 7.25, was added to each flask. We did not autoclave the feathers in PBS because in preliminary trials this appeared to contribute to feather dissolution. Ethylene oxide gas sterilization does not subject feathers to the high temperature and pressure of autoclaving. When sterilized with ethylene oxide gas, feathers were sterilized dry and then placed in flasks containing 50 ml sterile PBS. For both sterilization methods, flasks were kept at room temperature for 24 hrs after the addition of PBS to allow soluble proteins associated with the feathers to enter solution and to allow any potential contaminants to grow. We then sampled the feather media to take an initial reading of soluble protein concentration before experimental bacterial inoculation, and plated 20 ul of the solution on trypticase soy agar (TSA), a general growth media, to ensure that the flasks were not contaminated. Three flasks of unpigmented feathers and three of melanized feathers were then inoculated with FDB, resulting in a total of three treatment (inoculated with bacteria) and three control (not inoculated with bacteria) flasks per feather pigment type, per trial. Treatment feathers in trials were inoculated with 500,000 cells. To accomplish this, the FDB were streaked on TSA one or two days before feathers were inoculated, depending on the bacterium (*K.*
*rhizophila* grows more slowly than *B. licheniformis* on TSA, and thus required a longer incubation period), and were incubated at 35° C. Pure isolated colonies were removed from the plates with a sterile cotton swab and used to inoculate a solution of sterile PBS. The concentration of bacteria in the solution was determined by measuring the optical density at 600 nm with a Bio-Rad Smart Spec 3000 (Bio-Rad, Inc. Hercules California), and this concentration was used to calculate the volume required to inoculate the flasks with the target number of bacteria. Among trials the volume of bacterial solution added to treatment flasks ranged from 1-4 ul, and, thus, did not significantly affect the volume of solution in treatment flasks. After inoculation, all flasks were placed in a 37° C incubator with agitation at 120 rpm.

**Protein sampling and concentration assay**

Every 48 hours, over a 12-day period, we sampled 500 ml of feather solution from each flask and froze the solution at -20° C. Upon thawing for protein concentration assay, samples were centrifuged at 10,000 rpm for 5 min to remove particulate feather matter and bacteria from the supernatant. The soluble protein content of each sample was determined by measuring the absorbance of the supernatant at 280 nm (Lucas et al. 2003) using a Nanodrop spectrophotometer (Nanodrop Technologies, Inc., Wilmington, DE).

**Viable cell counts**

Bacteria were quantified every 48 hrs, at the time of protein sampling, using the plate count method. For each flask, 20 ul of feather solution were plated on TSA from the
following dilutions: $1, 10^{-1}, 10^{-10}, 10^{-100}, 10^{-1000}, 10^{-10000}$, and $10^{-100000}$. Plates were incubated at 35°C for 48 hours ($K. rhizophila$) or 24 hrs ($B. licheniformis$).

*Change in feather mass*

Before trials, the feathers to be placed in each flask were dried for 48 hours at 55°C and weighed to the nearest 0.1 mg on an Ohaus AS60 analytical balance (Ohaus, Inc.). After trials, the feathers were again dried, this time for 72 hrs (we found that this increase in drying time was necessary to remove all moisture after the feathers had been suspended in aqueous solution during trials) and weighed to the nearest 0.1 mg.

*Statistical analyses*

We used repeated measures analysis of variance (ANOVA) for all analyses unless otherwise noted. Analyses performed on bacterial growth data were log transformed to improve normality and reduce disparities in variance among groups. All statistical tests were performed using the R statistical programming package (v. 2.4.1) employing two-tailed tests of probability.

*Results*

*Feathers inoculated with* Kocuria rhizophila

Soluble protein in feather media of inoculated unpigmented feathers decreased initially, but changed little after 48 hrs (Figure 1). The initial decrease in protein concentration was accompanied by rapid bacterial growth (Figure 2), suggesting that the bacteria were feeding on the protein already in solution, and not on the feathers themselves.
Interestingly, after the soluble protein had been consumed to a concentration of about 0.3 mg/ml, the populations of *K. rhizophila* on unpigmented feathers crashed (Figure 2). Little change in soluble protein content was seen for media of the controls or the inoculated melanized feathers (Figure 1), and little bacterial growth was seen on melanized feathers (Figure 2). The change in protein concentration around inoculated unpigmented feathers was significantly different from that around control or inoculated melanized feathers (feather pigment x treatment x time interaction, $F_{(7,56)} = 3.5605, P = 0.003$). Bacterial growth on melanized feathers was significantly less than in the presence of unpigmented feathers (feather pigment x time interaction, $F_{7,28} = 3.7586, P = 0.005$).

Unpigmented feathers lost more mass than melanized feathers, whether or not they were inoculated with bacteria (feather pigment x time interaction, $F_{1,8} = 29.347, P = 0.0006$) (Figure 3). There was a pattern in which inoculated feathers appeared to lose more mass than control feathers (Figure 2), although the relationship was not significant (treatment x time interaction, $F_{1,8} = 2.8291, P = 0.131$).

The observations that unpigmented feathers lost more mass than melanized feathers (Figure 3) and that the initial protein concentration in media surrounding unpigmented feathers was higher than that around melanized feathers (Figure 1), led us to hypothesize that autoclaving may have a greater effect on unpigmented than melanized feathers. This difference could confound our results and those of others by unwittingly making unpigmented feathers weaker and easier to degrade by FDB. Thus, we conducted feather degradation trials with *B. licheniformis* using both autoclave and ethylene oxide sterilization to determine if autoclaving differentially affects unpigmented and melanized
feathers. We did not continue trials with *K. rhizophila* as this bacterium did not appear to degrade feathers.

*Autoclaved feathers inoculated with* Bacillus licheniformis

The soluble protein content around inoculated unpigmented and melanized feathers increased over time (Figures 4,5), suggesting that *B. licheniformis* was digesting feathers and not the available soluble protein as did *K. rhizophila*. Unpigmented feathers showed clear signs of degradation after 192 hours based on non-overlap of 95% confidence intervals of protein content for inoculated versus control feathers (Figure 4). Using the same criterion, melanized feathers demonstrated clear signs of degradation at 240 hours. Media around control melanized feathers increased in soluble protein content steadily over the course of the trial, suggesting that incubation conditions were affecting melanized feathers, although the same trend was not seen for control unpigmented feathers. *B. licheniformis* grew to higher densities on unpigmented feathers (repeated measures ANOVA, $F_{5,20} = 3.2791$, $P = 0.02529$) (Figure 5). Plate counts from 192 hours were removed from this analysis because the counts were done incorrectly, as were plate counts from 336 hours because one flask could not be counted and thus led to statistical imbalance. Unpigmented feathers lost more mass than unpigmented control or either group of melanized feathers. One melanized control flask and one melanized treatment flask had to be removed from the mass data set because an error occurred in their measurement, so we were unable to statistically compare feather mass loss across treatments and pigments.
Ethylene oxide sterilized feathers inoculated with Bacillus licheniformis

Based on soluble protein content in solution around feathers, unpigmented feathers showed clear signs of degradation at 240 hours (Figure 8). For melanized feathers, however, no clear sign of degradation was present (Figure 9). 95% confidence intervals of melanized control and inoculated feathers overlap heavily across the entire time series. Again, soluble protein content in solution around melanized control feathers increased steadily over time. Bacteria also reached higher densities on unpigmented feathers compared to melanized feathers (repeated measures ANOVA, $F_{7,28} = 4.9242, P = 0.001$) (Figure 10), and lost significantly more mass (Figure 11). There was no difference in mass loss between melanized control and inoculated feathers.

Discussion

The bacterium Kocuria rhizophila is reported to have feather-degrading abilities (Shawkey et al. 2003); however, in our trial, K. rhizophila did not appear to degrade feathers. Instead, this bacterium consumed the available soluble protein already in solution (Figure 1), which was accompanied by a spike in bacterial density (Figure 2). There also appears to be a threshold of soluble protein concentration (approximately 0.3 mg/ml) below which K. rhizophila can no longer subsist (Figure 1). Soluble protein in flasks with unpigmented feathers was consumed until the concentration reached this threshold, at which point the bacterial population crashed (Figure 2), with little variation in the threshold level among flasks as indicated by the small standard error with only three replicates (Figure 1). This threshold level also explains why there was little change
in protein concentration in inoculated flasks with melanized feathers. The initial protein concentration in these flasks was too low to be utilized by the bacteria (Figure 1).

Based on results from the *K. rhizophila* trial, we felt there was evidence that autoclaving weakens unpigmented feathers, a phenomenon that could confound the results of our study and those of Goldstein et al. (2004) and Grande et al. (2005). Initial protein concentrations (time = 0 hrs) were higher in flasks with unpigmented compared to melanized feathers, and unpigmented feathers lost significantly more mass than melanized feathers, irrespective of treatment (Figure 3). We conducted subsequent feather degradation trials with *B. licheniformis* with feathers sterilized by either autoclaving or ethylene oxide gas, a method that does not subject feathers to high temperature and pressure.

Irrespective of the feather sterilization method used, unpigmented feathers appeared to be degraded more readily than melanized feathers. In both trials, inoculated unpigmented feathers lost more mass than melanized feathers, grew bacteria to higher densities, and had a greater increase in soluble protein content around feathers relative to control feathers. These results support the conclusion of Goldstein et al. (2004), and not Grande et al. (2004). Melanized feathers do appear to resist the action of FDB, or at least the strain of *B. licheniformis* used by us and Goldstein et al. (2004). However, we also demonstrated that the feather sterilization method used can qualitatively influence the outcome of feather degradation experiments. Based on soluble protein content, unpigmented feathers showed signs of degradation 48 hours earlier in the autoclave compared to the ethylene oxide trial. Furthermore, using the soluble protein metric, melanized feathers showed clear signs of degradation in the autoclaved trial, but not in
the ethylene oxide trial. Autoclaving was used in the studies of Goldstein et al. (2004) and Grande et al. (2005). Furthermore, we found evidence that incubation conditions can influence feathers. Soluble protein content around control melanized feathers increased steadily over both *B. licheniformis* trials. Had we not had controls for melanized feathers nor measured bacterial growth or change in feather mass, as was the case in the study by Goldstein et al. (2004), we would have concluded that melanized feathers degrade more rapidly than unpigmented feathers.

Based on these results, we suggest that any future work on feather degradation rates avoid sterilizing feathers with autoclaving, and use methods, such as ethylene oxide gas or γ-ray sterilization (Shawkey et al. 2007), that do not subject feathers to structure-altering heat and pressure. Further, controls must be prepared for all feather types, and multiple metrics of bacterial activity should be measured to corroborate results.

Currently, there is no clear mechanism for the FDB resistance of melanized feathers (Goldstein et al. 2004). Goldstein et al. (2004) suggest that the incorporation of melanin granules into a feathers’ keratin matrix may force keratin rods into close proximity with one another, thus catalyzing the production of more disulfide bonds between adjacent keratin molecules. This could have the effect of slowing the action of keratinase enzymes that hydrolyze feather keratin. However, intuitively, one would assume that interactions among keratin molecules would be reduced if melanin granules were interspersed among them. At present, there is not enough known about the microstructure of melanized versus unpigmented feathers to determine if this is a reasonable hypothesis.
A simpler model by which melanins could inhibit FDB is by binding to the bacterial proteolytic keratinases that hydrolyze keratins, and there is experimental evidence to support this. Melanized fungal mycelia resist microbial enzymatic digestion, and to test the mechanism of enzymatic resistance, Kuo and Alexander (1967) measured the efficiency of several microbial enzymes in the presence of melanin. One of the enzymes they assayed was a protease of the bacterium *Bacillus subtilis*, a microbe closely related to *B. licheniformis*. The activity of the *B. subtilis* protease was reduced by 49% to 69% in the presence of various concentrations of melanin, and melanin itself was found to be resistant to microbial degradation (Kuo and Alexander 1967). Thus, it seems reasonable to suggest that melanins may protect feathers by binding to and inactivating the proteolytic keratinases of FDB before they can hydrolyze feather keratins.

The next step is to determine whether or not the resistance of melanized feathers to FDB is relevant to wild birds. Burtt and Ichida (2004) began to address this question by comparing the prevalence (number of individuals infected) and feather-degradation rates of *B. licheniformis* from light and dark subspecies of song sparrow (*Melospiza melodia fallax* and *Melospiza melodia morphna*, respectively). There was no difference in the prevalence of FDB between the populations, but their data suggest FDB on the darker sparrow population degrade unpigmented chicken feathers more quickly than those isolated from the lighter population (Burtt and Ichida 2004). The authors interpret this as evidence that more damaging bacteria are selecting for more melanin deposition in feathers. However, they quantified bacterial degradation subjectively in a manner similar to Grande et al. (2004), and they note that feather autoclaving before inoculation damaged feathers in flasks, and to different degrees in different flasks. Furthermore, it is
unclear how the degradation of melanized chicken feathers relates to the degradation of melanized sparrow feathers. If feather melanization puts selective pressure on FDB, then FDB would adapt to degrade feathers more readily in the presence of melanin, which, depending on the mechanism of melanic inhibition, would not necessarily influence the degradation rates of unpigmented feathers. Roulin (2007) found that the rate of preening in barn owl (*Tyto alba*) nestlings decreased with plumage darkness. Preen oil can inhibit the growth of FDB (Shawkey et al. 2003), and the physical act of preening may dislodge bacteria (Clayton 1999). If this is the case, then more melanized individuals may not need to preen as regularly to combat FDB. At present, well controlled field experiments are needed to test the hypothesis that melanized feathers resist degradation by FDB. However, our series of laboratory tests, and those by Goldstein et al. (2004), indicate that melanin deposition does make feathers relatively more resistant to degradation by FDB. Therefore, it is possible that FDB can impose selection pressures on the evolution of avian feather coloration and the physical abilities of feathers to resist abrasion and damage.

**Acknowledgements**

I would like to thank A. Frame for help in carrying out the experiments, J. Swaddle, M. Forsyth, D. Cristol, and G. Gilchrist for helpful discussions and comments on the project, and G. Gilchrist for statistical advice. This research was funded by graduate research grants from the College of William and Mary, an NSF CAREER award to J. Swaddle, and an NIH AREA grant to M. Forsyth.
References


Figure 1. Change in soluble protein content of feather solution during the same trial. Points and bars indicate means +/- SE.
Figure 2. Growth of *Kocuria rhizophila* on domestic goose feathers sterilized by autoclaving.
Figure 3. Change in feather mass of feathers in the *K. rhizophila* degradation trial. Note that the sign of the y-axis is negative. Mean +/- SE is reported.
Figure 4. Soluble protein content (+/- st. err.) of media surrounding unpigmented, autoclaved domestic goose feathers inoculated with *B. licheniformis*. All means have had the initial protein concentration (Time 0) subtracted from them. Bars indicate +/- two standard errors.
Figure 5. Soluble protein content (+/- st. err.) of media surrounding melanized, autoclaved domestic goose feathers inoculated with *B. licheniformis*. All means have had the intitial protein concentration (Time 0) mean subtracted from them. Bars indicate +/- two standard errors.
Figure 6. Growth of *B. licheniformis* on domestic goose feathers sterilized by autoclaving.
Figure 7. Loss of feather mass of domestic goose feathers sterilized by autoclaving. Bars represent +/- one standard error.
Figure 8. Soluble protein content of media surrounding unpigmented domestic goose feathers sterilized with ethylene oxide gas and inoculated with *B. licheniformis*. All means have had the initial protein concentration (Time 0) mean subtracted from them. Bars indicate +/- two standard errors.
Figure 9. Soluble protein content of media surrounding melanized domestic goose feathers sterilized with ethylene oxide gas and inoculated with \textit{B. licheniformis}. All means have had the initial protein concentration (Time 0) mean subtracted from them. Bars indicate $\pm$ two standard errors.
Figure 10. Growth of *B. licheniformis* on domestic goose feathers sterilized with ethylene oxide gas.
Figure 11. Change in feather mass of domestic goose feathers sterilized with ethylene oxide gas. Bars represent +/- one standard error.
Chapter 3. Evidence that plumage bacteria influence feather coloration and individual health of eastern bluebirds (Sialia sialis)
Introduction

Sexual selection theory predicts that the expression of sexually selected secondary sex characteristics can be influenced by parasites. In such situations, parasites may indirectly influence individual mating success by altering the traits upon which mating decisions are made (Andersson 1994). There are three models of parasite-mediated sexual selection (PMSS) (Clayton 1991). First, the “good genes” model of PMSS dictates that there is heritable variation in parasite resistance among individuals (usually males), and that by mating with these resistant individuals, females pass on the genes for parasite resistance to their offspring. Second, the “transmission avoidance” model suggests that females mate with males with low parasite loads to avoid acquiring parasites from their mates. In this situation, acquired parasites could directly influence female health and, thus, indirectly influence reproductive output and/or the health and survival of subsequent offspring that acquire the parasites from the infected female. Finally, the “resource acquisition” model of PMSS posits that, in mating systems where males provision young and/or females, males with low parasite loads should provision at higher rates or provision higher quality food than males with high parasite loads. In all three models of PMSS, the sex (or possibly both sexes in situations of mutual mate choice) under parasite-mediated sexual selection must exhibit a trait that is a reliable indicator of parasite load.

Birds have served as important models for the study of PMSS, and a number of avian systems have demonstrated how parasite infection can influence the expression of a sexually selected signal. Much of this work has focused on
endogenous parasites of the blood (Hoglund et al. 1992, Horak et al. 2001) or gastrointestinal tract (e.g., Horak et al. 2004, Costa and Macedo 2005, Mougeot et al. 2005), or on exogenous parasites that feed on blood (Fitze and Richner 2002, Doucet and Montgomerie 2003). However, birds also harbor ectoparasites that inhabit the plumage and feed on feathers and skin. Unlike endogenous parasites that affect signal expression by influencing health, these integumentary ectoparasites can directly alter signals by damaging the structures producing them. For instance, lice can create lesions on the air sacs of male sage grouse \((Centrocercus urophasianus)\), that are used in sexual displays (Boyce 1990). In mate choice trials, females preferred males without experimentally created lesions (Boyce 1990). Female rock doves \((Columba livia)\) prefer males that are uninfected by feather-feeding lice, although the mechanism by which females assess parasite load is unclear (Clayton 1990). The sexually selected white tail spots on barn swallows \((Hirundo rustica)\) are more often damaged by feather-feeding lice than surrounding areas, and the size of the tail spots may reliably indicate the intensity of infection with lice (Kose et al. 1999, Kose and Moller 1999).

Recently, bacteria that can degrade feathers have been isolated from the plumage of wild birds (e.g. Burtt and Ichida 1999, Whitaker et al. 2005). These bacteria have the potential to influence avian health, feather condition, and sexual selection as do other avian ectoparasites. Feather-degrading bacteria (hereafter FDB) are a polyphyletic group of microorganisms recognized solely by their ability to hydrolyze the protein \(\beta\)-keratin, which constitutes over 90% of feather mass (Ramnani et al. 2005). Burtt and Ichida (1999) were the first to isolate FDB from the
plumage of birds, and since that time FDB have been detected on a wide range of avian hosts (Burtt and Ichida 1999, Shawkey et al. 2003, Whitaker et al. 2005, Shawkey et al. 2007). While FDB appear common on wild birds, there is no evidence that they influence plumage condition or individual fitness; however, this may be a result of the fact that few studies have attempted to directly detect feather degradation on live birds. Cristol et al. (2005) inoculated captive birds in an outdoor aviary with high numbers of the well-studied FDB _Bacillus licheniformis_ (OWU 138B), a strain originally isolated from the plumage of a willow flycatcher (_Empidonax traillii_) (Ichida et al. 2001). They found no evidence of increased feather damage on inoculated compared with uninoculated birds. However, one experiment was conducted during the winter, on northern cardinals (_Cardinalis cardinalis_), when low temperatures may have inhibited bacterial activity. Their second experiment was conducted during the summer under experimentally increased humidity; however, they inoculated European starlings (_Sturnus vulgaris_), a bird with highly melanized black feathers which are likely resistant to the action of FDB (Goldstein et al. 2004, Gunderson et al. unpub. data).

Shawkey et al. (2007) attempted to detect the action of FDB on live birds by looking for an association between feather coloration and individual FDB load. They experimentally demonstrated that, _in vitro_, FDB can alter the color characteristics of structurally colored feathers. They inoculated blue rump feathers of male eastern bluebirds (_Sialis sialis_) with a FDB (originally isolated from the plumage of a house finch (_Carpodacus mexicanus_), and closely related to _Bacillus pumillis_) and found that the FDB brightened the feathers, but decreased UV chroma (relative reflectance...
in the UV portion of the spectrum, which is visible to birds (Hart and Hunt 2007)).
The change in reflectance appears to be the result of FDB thinning the outer cortex of feathers and allowing more light to pass to and from the color-producing medullary layer of the feathers (Shawkey et al. 2007). Within a wild population of eastern bluebirds, however, neither male rump brightness nor UV chroma correlated with plumage FDB load, suggesting that FDB are not degrading feathers on these birds, although male brightness was positively correlated with non-FDB plumage load (Shawkey et al. 2007).

FDB are assumed to be parasites (Burtt and Ichida 1999, Clayton 1999, Burtt and Ichida 2004, Goldstein et al. 2004), but the result that FDB can increase the brightness of plumage in vitro suggests that this need not be the case (Shawkey et al. 2007). Rump coloration of male eastern bluebirds appears to be a sexually selected trait. Rump coloration correlates with reproductive success (Siefferman and Hill 2003), predicts the outcome of competition for nesting sites (Siefferman and Hill 2005b), and generally appears to be involved in male-male competition as opposed to female choice (Liu et al. 2007). Thus, if FDB can increase the strength of this signal, they may have positive effects on individual bluebird fitness (Shawkey et al. 2007). Shawkey et al. (2007) found no correlation between feather-color expression and feather brightness on bluebirds in the wild. However, they analyzed color characteristics of individuals at only one point in time as opposed to color change over the breeding season. Bluebird feather coloration can be influenced by a number of factors such as age (Siefferman et al. 2005) and reproductive output during the previous breeding season (Siefferman and Hill 2005a). Thus, the effect of FDB could
easily be masked by unconsidered covariates. In short, within-individual changes in feather color due to FDB may not be correlated with among-individual color variation at any one time.

The goal of our study was to determine if within-individual feather color change over the course of the breeding season was associated with individual FDB load. To do so, we quantified FDB and non-FDB load within the plumage of nesting pairs of adult eastern bluebirds and objectively measured their feather coloration at two points during the breeding season to look for correlations between FDB load and feather color change. We predicted that FDB would be associated with increased feather brightness and decreased UV-chroma of individual birds. If an association between feather color change and FDB load is found, it would be the first evidence consistent with FDB degrading the feathers of live birds. If the association between FDB and color change is positive, as we predict, then it would suggest that FDB can have a positive influence on birds in the wild (at least those birds with sexually selected structural feather coloration), and thus it may not be appropriate to describe FDB as parasites. If the association between FDB and color change is negative, it would be consistent with a parasitic effect of FDB.

We also looked for evidence of parasite-mediated natural selection via FDB. Under parasite-mediated natural selection, the distribution of parasites among individuals should be aggregated, with most individuals possessing few parasites and a few individuals possessing many parasites, indicated by a high mean/variance ratio in individual parasite load within the population (Goater and Holmes 1997). Further, one expects a negative correlation between parasite load and host fitness metrics.
(Goater and Holmes 1997). To date, most studies of FDB in wild populations have only reported the percentage of individuals carrying FDB (Burtt and Ichida 1999, 2004, Whitaker et al. 2005) and have not addressed individual variation in FDB load (but see Shawkey et al. 2007), and no study has reported associations between FDB load and individual health or reproductive success. This information is essential to determine whether FDB, or plumage bacteria in general, are influencing natural populations of birds (Clayton 1999). We looked for an association between FDB load and body condition of the adult bluebirds monitored in this study.

**Methods**

*Field site and first capture of adult bluebirds*

Adult male and female bluebirds were captured using nest box traps (small trap doors set above the entrance holes of breeding boxes) from May 1 to May 31 2007 within York and James City counties, Virginia, USA. All birds were captured during the nestling phase of their first nesting attempt, when eggs had hatched and adults were feeding young. Each bird was banded with a U.S. Geological Survey metal leg band and a unique combination of three plastic colored leg bands. Mass was measured to the nearest 0.1g using an electric balance and wing length was measured to the nearest 0.1 mm using dial calipers. We collected nine feathers from the blue rump patch of each individual for spectrophotometric color analysis. The feather samples were randomly collected from either the right or left half of the rump patch of each individual. We did not take feathers from the entire rump area because we intended to recapture each bird and collect a second feather sample to address color change over
the breeding season in relation to FDB load. Second feather samples were taken from the side not sampled during the first capture (see below). By leaving one half of the rump undisturbed, and taking our second feather sample from that half, we reduced the chance that feathers collected the second time would be freshly grown and thus not reflect color change of feathers that had been present since fall molt. Latex gloves were worn during sampling and all further processing of feathers to avoid alteration of feather color characteristics due to oils from human skin.

Two samples of plumage bacteria were collected from each bird. One sample was collected from rump feathers (“rump bacteria”) and another was taken from feathers over the rest of the body (“body bacteria”). Rump bacteria were sampled from only one side of the rump patch, the same side from which rump feathers were collected. Bacterial samples were collected by dipping a sterile cotton-tipped applicator in 2 ml sterile phosphate buffered saline (PBS) with 0.01% Tween-80 (pH 7.25) contained in a sterile 15 ml falcon tube, and running the applicator, while rotating it, through the plumage. The applicator was then placed back in the PBS until bacterial plating. All bacterial samples were collected by ARG, and all birds were sampled in an identical manner. A new pair of sterile latex gloves were worn while processing each bird, and were put on only immediately before handling to ensure that all bacteria collected were derived from the plumage. All birds were released immediately following this sampling and none of them abandoned their nests.

One to three hours after collection, bacteria were removed from the cotton applicator by vortexing, and were grown on two different growth media; trypticase soy agar (TSA), a general microbial growth medium, and feather meal agar (FMA), a
medium upon which only microbes that can hydrolyze keratin can grow (Sangali and Brandelli 2000). 100 ul of sample was plated onto each medium from both the rump and body samples of each bird. Plates were incubated for 5 days at 35 °C, after which the colony forming units (cfu) present on each plate were counted. All bacterial plating and counting was completed by ARG without knowledge of the color or body condition of individual birds.

Second capture of adult birds

A minimum of 30 days after the initial capture, adult bluebirds were recaptured and a second rump feather sample was collected for color analysis (resamples collected between June 20 and July 28 2007). The second feather sample was taken from the opposite side of the rump from which the initial sample was taken (see above). At the same time we re-recorded all body measurements.

Color analysis

The nine feathers collected from each individual bird were stacked on top of each other to mimic their placement on the bird, and were placed over a standard matte black surface. Color analyses were conducted with an Ocean Optics S2000 uv-vis spectrometer (range 300-700 nm) with a PS2 pulsed xenon light source. The probe was held at a 90 degree angle to the feathers inside a metal sheath to exclude external light, with the distance from the feathers adjusted to sample an area with a diameter of 3 mm. One sample with the spectrometer consisted of the average of 20 reflectance spectra taken sequentially. We sampled the feathers from each bird three times in this
way, and averaged the three outputs to obtain the reflectance spectra for the rump patch of each bird used in analyses.

We calculated hue (wavelength of maximum reflectance), brightness (total area under the reflectance curve from 300-700 nm), UV-chroma (proportion of total reflectance from 300-400 nm), and color purity (proportion of reflectance within +/- 50 nm of the hue). Because of strong covariance among these color metrics, we entered the color scores into a principal components analysis (PCA) to reduce the number of variables. We ran color PCA’s for males and females separately because the reflectance spectra of the sexes, while similar, do differ markedly.

**Body Condition**

Individual body condition, here defined as mass corrected for body size, was the residuals of mass regressed over wing length. Because of sexual size dimorphism, regressions were conducted on males and females separately.

**Statistical Analyses**

Data were analyzed using linear regressions and mixed model analysis of variance (ANOVA) with two-tailed tests of probability. All statistical analyses and computation of color characteristics were done using the R statistical programming package (v. 2.4.1). Data on bacterial abundance were log transformed to improve normality.
Results

Distribution of FDB within the population

We detected FDB on 99% (67/68) of the adult bluebirds sampled. To determine the total FDB load of each individual, we summed the number of FDB cfu’s detected from the rump and body. The distribution of FDB load on individual adult bluebirds was highly skewed to the right (mean/variance ratio = 886.88), with most individuals having relatively few FDB, and some individuals having a high FDB load (Figure 1). A similar pattern was seen for non-FDB (mean/variance ratio = 781.983) (data not shown).

Principal Components Analysis with plumage bacteria loads

We combined individual FDB and non-FDB loads using two separate principal components analyses (PCA). In one PCA, we included FDB and non-FDB collected only from the rump feathers of each bird. Rump bacteria PC1 loaded positively for FDB and non-FDB, and loaded most highly for FDB (Table 1). Thus, individuals with a high rump bacteria PC1 score had high loads of FDB and non-FDB. In rump bacteria PC2, FDB and non-FDB loaded in opposite directions (Table 1). In the other PCA, we summed the FDB and non-FDB counts from the rump and body samples to create a metric of full-body plumage bacteria load. Body bacteria PC1 explained 80.9% of the variance in the data, and loaded positively for both FDB and non-FDB, and loaded most highly for FDB (Table 2). Thus, individuals with a high body bacteria PC1 score have many FDB and many non-FDB. PC2 explained 19.1% of the variance, and loaded in opposite directions for FDB and non-FDB. Individuals with a
high PC2 score had an inordinately large non-FDB load relative to their FDB load. All bacterial counts were log-transformed to improve normality before entering them into the PCA’s. PC1 scores were used in all further analyses as individual FDB and total bacteria load.

*Feather color change and FDB*

We recaptured 32 adult bluebirds ($n_{\text{males}} = 15$, $n_{\text{females}} = 17$) during their second nesting attempt. Principal components analysis with female color scores yielded two PC’s that explained 90.3% of the variance in the data (Table 3). PC1 loaded positively for brightness, saturation, and UV-chroma, but negatively for hue. Thus, females with a high PC1 score have brighter, more saturated, and more UV-rich rump feather coloration with a left-shifted hue. PC2 loaded negatively for brightness, hue, and UV-chroma, but positively for saturation. Females with high PC2 scores have highly saturated dull rump feathers with less UV-chroma and a left-shifted hue.

Change in female coloration over time was not associated with rump bacterial load. Rump bacteria PC1 was not associated with change in either female color PC1 ($r^2 = 0.0271$, $P = 0.5278$) (Figure 2) or color PC2 ($r^2 = 0.0847$, $P = 0.2569$) (Figure 3).

Principal components analysis with male color scores yielded two PC’s that explained 86.5% of the variance in the data (Table 4). Male color PC1 loaded positively for brightness, hue, and UV-chroma, but negatively for saturation (Table 4). Thus, males with a high PC1 score have brighter, more UV-rich feather coloration with right-shifted hues and low saturation. PC2 (Table 4). Male color PC2 loaded negatively for brightness, saturation and UV-chroma, but negatively for hue. Males
with high PC2 scores have duller, less UV-rich, and less saturated rump coloration with right-shifted hues. Rump bacteria PC1 was significantly associated with change in male color PC1 over time ($r^2 = 0.3333$, $P = 0.02424$) (Figure 4). Males with more FDB had increased UV-chroma, hue, and brightness. Interestingly, the PC1 scores of some males went down over time. Thus, FDB appear to not only increase, but mitigate the reduction of, the aforementioned color characteristics. Change in male color PC2 was not associated with rump bacteria PC1 ($r^2 = 0.0138$, $P = 0.6757$) (Figure 5).

**Associations of plumage bacteria with body condition**

We included body bacteria PC1 as a factor in a mixed model ANOVA with body condition as the dependent variable and several other independent variables that we felt may influence individual condition (date of sampling, number of chicks in the nest, and sex) (Table 5). All terms and interactions were initially included in the model, and non-significant terms were removed until the most parsimonious model was found. Body bacteria PC1 was used instead of rump bacteria PC1 because body condition would likely be affected by plumage bacteria throughout the entire body, and not just those on the rump. None of the independent variables significantly predicted body condition alone. There was however, a highly significant sex x bacteria PC1 interaction ($F_{1,56} = 10.4207$, $P = 0.002$). Body condition correlates positively with PC1 in males ($r^2 = 0.1993$, $P = 0.0104$) (Figure 6), but negatively with PC1 in females ($r^2 = 0.1265$, $P = 0.0332$) (Figure 7).
Discussion

We found the highest prevalence (% individuals infected) of FDB ever reported in a wild bird population (99%). Previous multispecies surveys of the prevalence of FDB on wild birds by Burtt and Ichida (1999) and Whitaker et al. (2005) found mean prevalences of 7% and 39%, respectively, while Shawkey et al. (2007) found FDB on 89% of male eastern bluebirds in a population in Alabama. Our data suggest that FDB may be a ubiquitous feature of avian plumage, at least in some species.

Furthermore, we found that FDB have an aggregated distribution within the bluebird population. Most individual bluebirds have few FDB in their plumage, while a few bluebirds carry many FDB (Figure 1). A highly aggregated parasite distribution on hosts is expected if parasites are mediating natural selection because the influence of parasites should select for individuals with high parasite resistance (Goater and Holmes 1997), thus shifting the distribution of individual parasite loads to the left within a population.

For parasites to mediate natural selection, they must also influence host fitness. We found correlational evidence that plumage bacteria affect body condition, a trait ultimately thought to affect individual reproductive success (Jensen et al. 2004, Blums et al. 2005, Dyrcz et al. 2005, O'Dwyer et al. 2006). Instead of having strictly negative effects, however, plumage bacteria appear to benefit males while harming females. Sex biases in parasitism are common, with prevalence of infection and susceptibility to parasites often being greater in one sex (Zuk and McKean 1996, Schalk and Forbes 1997, McCurdy et al. 1998, Klein 2000). We are unaware of any cases, however, in which reputed parasites positively influence one sex while
negatively influencing the other. These data suggest that plumage bacteria cannot be generally thought of as parasites. Interestingly, plumage bacterial loads of males and females within nesting pairs are highly correlated ($r^2 = 0.2379, P = 0.0098$) (Figure 8). Thus, it appear that there may be different trade-offs for each sex in terms of plumage bacteria. Females may benefit from low bacteria loads for themselves, while simultaneously benefiting from high loads for their mates, assuming male condition is correlated with mate quality. Males should benefit from high plumage bacteria loads for themselves, but low loads for their mates, assuming female condition correlates with reproductive parameters such as increased egg mass or number. However, because male and female plumage bacteria loads correlate, it appears that males and females cannot both have optimal plumage bacteria loads. The costs and benefits of these potential trade-offs will depend on how bacteria influence individual fitness, whether birds acquire plumage bacteria from mates or other environmental sources, and what specific species of bacteria occur within the plumage of each sex. At present, these questions have yet to be addressed.

We also found that within individual color change over the breeding season in relation to FDB load differs between the sexes. Change in rump feather coloration of females was not related to rump feather FDB load. However, in males, FDB were associated with an increase in UV-chroma, hue, and brightness of rump feathers. These results are important for two reasons. First, they are the first evidence consistent with FDB altering the feathers of live birds. This suggests that FDB may indeed be an important and understudied facet of avian biology. Second, these results suggest that FBD can influence the expression of sexually selected signals, and
perhaps not in a strictly negative manner (Shawkey et al. 2007). The blue coloration of bluebirds appears to be a sexually selected and involved in male-male interactions (Siefferman and Hill 2005b, Liu et al. 2007). Males with high UV-chroma nest earlier and fledge more offspring than those with low UV-chroma (Siefferman and Hill 2005b), although males with low hue values have also been shown to fledge more offspring (Siefferman and Hill 2003). Because FDB appear to increase UV-chroma and hue, we cannot tell if bacterial degradation enhances or reduces the color signal. What seems clear, however, is that the color status of males within the population will likely change due to the action of FDB. This dynamic could influence the relative female perception of males over time, which could lead to variation in male reproductive success as a result of extra-pair copulations.

Because of the correlational nature of our data, we can only speculate as to what mechanisms could cause the sexes to differ in their response to plumage bacteria. It is unlikely that the affect of plumage bacteria on individual condition is mediated by FDB specifically, because it is difficult to imagine a scenario in which feather degradation could improve male body condition. Instead, the relationship could be mediated by other members of the plumage microbial community and/or by behavioral differences between males and females. If other members of the plumage bacteria are responsible, they would have to work endogenously. Birds preen their entire plumage with their bills on a daily basis and, thus, could be constantly inoculating themselves with plumage bacteria. It is well known that certain bacteria can positively influence hosts by aiding in digestion and nutrient uptake. Perhaps
male plumage harbors these beneficial bacteria, while female plumage has higher numbers of detrimental bacteria.

Male and female bluebirds differ markedly in reproductive behaviors, which could lead to differences in plumage bacterial communities and ultimately to differences in the effect of plumage bacteria on condition. Both sexes defend territories and provision young, but females primarily build nests and spend two weeks incubating eggs in the nest box. This reduction in female activity could be associated with reduced preening and self-maintenance, and the humid, dark environment of the nest box may increase bacterial activity. Furthermore, the nesting substrate itself may be a source of bacteria for females but not for males, leading to differences in plumage bacterial communities between males and females.

The effect of FDB on feather coloration is thought to result from FDB thinning the outer cortex of feather barbules (Shawkey et al. 2007). This could explain the change in coloration of males as a result of FDB, but it does not explain the lack of association between FDB and color change in females. The blue of male and female feathers is produced by the same mechanism, namely the coherent refraction of light through the feather microstructure. However, female rump feathers are duller than those of males, and thus female feathers may contain higher concentrations of the pigment melanin, which imparts feathers with resistance to bacterial degradation (Goldstein et al. 2004, Gunderson et al. unpub. data). Male and female feathers may also differ in other structural aspects, although this is only speculation. As with body condition, the difference may also be the result of differences in the composition of male and female plumage microbiota.
Our results are the first consistent with FDB damaging the feathers of live birds in a wild population. This has broad implications for all avian systems, as FDB appear to be cosmopolitan and occur on a wide range of bird species (Burtt and Ichida 1999, Whitaker et al. 2005). They also suggest that FDB can influence feather coloration, an important component of avian communication. We have also shown that plumage bacteria may influence the health of individual birds, although with opposite effects for males and females. Clearly, microbial ecologists and researchers working with avian systems should begin to explore the microbial communities of birds’ plumage, as they may harbor important parasites and/or symbionts that have contributed to avian evolution.

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References


Figure 1. Distribution of feather-degrading load among adult bluebirds.
### Table 1. Principal components loadings for bacteria loads on rump feathers (variance explained by each PC in parentheses).

<table>
<thead>
<tr>
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<th>PC1 (83.8%)</th>
<th>PC2 (16.2%)</th>
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<tr>
<td>FDB</td>
<td>0.7798</td>
<td>0.6260</td>
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<tr>
<td>Non-FDB</td>
<td>0.6260</td>
<td>-0.7798</td>
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### Table 2. Principal components loadings for whole-plumage bacteria loads (variance explained by each PC in parentheses).

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<th>PC1 (80.9%)</th>
<th>PC2 (19.1%)</th>
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<tr>
<td>FDB</td>
<td>0.7670</td>
<td>-0.6416</td>
</tr>
<tr>
<td>Non-FDB</td>
<td>0.6416</td>
<td>0.7670</td>
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</table>

### Table 3. Principal components loadings for female color scores (variance explained by each PC in parentheses).

<table>
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<th>PC1 (56.1%)</th>
<th>PC2 (34.2%)</th>
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<tr>
<td>Brightness</td>
<td>0.3775</td>
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<td>Hue</td>
<td>-0.3509</td>
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<td>Saturation</td>
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<td>UV-Chroma</td>
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### Table 4. Principal components loadings for male color scores (variance explained by each PC in parentheses).

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<tr>
<th></th>
<th>PC1 (56.9%)</th>
<th>PC2 (29.6%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brightness</td>
<td>0.4174</td>
<td>-0.5782</td>
</tr>
<tr>
<td>Hue</td>
<td>0.6047</td>
<td>0.2519</td>
</tr>
<tr>
<td>Saturation</td>
<td>-0.3232</td>
<td>-0.7349</td>
</tr>
<tr>
<td>UV-Chroma</td>
<td>0.5963</td>
<td>-0.2490</td>
</tr>
</tbody>
</table>
Figure 2. Within-female change in female color PC1 in relation to rump bacteria load (rump bacteria PC1).
Figure 3. Within-female change in female color PC2 in relation to rump bacteria load (rump bacteria PC1).
Figure 4. Within-male change in male color PC1 in relation to rump bacteria load (rump bacteria PC1).
Figure 5. Within-male change in male color PC2 in relation to rump bacteria load (rump bacteria PC1).
Table 5. Mixed model ANOVA of factors contributing to individual body condition.

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>bacteria PC1</td>
<td>1</td>
<td>0.2164</td>
<td>0.1253</td>
<td>0.7247</td>
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<tr>
<td>sex</td>
<td>1</td>
<td>1.1105</td>
<td>0.6430</td>
<td>0.4259</td>
</tr>
<tr>
<td>date</td>
<td>1</td>
<td>0.2578</td>
<td>0.1493</td>
<td>0.7007</td>
</tr>
<tr>
<td># chicks</td>
<td>1</td>
<td>0.3339</td>
<td>0.1934</td>
<td>0.6618</td>
</tr>
<tr>
<td>PC1 x sex</td>
<td>1</td>
<td>17.9973</td>
<td>10.4207</td>
<td>0.0022**</td>
</tr>
<tr>
<td>PC1 x date</td>
<td>1</td>
<td>1.2894</td>
<td>0.7466</td>
<td>0.3912</td>
</tr>
<tr>
<td>sex x date</td>
<td>1</td>
<td>8.475</td>
<td>4.9072</td>
<td>0.0308*</td>
</tr>
<tr>
<td>PC1 x sex x date</td>
<td>1</td>
<td>6.527</td>
<td>3.7795</td>
<td>0.0569</td>
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<tr>
<td>Residuals</td>
<td>56</td>
<td>96.716</td>
<td>1.7271</td>
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</tr>
</tbody>
</table>
Figure 6. Association of male body condition with whole-plumage bacterial load (body bacteria PC1).
Figure 7. Association of female body condition with whole-plumage bacterial load (body bacteria PC1).
Figure 8. Association between whole-plumage bacteria load (body bacteria PC1) of males and females in nesting pairs.