Functional Ecology

Bacteria and the evolution of honest signals. The case of ornamental throat feathers in spotless starlings

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Summary

1. Mechanisms guaranteeing reliability of messages are essential in understanding the underlying information and evolution of signals. Micro-organisms may degrade signalling traits and therefore influence the transmitted information and evolution of these characters. The role of micro-organisms in animal signalling has, however, rarely been investigated.

2. Here, we explore a possible role for feather-degrading bacteria driving the design of ornamental throat feathers in male spotless starlings (*Sturnus unicolor*). We estimated length, bacterial load, degradation status and susceptibility to degradation by keratinolytic bacteria in those feathers, compared with non-ornamental adjacent feathers in males, as well as to throat feathers in females. In addition, the volume of the uropygial gland and its secretion was measured and the secretion extracted. We also experimentally evaluated the capacity of each secretion to inhibit growth of a keratinolytic bacterium.

3. The apical part of male ornamental throat feathers harboured more bacteria and degraded more quickly than the basal part; these patterns were not detected in female throat feathers or in non-ornamental male feathers. Moreover, degradation status of male and female throat feathers did not differ, but was positively associated with feather bacterial density. Finally, the size of the uropygial gland in both males and females predicted volume and the inhibitory capacity of secretion against feather-degrading bacteria. Only in males was uropygial gland size negatively associated with the level of feather degradation.

4. All results indicate differential susceptibility of different parts of throat feathers to keratinolytic bacterial attack, which supports the possibility that throat feathers in starlings reflect individual ability to combat feather-degrading bacteria honestly. This is further supported by the relationship detected between antimicrobial properties of uropygial secretion and the level of feather degradation.

5. Our results suggest that selection pressures exerted by feather-degrading bacteria on hosts may promote evolution of particular morphologies of secondary sexual traits with different susceptibility to bacterial degradation that reliably inform of their bacterial load. Those results will help to understand the evolution of ornamental signals.

Key-words: bacterial load, feather-degrading bacteria, ornaments, sexual selection, *Sturnus unicolor*, uropygial gland

Introduction

A variety of morphological, behavioural and physiological signals of animals that evolve in social contexts (Westneat 2012) allows individual senders to inform about certain characteristics that will influence decisions of receivers (Endler 1993; Ruxton & Schaefer 2011). Reliability of signals is mainly thought to depend on costs associated with their production, showiness and/or maintenance (Maynard-Smith & Harper 2003), and an important part of maintenance costs is related to resources allocated to prevent signal degradation. Any signal based on morphological structures is prone to deterioration due to abrasion (Burtt 1986) or damage induced by degrading organisms. Thus,

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these signals may require additional maintenance costs related to defending them from exposure to the physical and biological environment.

Micro-organisms include important degrading agents such as bacteria and fungi that digest the keratin (Burtt & Ichida 1999) modifying colour-based signals (Shawkey *et al.* 2007), as well as traits signalling the immune capacity of holders (Hamilton & Zuk 1982; Clayton 1991; Zuk 1992; Møller, Christe & Lux 1999; Zuk & Johnsen 2000). By evaluating traits that reliably signal immune capacity, receivers are able to choose less infected mates with genes conferring pathogen resistance that would be inherited by their offspring (Hamilton & Zuk 1982; Folstad & Karter 1992). Behavioural defences or non-specific innate immune responses, which represent the most important antibacterial barriers (Playfair & Bancroft 2004), are active against a wide range of micro-organisms, likely including those able to degrade signalling traits (Horrocks *et al.* 2012). Thus, the ability of animals to preserve signals against degrading micro-organisms may reflect their defence competence, not only because of the relationship between the exaggeration of signals and associated increase in immune-suppressive hormones in blood (Folstad & Karter 1992), but also because well-maintained, non-degraded structures will inform about the ability to fight degrading microbes (Shawkey, Pillai & Hill 2009; Ruiz de Castañeda *et al.* 2012).

Most of our knowledge on signal degradation due to the action of different environmental challenges including micro-organisms, and its effects on social interactions, come from studies with birds signalling phenotypic and/or genetic quality through plumage ornaments. Feathers are degraded by abiotic (abrasion or UV radiation, Burtt 1986; Bonser 1995) and biotic (ectoparasites, Proctor 2003; fungi and bacteria, Burtt & Ichida 1999) factors. In particular, important is the action of keratinolytic bacteria degrading plumage coloration of secondary sexual characters (Shawkey *et al.* 2007; Gunderson, Forsyth & Swaddle 2009; Shawkey, Pillai & Hill 2009). It has been shown that brightly coloured carotenoid-based and sexually selected feathers of house finches (*Carpodacus mexicanus*) harboured lower densities of feather-degrading bacteria (Shawkey, Pillai & Hill 2009). Moreover, the white parts of feathers forming the sexually selected white wing patch of pied flycatchers (*Ficedula hypoleuca*) are more easily degraded by keratinolytic bacteria than melanized parts of the same feathers (Ruiz de Castañeda *et al.* 2012). These and other studies suggest an important role for micro-organisms affecting the cost of signal maintenance and therefore assuring reliability of signals (Shawkey, Pillai & Hill 2009). Interestingly, we know that the level of innate immune response (Soler *et al.* 2011) and the size of the uropygial gland (Soler *et al.* 2012) are associated with bacterial loads of avian eggshells, and that size of the uropygial gland is negatively related to the density of feather-degrading bacteria in swallows (*Hirundo rustica*) (Møller, Czirjak & Heeb 2009). In addition, experimental manipulation of the microbial environment leads to changes in uropygial gland size and oil composition (Jacob *et al.* 2014). The uropygial secretion protects feathers from degrading bacteria (Shawkey, Pillai & Hill 2003; Møller, Czirjak & Heeb 2009; Vincze *et al.* 2013; but see Czirjak *et al.* 2013; Giraudeau *et al.* 2013), and antimicrobial components have been found in hoopoes (Martín-Platero *et al.* 2006; Ruiz-Rodríguez *et al.* 2009, 2013; Martín-Vivaldi *et al.* 2010). Therefore, the level of degradation of ornamental feathers may reflect immunocompetence of signal holders.

Costs of feather degradation can be reduced by developing feathers with a physical structure and/or pigments resistant to feather degradation (Burtt & Ichida 2004; Peele *et al.* 2009; Burtt *et al.* 2011). Yet, sexual selection theory predicts that feathers signalling an individual's capacity to combat microbes should be more easily degraded than non-signalling feathers (Ruiz de Castañeda *et al.* 2012). Signalling of defence capacity may therefore select for, and explain the evolution of, traits that are especially prone to degradation by micro-organisms if they reliably reflect the ability of individuals to cope with pathogens. Except for signals based on coloration, the hypothetical effects of micro-organisms driving evolution of special characters that may reliably reflect antimicrobial ability of holders has, as far as we know, never been investigated.

In the present study, we explore this by investigating the elongated throat feathers of spotless starlings (*Sturnus unicolor*). These feathers represent a sexually selected trait that is more developed in males than in females (Lezana *et al.* 2000; López-Rull, Celis & Gil 2007; Gil & Culver 2011). We estimated bacterial loads and evaluated bacterial degradability of the ornamental throat feathers of male spotless starlings (Fig. 1) and compared these estimates with bacterial load and bacterial degradability of non-ornamental feathers in males and throat feathers in females. In addition, we measured the uropygial gland of individuals and estimated the antimicrobial capacity against keratinolytic bacteria in their uropygial secretion.

The ornamental throat feathers are quite delicate and flexible (Fig. 1c), and males exhibit them very conspicuously during courtship (Fig. 1b). If throat feathers in males do reflect the ability to evict feather-degrading bacteria, we should find that throat feathers exhibited during courtship are more easily degraded than non-ornamental throat feathers. Thus, bacteria would likely be concentrated in those feathers more easily digested. Alternatively, males could devote more effort to cleaning the more susceptible feathers, and a lower bacterial load would be detected in this case. Moreover, if degradation of ornamental feathers and antibacterial defence mechanisms are negatively related, we should find that birds with less degraded feathers have larger uropygial glands. In addition, if the uropygial secretion directly inhibits bacterial growth, uropygial gland size should also be related to antimicrobial activity rate. Finally, degradation level of feathers should be positively related to the bacterial load, but negatively associated with uropygial gland size.
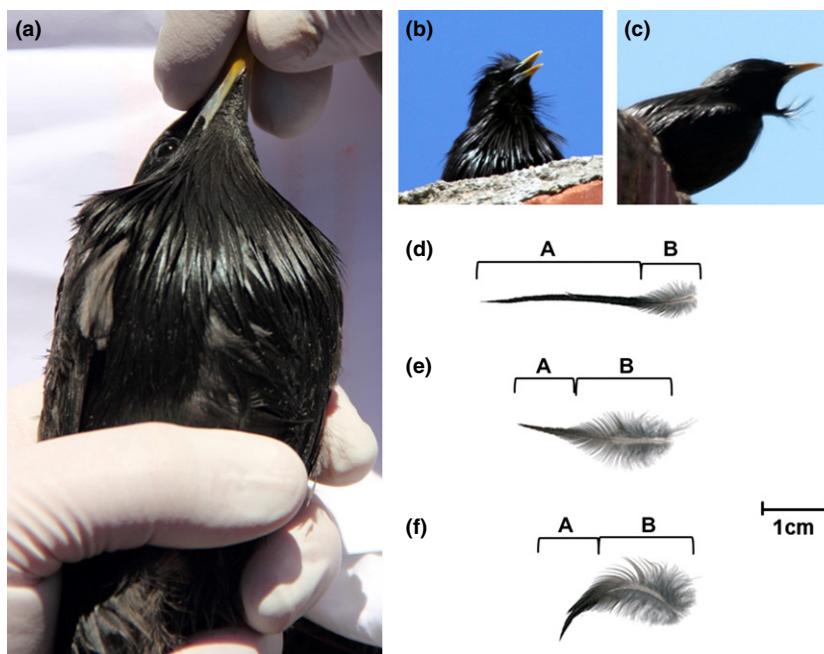
Fig. 1. (a) Starling male captured during the pre-breeding period with very apparent and conspicuous ornamental throat feathers (long and narrow). Picture (b) shows a singing starling male with bristled ornamental feathers, while Picture (c) shows the flexibility of ornamental throat feathers upon wind exposure. (d, e) and (f) Apical (A) and basal (B) parts of the throat feathers in males (ornamental, d; non-ornamental, f) and females (e).

Materials and methods

FIELD WORK AND STUDIED SPECIES

The study took place in a south-eastern region of Spain (Hoya de Guadix, 37°18′N, 3°11′W), where more than 100 cork nest boxes are available for starlings since 2005, attached to tree trunks or walls at a 3–4 m height above-ground.

Spotless starlings are slightly dimorphic (Lezana *et al.* 2000). Males present a group of ornamental feathers on the throat with enlarged and narrowed apical parts (Lezana *et al.* 2000) (Fig. 1), and much less developed in females (López-Rull, Celis & Gil 2007). The length of male throat feathers is associated with reproductive success, homozygosity levels (Aparicio, Cordero & Veiga 2001) and the strength of inhibitory effects of testosterone on macrophage phagocytosis (Gil & Culver 2011). During courtship, male starlings display throat feathers that are very conspicuous while singing in highly visible places (Fig. 1b).

In our population, starlings begin to lay eggs during early–mid April, but courtship activity starts more than 1 month earlier (J.J. Soler, G. Tomá, D. Martín-Gálvez, pers. obs.). On 27 March 2012, we captured all individuals used in the present study (24 females and 20 males). During the mating period, starlings roost in the nest boxes. One hour before dawn, we closed the entrance of all nest boxes in the study area and captured by hand all individuals roosting inside. To reduce stress related to captivity, captured birds were kept individually in cotton bags (previously washed) and were released immediately after sampling. The bags were hung on wood sticks, and starlings were quite still inside the bags. Moreover, to reduce the time that starlings were captive in the bag, all authors participated in sampling, and the time spent in the bag did not exceed 3 h. We know from previous captures that handled individuals breed in the population with no signs of negative effects from procedure (Soler *et al.* 2008). Birds were always handled with sterile latex gloves (in both capture and manipulation).

UROPYGIAL GLAND, SECRETION AND ANTIMICROBIAL ACTIVITY

From each individual male, we took at least three ornamental feathers from the throat and three non-ornamental feathers from the breast, close to the throat. From females, we collected three throat feathers (Fig. 1). We measured height, width and length of the uropygial gland of males and females and estimated gland volume following Martín-Vivaldi *et al.* (2009). The uropygial gland secretion was extracted by introducing a sterile micro-capillary (32 mm 9 μL^{-1}) into the gland opening and slightly pressing the gland to completely remove its secretion. Extraction of the secretion was always performed by the same researcher (M. Ruiz-Rodríguez), and the length (mm) of the capillary filled with secretion was used as a proxy of secretion volume. The extracted secretion was immediately placed on inhibition plates to assess antimicrobial capacity against the feather-degrading bacterium, *Bacillus licheniformis* (strain D13, from our laboratory collection).

Less than 8 h before tests were performed, inhibition plates were prepared as follows: 15 mL of a culture media previously prepared and sterilized (1·8% of brain–heart infusion (BHI) and 0·8% agar in 0·1 M pH 7 phosphate buffer) was smelted and then maintained at 50 °C for 10 min. Then, 100 μL of a 12-h culture of *B. licheniformis* was added to the media, vigorously vortexed and spread onto a Petri dish. When dry (about 20 min later), plates were transported in a portable cooler (4 °C) to the field. Plates with the uropygial secretion were transported to the laboratory in the portable cooler and later incubated for 12 h at 28 °C. Plates were then checked for inhibition halos, that is, transparent zones around the secretion drop in which the indicator bacterium (*B. licheniformis*) growth was inhibited by the secretion. Those halos were measured (in mm) from the limit of the drop to the end of the halo (i.e., where the indicator bacteria growth begins).

FEATHER PREPARATION

For all feathers of the three types collected, two different parts were easily distinguished: the apical part with closely assembled short barbs and the basal part with long, more separated barbs (Fig. 1). Each part was measured separately to the nearest mm. Visual differences in feather morphology were verified with statistical tests showing that the length of the two parts of ornamental feathers differed significantly from that of the other two types of feathers (repeated measures ANOVA, $F_{2,56} = 71·06$, $P < 0·0001$; Tukey *post hoc*, apical part: ornamental vs. (i) non-ornamental feathers in males, $P = 0·0001$, (ii) female feathers, $P = 0·0001$; basal part: ornamental vs. (i) non-ornamental feathers in males,

$P = 0.0001$, (ii) female feathers, $P = 0.0001$) (Fig. 2). Therefore, both parts of each feather were independently analysed in the laboratory.

Two feathers per female and two feathers of each type per male were cut under sterile conditions in a laminar flow cabinet to separate apical and basal parts (Fig. 1). Each part was placed in sterile Eppendorf tubes, previously weighed on a precision balance (AB135-5/FACT (±0.00001 g), Mettler Toledo, Spain), and then weighed again with the feather inside to calculate the mass of the piece. Each piece of each feather was used for estimation of (i) bacterial load and (ii) experimental degradability by bacteria.

FEATHER BACTERIAL LOAD

Two types of culture media were used to estimate the number of bacteria growing on the feathers: trypticase soy agar (TSA) and feather meal agar (FMA). The former is a highly nutritive, general medium in which a large variety of mesophylic bacteria can grow. FMA, on the other hand, is a highly selective medium where only keratinolytic bacteria can grow. FMA was prepared with 15 g L^{-1} of crunched feathers, 0.5 g L^{-1} NaCl, 0.3 g L^{-1} K$_2$HPO$_4$, 0.4 g L^{-1} KH$_2$PO$_4$, 15 g L^{-1} agar and 100 μg mL^{-1} of cycloheximide to inhibit fungus growth (Shawkey *et al.* 2005). One millilitre of sterile phosphate buffer (PB, 0.2 M; pH = 7.2) was added to each

tube containing the weighed feathers and vigorously mixed to release bacteria. 100 μL of the solution was spread on each media. Plates were incubated at 37 °C for 72 h, and colony forming units (CFU) were counted. The bacterial load was calculated as the coefficient between the CFU and the feather mass (CFU mg^{-1}).

FEATHER DEGRADATION LEVEL

Feathers were examined and photographed under a binocular lens (Nikon SMZ1500, Melville, NY, USA) connected to a camera (Nikon Digital Sight DS Fi1). Measurements from pictures were taken using the software NIS Elements F 3.1. For estimating feather degradation, we considered: (i) whether or not the tip of the feather was incomplete (Fig. 3a); (ii) the length (mm) of the feather tip that showed clear signs of degradation (apical part) (Fig. 3b); and (iii) the number of barbules that were degraded in 20 randomly chosen barbs of the basal part. In addition, photographs of different feathers and parts magnified 2× were prepared by M. Ruiz-Rodríguez such that the other authors could visually estimate degradation, while blinded to feather origin. Degradation levels were ranked from 0 (no degradation) to 3 (maximum degradation) (Fig. 3c). Repeatability of visual estimations was assessed using one-way ANOVA (Senar 1999), considering the estimates by different researchers for each photograph. Repeatability was relatively high ($F_{175,1232} = 10.79$, $P < 0.0001$, repeatability index = 0.83). Thus, we used mean values of all estimations for each part of the feathers. Moreover, to compare degradation levels of basal and apical parts of feathers, degradation values of each feather part were first standardized by subtracting mean values and dividing by standard deviation.

FEATHER DEGRADABILITY BY KERATINOLYTIC BACTERIA

Feathers previously separated and weighed in the Eppendorf tubes were sterilized in the autoclave before the experiment, following previous studies (e.g. Gunderson *et al.* 2008; Ruiz de Castañeda *et al.* 2012). A buffer was prepared by mixing 9.34 mM NH$_4$Cl, 8.55 mM NaCl, 1.72 mM K$_2$HPO$_4$, 2.92 mM KH$_2$PO$_4$, 0.49 mM MgCl$_2$-6H2O and 0.01% yeast extract in 100 mL of distilled water (following Goldstein *et al.* 2004). From this mix, 4 mL were deposited in each experimental glass tube and then sterilized. A colony of *B. licheniformis* previously isolated on TSA plates was introduced with a sterile loop in each experimental tube along with the sterilized feather piece. After vortexing, we collected
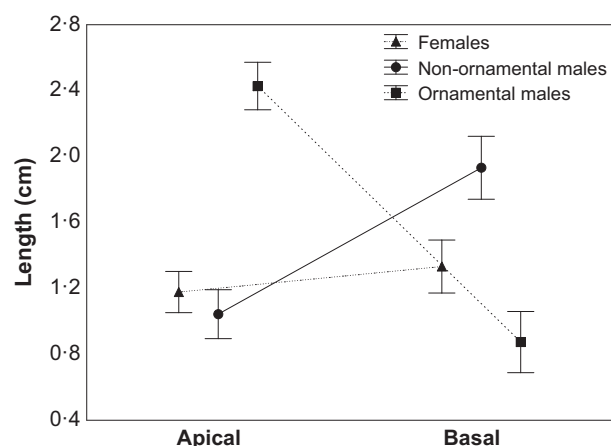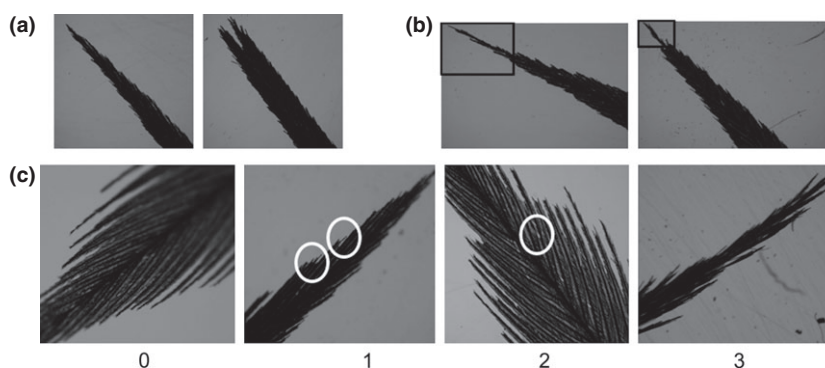
Fig. 2. Results of the repeated measures ANOVA comparing the length (cm) of both parts of each throat feather (females, ornamental and non-ornamental males).

Fig. 3. Estimation of feather degradation (pictures were taken at 2×): (a) an undamaged tip (left) and a broken tip (right); (b) squares delimit the piece of the tip considered more degraded than the rest; (c) examples of the different levels of degradation considered in evaluating the pictures (0: no degradation, 1: only the borders are slightly degraded (< 1/3 of the feather degraded), 2: not only on the borders, holes can also be observed inside the feathers (approximately 1/3-2/3 of the feather is damaged), 3: the degradation is extended to more than 2/3 of the feather).

1 mL from each tube as a basal measurement (i.e., 0 h of incubation) and kept it separately at 4 °C until measurement in the spectrophotometer (see below). Experimental tubes were incubated at 37 °C in constant agitation at 120 rpm, in an orbital agitator (VWR, Spain), and 1 mL was collected after 120 and 408 h. Samples were centrifuged to remove bacterial cells, and absorbance of the supernatant was then estimated using a spectrophotometer (Helios Zeta UV-Vis, Thermo Scientific, United Kingdom) at 230 nm (Goldstein *et al.* 2004). The oligopeptide concentration in the supernatant originates from keratin degradation and thus is directly related to the amount of feather degraded. Calibration curves of absorbance and oligopeptide concentration (from 0 to 300 μg mL^{-1}) were obtained by using bovine serum albumin (BSA) ($R^2 = 0.98$ in both curves), which allowed us to extrapolate the absorbances to values of concentration.

To assure that oligopeptide measurements were due to the degradation of feathers by *Bacillus* exclusively, we also prepared the following control samples: seven tubes of buffer only, 12 tubes of buffer and the feather (six apical and six basal parts, without bacteria) and seven tubes of buffer and bacteria but without the feather. Control tubes had significantly less oligopeptide concentration ($\bar{x} \pm SD = 8.35 \pm 5.66$ and $\bar{x} \pm SD = 34.94 \pm 9.07$) than experimental ones ($\bar{x} \pm SD = 28.36 \pm 12.7$ and $\bar{x} \pm SD = 56.96 \pm 14.91$) after both 120 h and 408 h of incubation (one-way ANOVA, $F_{1,148} = 61.73$, $P < 0.0001$; $F_{4,147} = 50.55$, $P < 0.0001$, respectively). Therefore, we can assume that oligopeptides measured in experimental samples were significantly influenced by the action of *Bacillus* degrading the feather.

STATISTICAL ANALYSES

Uropygial gland size, amount of secretion extracted and the inhibition halo of bacteria, as well as the total degradation index of the apical and basal parts of the feathers, and log-transformed abundance of mesophylic bacteria did not differ from normal distributions (Kolmogorov–Smirnov tests for continuous variables, $P > 0.1$). Oligopeptide concentrations resulting from the experiment of feather degradability were corrected for mass of the feather piece by using the residuals from a regression line. The average value of degradation level was then added to the residuals to remove negative values. Frequency distribution of this variable (oligopeptides corrected for mass) did not differ from normality.

Given that keratinolytic bacteria grew on ca. 50% of the plates with the highly restrictive FMA medium, we conservatively used detected vs. non-detected bacterial growth as a binomial variable in analyses.

Differences among sexes in uropygial gland size, secretion volume and antagonistic activity, as well as the relationship between uropygial gland size (predictor) and volume of secretion extracted from each individual and their antimicrobial activity (dependent variables) were evaluated through general linear models (GLM). Repeated measures ANOVAs were used to compare density of mesophylic bacteria and degradation state of apical and basal parts of different feathers. Differences between ornamental and non-ornamental feathers were analysed by only considering male feathers with type (ornamental or not) and part (apical/basal) of feathers as two different within effects. Sexual differences in bacterial abundance and the degradation index of feathers were explored by separately comparing ornamental and non-ornamental feathers of males with throat feathers of females. In these models, feather part (apical and basal) was considered the within effect, and sex as the between effect. Uropygial gland size was included as a continuous predictor in the models explaining degradation index.

We also used repeated measures ANOVA to compare susceptibility to degradation by *B. licheniformis* of ornamental vs. non-ornamental feathers of males, but here three variables were included as within effects: type of feather (ornamental or non-ornamental), part

(apical or basal) and time (0, 120 and 408 h of incubation). For comparisons between males and females, we used a similar design with sex as the categorical factor and part (apical vs.basal) and time (0, 120 and 408 h) as the within effects. This latter analysis was made separately for each type of male feather (ornamental or not).

Differences in the distribution of keratinolytic bacterial load (FMA) in the two parts of each type of feather were analysed through chi-square tests to assess whether prevalence differed between the two parts in each type of feather. Finally, relationships between bacterial load and degradation level of feathers were explored by means of GLMs with degradation index as the dependent variable and type (ornamental vs. non-ornamental) and part (apical vs. basal) of the feather as categorical predictors. Bacterial load was added to the models as an additional independent continuous predictor.

All analyses were performed in Statistica 7.1. (StatSoft 2005).

Results

UROPYGIAL GLAND, SECRETION AND ANTIMICROBIAL ACTIVITY

As expected due to the inhibition of microbe growth by uropygial secretion, volume of uropygial gland correlated with the volume of secretion extracted from each individual ($F_{1,41} = 10.69$, $\beta \pm SE = 0.45 \pm 0.14$, $P = 0.002$) and with size of its inhibition halo against keratinolytic bacteria ($F_{1,40} = 7.85$, $\beta \pm SE = 0.40 \pm 0.14$, $P = 0.007$). Yet, male and female starlings did not differ significantly in uropygial gland size (♀♀: $\bar{x} \pm SE = 372.66 \pm 57.42$ mm^3, ♂♂: $\bar{x} \pm SE = 393.20 \pm 56.36$ mm^3, $F_{1,41} = 1.38$, $P = 0.25$), volume of secretion (♀♀: $\bar{x} \pm SE = 4.51 \pm 3.52$ μL, ♂♂: $\bar{x} \pm SE = 3.22 \pm 2.40$ μl, $F_{1,41} = 1.87$, $P = 0.18$) or size of inhibition halos due to uropygial secretion (♀♀: $\bar{x} \pm SE = 1.09 \pm 1.04$ mm, ♂♂: $\bar{x} \pm SE = 1.32 \pm 1.15$ mm, $F_{1,40} = 0.46$, $P = 0.50$).

FEATHER BACTERIAL LOAD

Estimates of bacterial loads (CFU) of collected feathers in the two different growth media (TSA and FMA) were significantly correlated (Spearman correlation: $R = 0.34$, $t_{121} = 4.03$, $P < 0.001$, $N = 123$). Mesophylic bacteria were significantly more abundant in the apical than in the basal part of the three types of feathers (ornamental of males and non-ornamental of both sexes) (Table 1, Fig. 4). For males, mesophylic bacterial load of ornamental and non-ornamental feathers did not differ significantly (Table 1, Fig. 4). Moreover, although non-ornamental feathers of males and females harboured similar amounts of mesophylic bacteria, ornamental throat feathers of males had less than the throat feathers of females (Table 1, Fig. 4), which supports the prediction that males may devote more effort to cleaning those feathers. These effects were independent of the part of the feather considered (i.e., apical vs. basal) (see interaction terms in Table 1).

When considering bacterial load estimates in restrictive growth media for keratinolytic bacteria (FMA), bacteria were more often detected in the apical (13 out of 22) than

Table 1. Differences in degradability, mesophylic bacterial loads and degradation index of different types (♂s: male sexual feather; ♂ns: male non-sexual feather; ♀: throat feathers of females) and parts (apical and basal) of spotless starling feathers. Statistically significant relationships are in italics

|		Mesophylic bacteria			Degradation index			Degradability experiment		
		F	d.f.	P	F	d.f.	P	F	d.f.	P
♂s_♂ns	Type	0·12	1,15	0·74	1·89	1,14	0·19	*8·61*	*1,15*	*0·01*
	Part	*10·30*	*1,15*	*0·006*	<0·001	1,14	0·99	0·22	1,15	0·65
	Type*part	0·92	1,15	0·35	1·08	1,14	0·31	*16·39*	*1,15*	*0·001*
♂ns_♀	Sex	1·64	1,38	0·21	0·22	1,39	0·64	1·18	1,38	0·28
	Part	*0·12*	*1,38*	*0·03*	0·18	1,39	0·68	*7·59*	*1,38*	*0·009*
	Sex*part	0·12	1,38	0·73	0·81	1,39	0·37	0·25	1,38	0·61
♂s_♀	Sex	*5·12*	*1,41*	*0·03*	0·34	1,40	0·56	1·95	1,41	0·17
	Part	*14·55*	*1,41*	*<0·001*	<0·001	1,40	0·99	0·43	1,41	0·51
	Sex*part	1·06	1,41	0·31	1·88	1,40	0·18	*9·98*	*1,41*	*0·003*
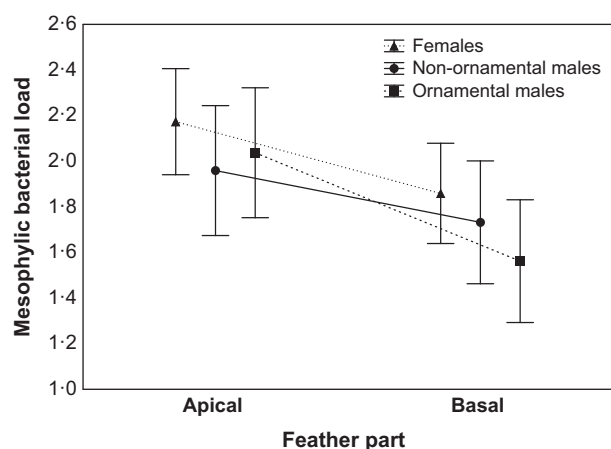
Fig. 4. Comparison of mesophylic bacterial load (log-transformed CFU corrected by mass) among the apical and basal parts of male feathers (ornamental and non-ornamental) and female spotless starlings. Vertical bars denote 0·95 confidence intervals.

in the basal part of sexual feathers of males (3 of 22) ($\chi^2_{21} = 0.82$, $P = 0.002$). However, prevalence of keratinolytic bacteria in the apical and basal parts of non-ornamental feathers of males (12 of 16 vs. 11 of 16) and of females (14 of 24 vs. 9 of 24) did not differ significantly ($\chi^2_{15} = 0.15$, $P = 0.69$ and $\chi^2_{23} = 2.09$, $P = 0.15$, respectively).

Time that birds spent in the bags was not related with the mesophylic bacterial growth for each part of each type of feather (all $R^2 < 0.16$, $P > 0.13$). Thus, the time that starlings spent in the bags did not bias our results.

FEATHER DEGRADATION LEVEL

Feather degradation levels of apical and basal parts of the same feather were positively correlated ($R^2 = 0.28$, $P < 0.0001$, $N = 59$). There were no significant differences in degradation level between apical and basal parts of feathers or between males and females (Table 1). Abundance of mesophylic, but not the presence of keratinolytic

bacteria, significantly explained feather degradation levels independently of the part and type of feathers considered (Table 2), which supports our prediction of bacteria causing feather degradation. Moreover, in accordance with the expected negative effect of preen oil on the degrader microbes, the size of the uropygial gland significantly explained feather degradation levels of apical and basal parts of all male feathers, but these associations were not detected for female feathers (Table 3). Males, but not females, with larger uropygial glands have less degraded feathers.

FEATHER DEGRADABILITY BY KERATINOLYTIC BACTERIA

Degradability of apical parts of ornamental and non-ornamental feathers of starling males was similar after incubation, independent of time in contact with *B. licheniformis* (Fig. 5). Basal parts did, however, degrade more in non-ornamental than ornamental feathers (Table 1, Fig. 5), which confirms different degradability of the two feather types. At the beginning of the experiment, no differences among the oligopeptide concentrations were found in males (Tukey HSD *post hoc* tests, all the comparisons among different type/parts, $P > 0.78$). Interestingly, degradability of female throat feathers was higher for the basal than for the apical part, opposite to the pattern found for ornamental feathers (see interaction effect between Part and Sex in Table 1, Fig. 5), although no differences were found before incubation (Tukey HSD *post hoc* tests, all the comparisons among different type/parts, $P > 0.97$). In addition, non-ornamental feathers of males and throat feathers of females did degrade at the same rate (see non-significant interaction between Part and Sex in Table 1, Fig. 5).

Discussion

In the present study, we found that the apical part of ornamental throat feathers of males was more likely to harbour

Table 2. Relationships between mesophylic and keratinolytic bacteria (detected vs. non-detected bacterial growth) load and degradation index in spotless starling feathers. Interaction terms among bacterial counts and the two parts (apical and basal) from each type of feather (male ornamental and non-ornamental feathers, and female feathers) are also given. Significant values are in italics

	Mesophylic bacteria				Keratinolytic bacteria			
	F	d.f.	P	β (SE)	F	d.f.	P	β (SE)
Bacterial load	*6·53*	*1,104*	*0·01*	*0·26 (0·10)*	0·55	1,108	0·45	0·08 (0·10)
Part	0·61	1,104	0·44	0·26 (0·33)	0·21	1,108	0·65	0·04 (0·10)
Type	1·42	2,104	0·25	0·23 (0·35)	0·67	2,108	0·52	0·04 (0·11)
Part*Bacterial load	1·05	1,104	0·31	−0·35 (0·34)	0·23	1,108	0·63	0·04 (0·09)
Type*Bacterial load	1·36	2,104	0·26	−0·33 (0·35)	1·46	2,108	0·24	−0·06 (0·11)

Table 3. Relationships between the uropygial gland volume and degradation index of feathers of male and female spotless starlings. Interaction terms among the gland size and the two parts (apical and basal) of all the feathers, as well as the two types of male feathers (ornamental or not) are also given. Significant values are in italics

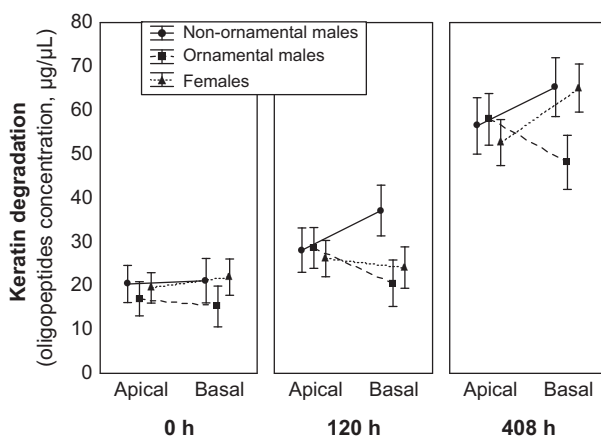|	Males				Females			
	F	d.f.	P	β (SE)	F	d.f.	P	β (SE)
Uropygial gland volume	*5·44*	*1,13*	*0·03*	*−0·49 (0·24)*	1·88	1,22	0·18	−0·25 (0·21)
Part	<0·001	1,13	0·98		0·007	1,22	0·93	
Type	0·32	1,13	0·58					
Part*gland volume	<0·001	1,13	0·98		0·05	1,22	0·82	
Type*gland volume	0·05	1,13	0·46					

Fig. 5. Repeated measures ANOVA comparing the keratin degradation (μg μL⁻¹ of oligopeptide concentration) of both parts (apical and basal) of each feather: both male feathers (ornamental and non-ornamental), and female feathers, before (0 h) and after incubation (120 h and 480 h). Vertical bars denote 0·95 confidence intervals.

keratinolytic bacteria and degraded more rapidly than the basal part; patterns that were not detected in female throat feathers or in non-ornamental feathers of males. Moreover, although the state of degradation of male and female throat feathers did not differ, this was associated with mesophylic bacterial density. Finally, the uropygial gland size in both sexes predicted volume and antagonistic activity of secretion against feather-degrading bacteria, and only in males was it negatively associated with throat feather degradation. Below we discuss these results in a scenario

where differential susceptibility of throat feathers of males to bacterial degradation reliably indicates their ability to overcome bacterial colonization.

The apical zone of throat feathers is more exposed to environmental degrading agents and thus should be more resistant to bacterial degradation than the basal zone. Interestingly, we found this pattern in non-ornamental feathers, but the opposite in ornamental feathers. During sexual display, singing spotless starling males adopt a hunch-backed posture with a lowered tail, and feathers erected on the crown, throat, upper breast and rump (Cramp 1998) (Fig. 1). Thus, receivers of the signal (both females and rival males) can evaluate feather state (degradation level) of displaying males and behave accordingly (Aparicio, Cordero & Veiga 2001). Consequently, males with better abilities to prevent feather degradation would gain a selective advantage in a sexual selection scenario.

The above scenario may predict selective advantage for males resistant to degradation of apical parts of sexually selected feathers, which were more easily degraded. Feathers that are more physically resistant to bacterial attack require a more robust structure that can impede the feathers' delicate movements that usually occur during singing (J.J. Soler, G. Tomá, D. Martín-Gálvez, pers. obs.). Therefore, we speculate that the mode of display of the sexual trait, particularly the need for feather flexibility (Fig. 1c), favours the evolution of sexual feathers with apical parts that are relatively more flexible and thus more susceptible to bacterial degradation than the basal part, allowing delicate movements of conspicuous feathers during display. Length of the apical part of the male ornamental feathers

is about double the size of non-ornamental throat feathers (Figs 1 and 2), which facilitates visual examination by females. These apical parts were more easily degraded and had a higher prevalence of feather-degrading bacteria than the basal parts, which was not the case in the non-ornamental feathers of either sex. Thus, having delicate, long sexual feathers would be a handicap for starlings mediated by feather-degrading bacteria. Only males with better capacity to prevent feather degradation by bacteria would show non-degraded, long and delicate feathers during courtship display.

Mesophylic bacterial load significantly explained degradation level in all feathers, that is, degradation level of feathers could be attributed, at least in part, to the action of micro-organisms. Given that starlings moult feathers after the reproductive period (July-October, Lezana *et al.* 2000), ornamental throat feathers have been exposed to the bacterial environment for several months before courtship. Thus, the degradation level of ornamental feathers would reliably inform about an individual's bacterial resistance in the long term. Not only maintenance but also the annual growth of feathers after moulting in birds is energetically costly (Zirpoli, Black & Gabriel 2013). Consequently, individual capacity to resist bacterial colonization also influences feather development and final signal structure. This information adds to that from other signals of individual phenotypic condition in the short term in spotless starlings such as carotenoid-mediated beak coloration (Navarro *et al.* 2010).

In accordance with the hypothesis that individual ability to combat microbes is related to feather degradation, we found that those males with larger uropygial glands had less degraded feathers, possibly due to the action of uropygial secretion. Uropygial gland size is correlated with amount of uropygial secretion as has been found in other species (Martín-Vivaldi *et al.* 2009) and predicted the intensity of antagonistic activity against a feather-degrading bacteria. Growth of the uropygial gland as well as production of uropygial secretion is energetically costly (Piault *et al.* 2008; Pap *et al.* 2013) and depend on the bacterial environment (Jacob *et al.* 2014). Both the amount of secretion produced (Vincze *et al.* 2013) and its chemical composition show sex-related differences (Amo *et al.* 2012). According to our results, male starlings able to invest more in gland development produce more uropygial secretions, therefore increasing protection of their feathers against feather-degrading bacteria. Thus, males with healthier ornamental feathers will signal their capacity to invest in defences against bacteria and thus their defence competence. Nonetheless, our results are descriptive and only with an experiment modifying uropygial secretion and studying its effect on ornamental feathers can the causal effect between uropygial secretion and degradation of ornamental feathers be confirmed.

Micro-organisms are a major force in shaping animal evolution (McFall-Ngai *et al.* 2013). Microbial parasites can induce selection on hosts for defence mechanisms such as immune system and behavioural traits (Poulin 1998). Our results show that the response of feather structure to keratinolytic bacterial attack differs between ornamental and non-ornamental feathers, in accordance with the possibility that sexual throat feathers of starlings reflect individual ability to combat feather-degrading bacteria. This is further supported by the detected relationship between antimicrobial properties of uropygial secretion and the level of feather degradation and may explain the evolution of fragile ornamental signals especially prone to bacterial degradation. Thus, our results suggest that selection pressures exerted by bacteria may promote the evolution of particular morphological structures in animals. In addition, special structures may have evolved to inform others (con- or heterospecific) about the capacity of individuals to cope with microbes; a possibility that, as far as we know, has never been suggested.

Data accessibility

Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.p69n4 (Ruiz-Rodriguez *et al.* 2014).

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