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Original Article

Feather bacterial load affects plumage condition, iridescent color, and investment in preening in pigeons

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Feathers are inhabited by numerous bacteria, some of them being able to degrade feathers, and thus potentially alter thermoregulation and visual communication. To limit the negative effects of feather bacteria on fitness, birds have therefore evolved antimicrobial defense mechanisms, including preening feathers with secretions of the preen gland. However, whether feather bacteria can alter feather condition and color signaling in vivo, and thus whether birds adjust their investment in preening according to feather bacterial load, has barely been investigated. Here, we experimentally decreased and increased feather bacterial load on captive feral pigeons *Columba livia* and investigated the effects on plumage characteristics and investment in preening. We found that birds of both sexes had a plumage in higher condition and invested less in preen secretion quantity and preening behavior when feather bacterial load was lower. It suggests that preen secretions may be used by pigeons to limit feather degradation by bacteria, but as they are probably costly to produce, their quantity is adjusted depending on feather bacteria load. Birds with lower bacteria load on feathers had brighter iridescent neck feathers, suggesting that feather bacteria may play an important role in the evolution of the signaling function of iridescent color in pigeons. Altogether, our study provides the first experimental evidence for in vivo effects of feather bacteria on plumage degradation and coloration and suggests that preening is an inducible antibacterial defense.

Key words: **bacteria, birds, iridescence, plumage, preen oil.**

INTRODUCTION

Bacteria are fundamental associates of animal bodies living in digestive, respiratory, and reproductive tracts. Bacteria live not just within but also on the surface of bodies, in skin, and feathers (Tannock 1995; Burtt and Ichida 1999; Shawkey et al. 2003). Some of these bacteria are opportunistic pathogens (Scott 2001; Cogen et al. 2008), while others are part of the normal microflora (Tannock 1995). Recently, several studies have highlighted the potential influence of bacteria on animal behavior and communication (Archie et al. 2007; Sharon et al. 2010; Ezenwa et al. 2012). However, beyond the effects associated with bacterial infection (Hart 1988), we understand little about bacteria's more routine contribution to host behavior and life history traits.

In birds, a small subset of feather bacteria is detrimental to the bird by degrading keratin (i.e., keratinolytic bacteria) and causing damage to feathers (Burtt and Ichida 1999). By breaking down the structures of feathers, feather-degrading bacteria may reduce bird fitness via the alteration of thermoregulation, flight, and signaling (Swaddle et al. 1996; Clayton 1999; Shawkey et al. 2007). For instance, structural parameters of feathers may determine their water repellency (Rijke 1970; Giraudeau et al. 2010; Eliason and Shawkey 2011), an important component of thermoregulation, and flight efficiency. Feather degradation may also reduce feather coloration, as feather microstructures or pigments are consumed or modified through microbial action (Shawkey and Hill 2004). Accordingly, in vitro experiments have shown that feather-degrading bacteria brighten structurally colored feathers in male eastern bluebirds *Sialia sialis* (Shawkey et al. 2007). However, experimental in vivo work on the effects of feather bacteria on plumage has rarely been done. The only experimental study on live birds has shown no change in feather damage after inoculation with one species of feather-degrading bacteria (Cristol et al. 2005). Given the complexity of plumage bacterial communities, more in vivo experiments are required to test for the impact of plumage bacterial communities on feather condition and coloration (Gunderson 2008).

Moreover, if maintaining feather condition and coloration is important in term of fitness, birds must have evolved a number of antibacterial defenses (Gunderson 2008). For instance, the deposition of melanin pigments in feathers can constitute such adaptation

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by increasing feathers resistance to bacterial degradation. Several behaviors, such as sunbathing (as sunlight may destroy bacteria), molting, or preening may also constitute adaptation to plumage bacterial communities (Gunderson 2008). During preening, birds deposit oily secretions of the preen gland (also called uropygial gland) onto the plumage. These oily secretions have numerous properties (Jacob and Ziswiler 1982; Hagelin and Jones 2007), one of which is to protect feathers against feather-degrading bacteria (Shawkey et al. 2003). In vitro experiments have shown that preen secretions of several species inhibit the growth of isolated bacteria (Shawkey et al. 2003; Reneerkens et al. 2008). Furthermore, in house sparrows *Passer domesticus*, preen gland removal led to increased load of feather bacteria (Czirják et al. 2013) and, in barn Swallows *Hirundo rustica*, the abundance of feather-degrading bacteria decreased with increasing size of the uropygial gland (Møller et al. 2009). Furthermore, if preen secretions or preening are costly, birds are expected to invest in them only when bacterial load is high (i.e., induced defense; Harvell 1990; Tollrian and Harvell 1999).

Here, we experimentally increased and decreased bacterial load on the plumage of captive feral pigeons *Columba livia* to test whether load of feather bacteria affects plumage condition (quality, water repellency efficiency, and iridescent color) and investment in preening (preen secretion quantity and preening behavior).

MATERIALS AND METHODS

Experimental design

In March–May 2013, 80 feral pigeons (43 females and 37 males) were captured at different locations in Paris, France. They were kept in 6 outdoor aviaries at the CEREEP field station (Centre de recherche en Ecologie Expérimentale et Prédictive – Ecotron Ile-de-France, UMS 3194, Saint Pierre lès Nemours, France) in similar conditions and fed ad libitum with a mix of maize, wheat and peas, and mineral supplements. Birds were kept in captivity for ca. 2 months for acclimation to obtain naturally representative pigeon physiology and behavior. After acclimation, birds were assigned to treatment (BACT−: decreased feather bacterial load, BACT+: increased feather bacterial load, and CO: control treatment), and they were weighed to the nearest g, wing length was measured to the nearest mm, and melanin-based color morph was recorded. Feral pigeons display a continuous variation in eumelanin-based coloration from white to black that display differences in several life history traits (Jacquin et al. 2011; 2013). Therefore, we equally distributed eumelanin-based coloration of pigeons among treatments (Kruskal–Wallis test: $H_3 = 0.65$, $P = 0.89$). We did the same for body mass (linear model: $F_{2,77} = 0.45$, $P = 0.64$) and body condition (linear model: $F_{2,77} = 1.10$, $P = 0.34$). Birds were weighed at day 15, day 28, day 42, day 56, and day 70 after onset of treatment.

In the BACT− treatment, birds from 2 aviaries ($n = 14$ females and 13 males) were sprayed twice a week with 0.02% chlorhexidine (Hibitane Irrigation®, MSD) in saline solution (0.9% NaCl solution). Chlorhexidine is an antiseptic, frequently used as a topical antiseptic skin scrub and topical disinfectant of wounds in hospitals and veterinary clinics. In the CO treatment, birds from 2 aviaries ($n = 14$ females and 12 males) were sprayed twice a week with saline solution. In the BACT+ treatment, birds from 2 aviaries ($n = 15$ females and 12 males) were sprayed twice a week with freshly cultivated bacteria in saline solution. Freshly cultivated bacteria came from feather bacteria sampled from Parisian feral pigeons and cultivated on Tryptic Soy Agar (TSA) plates and feather meal agar (FMA) plates. TSA allows the growth of both keratinolytic and non-keratinolytic bacteria, while FMA allows the growth of keratinolytic bacteria only. We used both agar media to ensure the inoculation of keratinolytic bacteria in BACT+ birds. Each day of treatment, a total of 1.5 L of solution per aviary was used to spray birds. Birds of the same aviary got the same treatment to avoid potential transmission of the treatment between birds by social interactions.

We checked the effect of treatment on feather bacterial load by cultivating feathers bacteria on whole flora agar slides (plate count agar + triphenyltetrazolium chloride + neutralizing dip slides; VWR BDH Prolabo), every fortnight for 2.5 months ($n = 6$ control date). Slides were pressed for 10 s onto the back feathers of 4 random birds of each treatment and then incubated for 24–48 h at 37 °C. Feather bacterial load was expressed as the number of bacterial colonies per slide.

Iridescent feather color

After 1.5 months of treatment, 5–10 feathers from the left side of the neck were cut at the base and stored at −20 °C until analyses. Pigeons display 2 kinds of iridescent neck feathers, the green and purple feathers, which show color changes in opposite ways when reflection angles vary (McGraw 2004; Yin et al. 2006; Yoshioka et al. 2007). Here, we focused on feathers that appeared green in color to the human eye at normal incidence. Neck feathers were mounted on a black velvet card and color was measured with a reflectance spectrometer (Ocean Optics USB2000), a Xenon light source (Ocean Optics PX-2), and a 200-µm fiber optic reflectance probe. The probe was inserted in a black tube in a way that the probe incidence angle was 90°, but that the probe could be slightly orientated to maximize reflectance. Reflectance was measured using SpectraSuite software (Ocean Optics, Inc.) and in relation to a dark and a white (Spectralon®, Labsphere) standard. The spectrometer was calibrated between each 15 measurements. Reflectance measurements were done blind according to treatments. For each bird, feather color was measured 3 times and the 3 spectra were then averaged to make a single measurement.

Iridescent neck feathers that appeared green in color to the human eye exhibited 3 full reflectance peaks (McGraw 2004), one in the UVB range (average $\lambda_{max} = 357 \pm 1$ nm), one in the violet range (average $\lambda_{max} = 431 \pm 2$ nm), and one in the green range (average $\lambda_{max} = 548 \pm 2$ nm). Two additional peaks—one in the UVB range and one in the red range—were frequently truncated when considering the 300–700 nm range (Figure 1).

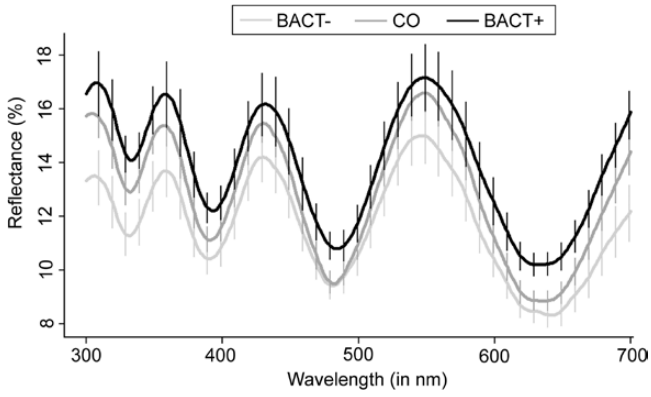

Figure 1
Mean reflectance spectra ± SE of iridescent neck green feathers in BACT−, CO, and BACT+ pigeons.

To assess whether birds from the 3 different treatments appeared dissimilar to their conspecifics, we modeled spectral sensitivities of pigeons and computed photoreceptor responses (quantum catches) and brightness (luminance) using models developed for the tetrachromatic visual system of birds. We modeled spectral sensitivities of cone photoreceptors between 300 and 700 nm according to the model of Hart and Vorobyev (2005), using oil droplet and single cone photoreceptor spectral parameters of pigeons published by Bowmaker et al. (1997), and the ocular media transmittance of pigeons measured by Lind et al. (2014). We computed photoreceptor responses and brightness using the Endler and Mielke's model (2005). We used the sum of the 2 longest-wavelength cones as the sensitivity data to calculate achromatic cone stimulation. We chose the standard illuminants D65 as a representative spectrum for open habitat midday ambient light. All spectral analyses were conducted with the "pavo" package (Maia et al. 2013) in R statistical software (R Development Core Team 2014).

Repeatability (intraclass coefficient) of the 4 quantum catches and brightness within birds was measured using the "ICC" package in R (Wolak et al. 2012) and was mean (95% confidence interval): 0.52 (0.39–0.64), 0.68 (0.57–0.77), 0.59 (0.46–0.70), 0.61 (0.49–0.72), and 0.45 (0.31–0.58), respectively.

Plumage condition and hydrophobicity

In order to measure plumage condition, we used a similar method as Moyer et al. (2003). After 2 months of treatment, around 5 feathers from the lower back of the birds were collected by cutting them at the base and stored in plastic bags until analyses. Plumage quality was then scored from 1 to 5 (1 = very poor condition, 2 = poor condition, 3 = fair, 4 = good, and 5 = very good; Figure 2), blind to treatment. Condition was scored twice independently. As scores were repeatable ($r = 0.60$, $P < 0.0001$; ICC = 0.61), analyses of plumage condition was performed using average values.

After 3 months of treatment, plumage water retention efficiency was tested using the protocol described in Giraudeau et al. (2010). Briefly, we measured the bird's dry weight (to the nearest 0.1 g), then we submerged the birds into a water bath (except the head) for an exact duration of 5 s, by gently holding them in a way that they could not open their wings. After 5 s, we lifted the birds from the water and waited for 5 s before the second weighing to let the extra water run-off of the surface of plumage. The difference between the 2 weights indicated the amount of water retained by the plumage and provided a measure of plumage wetness. Birds were kept without any access to water during 2 h before the test to be sure that their plumage was dry at the moment of the measurement.

Preen secretion and preening behavior

After 2.5 months of treatment, preen secretions were collected by gently pressing the gland and collecting the exudates in 20 µL glass capillaries. The gland was pressed until no more secretion went out. Secretion quantity into the capillary was measured as the total capillary length filled with secretion (to the nearest 0.5 mm) and then converted to µL. One bird had no gland, and another bird had a gland orifice which seemed to be blocked. An abnormal ball of preen oil appeared within the preen duct but no oil exuded when the gland was pressed. These 2 birds were excluded from the analyses on preen secretion quantity, preening behavior, and water repellency efficiency.

Preening behaviors were observed for 46 days after onset of treatment. Each bird was observed for 5 min once or twice a week ($n = 790$ focal sessions). Focal birds were randomly chosen but by alternating between treatments. Furthermore, when a bird was observed only once a week, it was observed twice in the following week. For each bird, we calculated the average percentage of time spent preening per 5-min observation. Birds were excluded from the analyses once they had laid eggs ($n = 14$ birds; 46 focal sessions).

Statistical analyses

The effect of treatment on log-transformed feather bacterial load was tested using a linear model with treatment and date as fixed effects. The effects of treatment on preen secretion quantity, percentage of time spent preening, log-transformed plumage water retention, plumage condition, and color (each of the 4 receptor

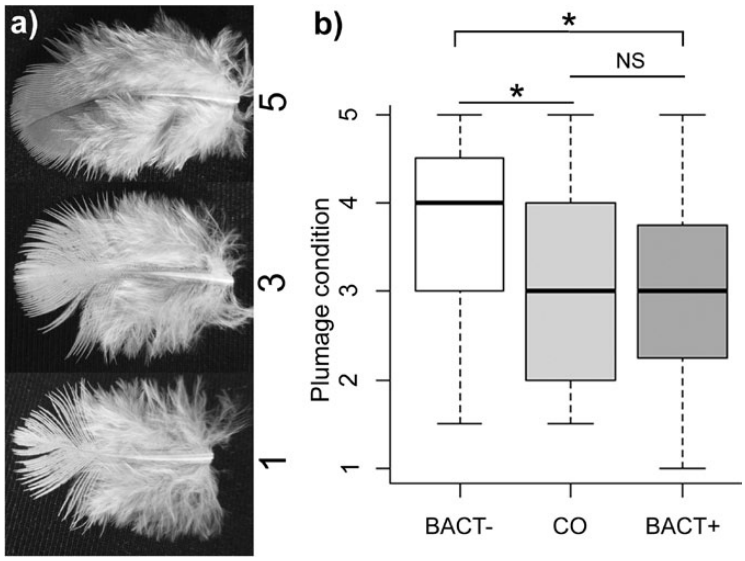

Figure 2
(a) Examples of back feather condition (1: very poor condition, 3: fair condition, and 5: very good condition). (b) Boxplot of back feather condition in BACT−, CO, and BACT+ pigeons. *$P < 0.05$, NS: $P > 0.05$.

quantum catches, and brightness) were tested using linear mixed models, with treatment, sex, color morph, and all double interactions as fixed effects. Body condition, measured as the residual of a regression between body mass and wing length, was also added as a fixed effect in the analyses. We did not use tarsus length as the index of body size as it strongly depended on the observer ($P < 0.0001$) and was not measured for all birds. In contrast, wing length was measured for all experimental birds and did not depend on the observer ($P > 0.10$). Wing length was slightly correlated with tarsus length (Pearson correlation: $r = 0.27$, $P = 0.017$). We began by including the aviary identity as a random factor in the models. However, it was significant in none of the analyses, and we excluded it from the models. When the treatment was significant, we used Tukey's tests to determine the treatments that significantly differ from each other. The effects of treatment on body condition was tested using linear mixed models with treatment, sex, morph and their interactions, and date as fixed effects. Bird identity was included as a random effect. Body condition at onset of treatment was added as a covariate. Model selection was performed by backward dropping nonsignificant terms (unless they appeared in higher order interaction terms) using a stepwise elimination procedure.

All statistical tests were conducted with SAS, version 9.1 (SAS Institute, Cary, NC). We used 2-tailed Type 3 tests for fixed effects with significance level set at $\alpha = 0.05$.

RESULTS

Feather bacterial load varied among treatments ($F_{2,64} = 29.89$, $P < 0.0001$; Figure 3). BACT+ birds had higher bacterial load than BACT− birds ($P < 0.0001$), while CO birds had intermediate bacterial load (CO vs. BACT+: $P < 0.0001$ and CO vs. BACT−: $P = 0.037$; Figure 3).

Body condition did not differ among treatments ($F_{2,73} = 1.56$, $P = 0.22$), but it depended upon the interaction between sex and color morph ($F_{1,75} = 5.73$, $P = 0.019$). Darker females are in better condition than paler females ($F_{1,40} = 8.42$, $P = 0.006$), while body condition did not differ among color morphs in males ($F_{1,34} = 0.26$, $P = 0.61$).

Quantity of preen secretion differed among treatments ($F_{2,74} = 6.88$, $P = 0.0018$; Figure 4a). BACT− birds had a lower quantity of preen secretion than CO and BACT+ birds ($P = 0.03$ and $P = 0.002$; Figure 4a), while BACT+ and CO birds had a similar quantity of secretion ($P = 0.57$; Figure 4a). Birds in better condition had a lower quantity of preen secretion than birds in lower condition ($F_{1,74} = 5.83$, $P = 0.018$).

Duration of preening differed among treatments ($F_{2,73} = 4.14$, $P = 0.02$; Figure 4b). BACT− birds preened less often than BACT+ birds ($P = 0.018$; Figure 4b). Duration of preening of CO birds was intermediate, but not significantly different from that of BACT− ($P = 0.13$) and BACT+ birds ($P = 0.72$; Figure 4b). Males preened for longer than females ($F_{2,73} = 4.49$, $P = 0.038$), and darker pigeons preened for longer than paler pigeons ($F_{2,73} = 5.89$, $P = 0.018$).

Condition of back feathers differed among treatments ($F_{2,76} = 6.81$, $P = 0.0019$; Figure 2b). BACT− birds had back feathers in higher condition than CO and BACT+ birds ($P = 0.045$ and $P = 0.0016$, respectively; Figure 2b), while feather condition of BACT+ birds did not significantly differ from that of CO birds ($P = 0.51$; Figure 2b).

Plumage water retention did not differ among treatments ($F_{2,74} = 2.47$, $P = 0.09$; Figure 5). Water retention was higher in birds in lower body condition than in better body condition ($F_{1,76} = 4.35$, $P = 0.040$).

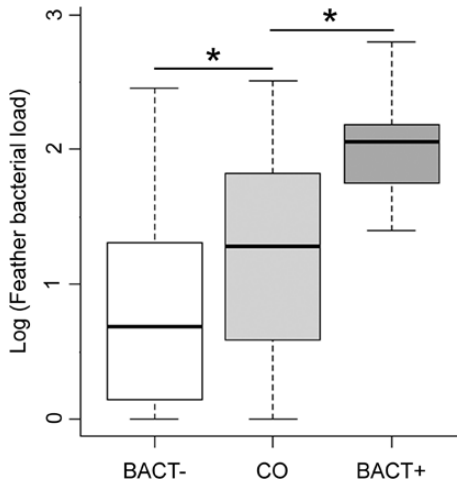

Figure 3
Boxplot of feather bacterial load in BACT−, CO, and BACT+ pigeons. *$P < 0.05$.

Brightness of iridescent neck feathers differed among treatments ($F_{2,71} = 4.36$, $P = 0.024$; Figure 1). BACT− birds had lower brightness than BACT+ birds ($P = 0.029$; Figure 1), while CO birds had intermediate brightness but not significantly different from BACT− and BACT+ birds (CO vs. BACT−: $P = 0.41$, CO vs. BACT+: $P = 0.37$; Figure 1). Interestingly, we found a significant interaction between sex and color morph on brightness ($F_{1,71} = 13.10$, $P < 0.0001$). In males, paler pigeons had lower brightness than darker pigeons ($F_{1,34} = 5.66$, $P = 0.023$), while in females, paler pigeons had higher brightness ($F_{1,39} = 5.94$, $P = 0.019$). The Q4 quantum catch (the long-wavelength-sensitive photoreceptor response) differed between neck feathers of males and females (mean ± SE: 0.24 ± 0.00 and 0.26 ± 0.01; $F_{1,75} = 6.05$, $P = 0.016$), and the Q1 quantum catch (the violet-sensitive photoreceptor response) tended to differ between neck feathers of males and females ($F_{1,75} = 3.15$, $P = 0.08$).

DISCUSSION

As predicted, pigeons with lower bacterial load on feathers had higher quality plumage. Avian feathers host keratinolytic bacteria (Burtt and Ichida 1999; Whitaker et al. 2005), which have the ability to degrade feathers in vitro (Burtt and Ichida 1999; Cristol et al. 2005; Shawkey et al. 2007; Ruiz-De-Castaneda et al. 2012). However, whether they degrade feathers in vivo has remained unknown. So far, the only experimental study on live birds has tested only one strain of keratinolytic bacteria and has shown no effect of the bacteria on feather condition (Cristol et al. 2005). Our study provides the first in vivo experimental evidence of a negative effect of plumage bacteria on feather condition, hence validating previous in vitro experiments.

As predicted, pigeons with lower feather bacterial load had a lower quantity of secretion within the preen gland and preened their feathers less often. Although pigeons have small preen gland compared to other birds (Montalti et al. 2005), our study seems to validate its functionality in this species, as found in a previous experiment showing positive effects of preen oil on feather condition in pigeons (Moyer et al. 2003). As suggested in previous studies

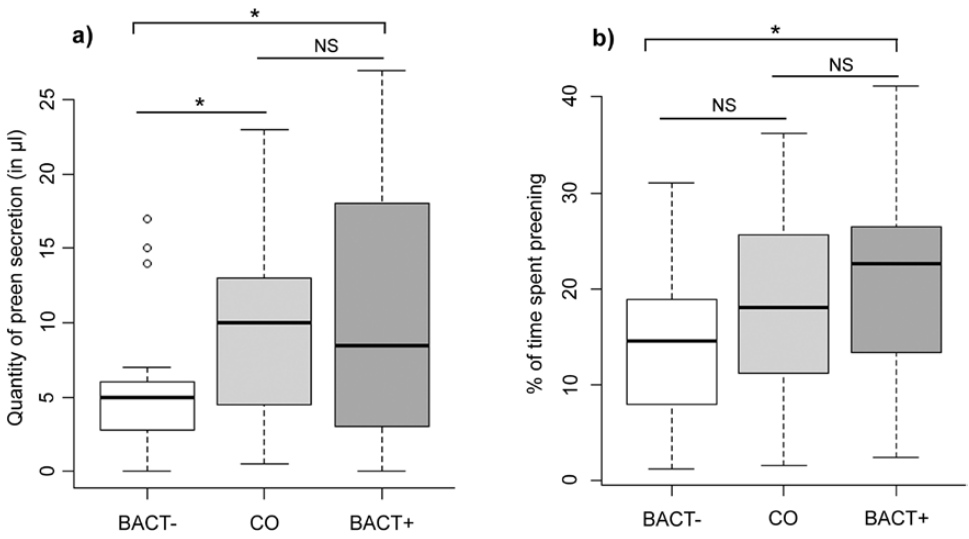

Figure 4
(a) Boxplot of quantity of preen secretion within the gland and (b) Boxplot of percentage of time spent preening in BACT−, CO, and BACT+ pigeons. *$P < 0.05$, NS: $P > 0.05$.

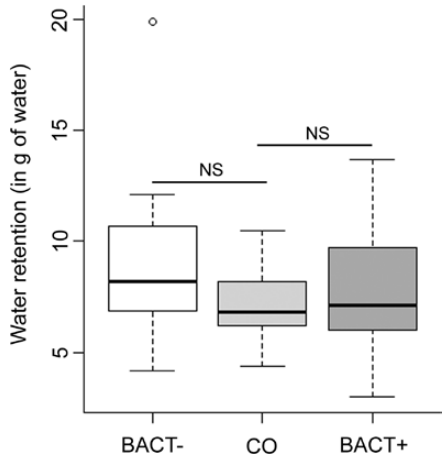

Figure 5
Boxplot of water retention (grams of water retained in plumage) in BACT−, CO, and BACT+ pigeons. NS: $P > 0.05$.

(Reneerkens et al. 2008), preen oil may reduce bacterial load by acting as a physical barrier that prevents bacteria to reach feathers. Nonexclusively preen oil may contain antibacterial substances, either lipids (Bandyopadhyay and Bhattacharyya 1996; Jacob et al. 1997) or peptides produced by bacteria hosted in the gland (Martín-Vivaldi et al. 2010). Our result shows that the production of preen oil may be induced by the presence of environmental pressures (i.e., high plumage bacterial load). According to the theory of inducible defense (Harvell 1990), if preen secretion and preening behavior are inducible, they may be costly. This cost may arise from the physiological costs of producing preen secretion or large preen gland or from the fact that preening, being time-consuming, may reduce time devoted to other behavior such as feeding or sleeping (Christe et al. 1996). In tawny owls *Strix aluco*, birds stimulated to produce an immune response have smaller preen gland (Piault et al. 2008), and in apapanes *Himatione sanguinea*, birds infected by *plasmodium* preen less frequently (Yorinks and Atkinson 2000). Whatever the mechanism behind the cost, the consequence of this induced response may have important consequences in life history strategies. Birds with low bacterial load on feathers may save energy from this costly response for other life history traits. However, the cost of this response needs to be tested experimentally by distinguishing the negative effects of bacterial load from the induced response. It is the next methodological challenge to address this question for future studies.

Using an avian visual model, we found that birds with lower bacteria load on plumage had less bright neck feathers. In eastern bluebirds, feather bacteria load is positively related to brightness of structural UV-blue plumage color and in vitro experiments has confirmed that keratinolytic bacteria degrade fathers and brighten them. Bacterial degradation of the light-absorbing cortex of bluebirds' feathers may cause greater reflection of light and hence higher brightness (Shawkey et al. 2007). In contrast to bluebirds, structural colors of pigeons originate from the thin-film interference of the top keratin cortex layer, while brightness partly originates from the medullary layer (Yin et al. 2006). It suggests that changes in brightness coupled with lack of change in chromatic color are probably not associated with bacterial degradation of the barbule cortex layer. Further studies should examine whether changes in brightness in pigeons' feathers are due to modifications of feather structure. Alternatively, decreased preen secretion onto the plumage of birds with lower bacterial load may have directly decreased feather brightness. However, evidence in mallards and tawny owls suggest that preen oil decreases, rather than increases, integument brightness (Delhey et al. 2008; Piault et al. 2008). Although the importance of neck feather coloration in signaling in pigeons is unknown, several observations suggest that it may play a role in sexual selection. During agonistic and courtship behavior, males display their neck feathers at conspecifics (Johnston 1992). Furthermore, our results show that males and females differ in the chromatic coloration of neck feathers, and that this difference occurs mainly in the long wavelength range. To humans, females seem also less iridescent than males (Johnston 1992), although confirmation of this observation would require measurements of iridescence properties and quantity of iridescent feathers. Feather bacteria may therefore play an important role in the evolution of the signaling function of neck feather color in pigeons. Neck feather color might reveal bacterial damage and therefore be scrutinized by conspecifics during mate choice or competition.

Feather bacterial load did not seem to affect water repellency efficiency of the plumage. Water repellency efficiency of the plumage depends on feather structure (Rijke 1970), but also on the quantity of preen secretions (van Rhijn 1977), which contain hydrophobic compounds (Montalti et al. 2005; Leclaire et al. 2011; Campagna et al. 2012). In our study, plumage quality is negatively related to preen secretion quantity and preening. If both factors affect water retention in pigeons, they may compensate each other, which result in a lack of differences between treated and control birds. Studies including experimental increase of preen secretion on feathers of varying condition, and hydrophobicity measurements using, for instance, contact angle between water droplet and feathers (see Eliason and Shawkey 2011) would be needed to test this hypothesis.

To experimentally decrease bacterial load on feathers, birds were sprayed twice a week with a chlorhexidine solution. Chlorhexidine has not been shown to have adverse effects, and it is not irritating to the skin when concentration is lower than 2%. However, it might remove natural oils and emollients and therefore frequent washing may result in skin dryness. Birds sprayed with chlorhexidine would therefore have been expected to invest more in preening and to have degraded feathers. This alternative explanation is however unlikely as our results show the opposite pattern, suggesting that the observed effects are not due to chlorhexidine per se, but on its effect in decreasing bacteria load.

In conclusion, our study demonstrates, for the first time in vivo, that feather bacteria degrade feathers and alter iridescent coloration, thus potentially affecting visual signals involved in sexual or social competition. It further suggests that birds may invest in preening—a likely costly defensive traits—depending on the load of feather bacteria. Feral pigeons live in highly urbanized habitats which are known to harbor high bacterial densities (Shaffer and Lighthart 1997). Further studies should now evaluate whether wild urban pigeons have elaborated strategies, such as enlarged preen gland, to prevent feather degradation. Another strategy may be the elaboration of a darker plumage, as melanin-colored feathers are more resistant to bacterial degradation than unmelanized feathers (Ruiz-De-Castaneda et al. 2012). Urban feral pigeons display a darker melanin-based coloration than rural individuals (Johnston and Janiga 1995), but whether it reflects adaptation to habitat with high bacterial load needs now to be studied.

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