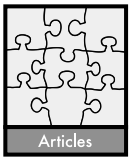

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Seasonal, sexual and developmental differences in hoopoe *Upupa epops* preen gland morphology and secretions: evidence for a role of bacteria

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The uropygial glands of birds serve multiple functions, and there is great interspecific variability in the composition and properties of their secretions. A special case is the secretion in the hoopoes *Upupa epops*, and green woodhoopoes *Phoeniculus purpureus*, which, contrary to the commonly white and odourless secretions, are dark with pungent odour. Recently, bacteria have been isolated from glands of both woodhoopoes and hoopoes and here we test the hypothesis that bacteria are responsible of some of the special properties of glands and secretions of this group of birds. We explore natural seasonal changes and intersexual differences in the properties of hoopoe glands and secretions, check the natural occurrence of bacteria within secretions, and analyse the effect of experimental injection of antibiotics on uropygial gland properties. Male glands underwent no seasonal changes, and their secretions were invariably white and odourless, very similar to female glands outside the breeding season. However, in comparison to the uropygial gland of non-breeding females, those of incubating females showed a marked increase in size and volume of secretion produced, which became dark and pungent. All these parameters increased until the hatching date and returned to values similar to those in the prelaying phase towards the end of the nestling period. Nestling glands produced secretions similar to those of females in colour and odour. Gland size of both females and nestlings predicted the amount of secretion produced. Microscopic techniques confirmed the presence of bacteria at high density and in active division in all dark secretions examined. The antibiotic treatment significantly reduced the load of enterococci in nestling glands, did not affect size of glands, but diminished the volume of secretion, which was lighter in colour than that of control nestlings. In nesting females, the experimental injection of antibiotic affected some measurements of gland size and secretion colour. Because the experiment did not affect general health estimates (immunocompetence, body condition or growing) of nestlings, our results suggest that some of the special properties of hoopoe glands are mediated by the presence of symbiotic bacteria.

Apart from flight feathers and powered flight, the preen (or uropygial) gland, is a prominent and particular attribute of birds. The uropygial gland is a holocrine gland in the integument of the bird that lies dorsally and medially in the synsacrocaudal region (Jacob and Ziswiler 1982). Despite intensive research on several aspects of uropygial glands, neither the origin or phylogeny, nor the functions of this organ are fully understood (reviewed in Elder 1954, Jacob and Ziswiler 1982, Reneerkens et al. 2006). Contrary to suggestions (Elder 1954), Jacob and Ziswiler (1982) found evidence for fully developed glands or rudiments in all groups of extant birds, and thus considered that it probably appeared early in the phylogeny of birds (see Mayr 2006). It is clear that uropygial secretions are used by most birds and serve an extremely diverse array of habits or ecologies. This correlates with a complex and variable chemical composition of uropygial secretions, which can differ taxonomically (Jacob and Ziswiler 1982, Sweeney et al. 2004), sexually (Jacob et al. 1979) or seasonally (Bhattacharyya and Chowdhury 1995, Reneerkens et al. 2002, 2006, 2008, Soini et al. 2007). The list of functions proposed for whole secretions include waterproofing or flexibilization of feathers (Jacob and Ziswiler 1982), antimicrobial effects (Bandyopadhyay and Bhattacharyya 1996, Shawkey et al. 2003, Burger et al. 2004, Reneerkens et al. 2008), pheromonal activity (Bohnet et al. 1991, Soini et al. 2007), sexual signalling (Piersma et al. 1999, Surmacki and Nowakowski 2007), or even diminishing predation risk (Burger et al 2004, Reneerkens et al. 2005).

Uropygial secretions are typically composed of mono-, di- or triester waxes of aliphatic alcohols and fatty acids, with some cases of triglycerides, sterols and hydrocarbons. Although there is an enormous variety in the substitutions, chain length, or branches in these molecules, the secretions

of most birds lack any other kind of chemical compound (Jacob and Ziswiler 1982). This uniformity highlights the special character of the secretions of the few species where researchers have found other substances. This is the case of green woodhoopoes *Phoeniculus purpureus,* in whose secretions Burger et al. (2004) have identified phenol, indole, benzaldehyde, etc. and the genus *Pitohuis* which has been suggested to include toxic alkaloids in its preen oil (Jonsson et al. 2008). The scarcity of these cases suggests that the special compounds are not synthesised by birds but instead obtained from special sources. Indeed, in the case of *Pitohuis,* the batracotoxin seems to be sequestered from ingested beetles (Dumbacher et al. 2004, Jonsson et al. 2008). The origin of the aromatic compounds in woodhoopoe glands is unknown, although indole and phenol are known metabolites of bacteria.

Indole may act as a bacteria signalling molecule involved in regulation of adhesion and biofilm-promoting factors (Martino et al. 2003) and its presence in woodhoopoe secretions, as well as the detection of a new species of bacteria *Enterococcus phoeniculicola* within its uropygial gland (Law-Brown and Meyers 2003), suggests a role of bacteria in the production of these chemical products. Recently different species of enteroccoci have been isolated from the gland of the European hoopoe *Upupa epops* (Martín-Platero et al. 2006; Soler et al. 2008) from the gland of a European hoopoe nestling. Woodhoopoes (Phoeniculidae) and hoopoes (Upupidae) are two closely related families of birds classified together in the order Upupiformes (Feduccia 1975, Mayr 2000, 2006). In both cases, the uropygial gland exhibits special properties that most species of birds lack. The uropygial gland secretions of woodhoopoes and hoopoes are dark and pungent, and the glands are unusually large (Cramp 1998, Ligon 2001). To our knowledge there is no other bird family with dark and pungent uropygial secretions, and there is no other case of isolation of bacteria from the uropygial gland of a bird species. Thus, it is possible that the presence of *symbiotic* bacteria in the gland of Upupiformes is one of the factors promoting the special gland properties of this group of birds.

The term s*ymbiosis*, in the original sense, i.e. ''living together of different named organisms'' (De Bary 1879) does not imply benefits for any of the counterparts. Throughout the paper we use the term symbiosis with that broad sense and, consequently indicate a symbiont as ''an organism that lives intimately throughout its lifetime or a major life-history stage on or within a host individual'', which includes parasites, commensals, and mutualistic symbionts (Thompson 2005). A direct role of symbiotic bacteria in the properties of woodhoopoe uropygial gland secretions has already been explored. After injection of broad-spectrum antibiotics within the glands of three adult woodhoopoes kept in captivity, Law-Brown and Meyers (2003) found a change in the color, odour and viscosity of the secretion in comparison to three control birds. Therefore, all the available evidence suggest that in the case of woodhoopoes, a specialist symbiotic bacteria (novel species not found in other environments) lives within its gland and affects properties of the secretion. The system of the European hoopoe however has not been explored in detail, although the special properties of secretions appear not to be present in females out of the breeding season, and never in males, which are instead whitish and odourless (Cramp 1998, pers. obs.). This is a remarkable and very interesting difference with the system of woodhoopoes, which always produce dark secretions.

Here we provide detailed information on natural variation of the gland and secretion, examine dark secretions microscopically and, by injecting antibiotics in the glands of both female and nestling hoopoes and comparing them with control individuals, examine the effects of bacterial clearance on both uropygial gland morphology and secretion characteristics.

Material and methods

Study area, study species and general procedures

The study was performed between 2003–2006, in the Hoya de Guadix (37° 18′ N, 38° 11′W), southern Spain, where hoopoes breed in farmlands, forests, and gullies within nest-boxes placed in trees or buildings (for a more detailed description of the study area, see Martín-Vivaldi et al. 1999).

Hoopoes nest in a variety of holes that are re-used successfully by the same or different individuals for many years (Cramp 1998). Females lay one or, at most, two clutches over the breeding season. Incubation (17 d) and brooding is performed only by the female, which stays the whole day within the nest until nestling are about eight d old. During this period of approximately three weeks, the male provides all the food for both the female and the nestlings. Both pair members provision the chicks until fledging at 24–30 d (Baldi and Sorace 1996, Cramp 1998, Martín-Vivaldi et al. 1999). Incubation usually starts with the first or second egg, followed by complete hatching asynchrony in which eggs hatch at 24 h or even greater intervals (Bussman 1950, Gupta and Ahmad 1993, Baldi and Sorace 1996, Cramp 1998). This generates a marked size hierarchy within the brood that can be used to deduce hatching order (Martin-Vivaldi et al. 2006).

Each breeding season the nest-boxes were visited twice per week from mid February to the end of July to record laying dates, clutch size and hatching dates. In 2005–2006, nestlings were measured soon after hatching to estimate their age from the initial weight hierarchy. Nestlings were individually painted with permanent markers on their tarsus every two days until they were banded with numbered metal rings. When the brood reached 19–21 d old, nestlings were measured again.

Adults were caught with mist-nets throughout the whole breeding season, but during nestling period we used traps for nest-box entrances. Incubating or brooding females were caught by hand within the nest-box and after manipulation were released again within it to reduce disturbance. For individual recognition, all adults were ringed with numbered (Spanish Ministerio de Medio Ambiente) and colour rings. However, to reduce manipulation time and disturbance, incubating females were marked only with the numbered metal ring.

Gland measurements

The uropygial gland of birds has two secretor lobes that flow into a papilla where the secretion accumulates. In the case of the hoopoe the papilla has a single orifice to the exterior, surrounded by a circlet of feathers forming a tuft (Jacob and Ziswiler 1982). The papilla varies greatly in size, which depends mainly on the amount of secretion stored. Walls of the papilla are elastic and thin, making its measurement difficult once the secretion has been extracted. Thus, for the most reliable measurements possible, we characterized the lobe area, where the secretion is produced. Briefly, with a digital calliper (Mitutoyo) we took three linear measurements of the lobes to the nearest 0.1 mm: gland width, gland length, and gland height (Fig. 1). The product of the three linear measurements was used as a composite measure of gland volume.

Adult glands were measured in all captures to describe changes in their size over the breeding season. Throughout the paper, we distinguish the following stages for females: (1) non-reproducing female: before laying, with white and odourless secretion. (2) prelaying females: few days before laying, with odorous secretions turning brown in colour, and (3) nesting females: laying, incubating or brooding females with brown and odorous secretions. Nestling glands were measured at 11–14 d and 19–21 d old.

We estimated the repeatability of the three linear measurements of glands with adult hoopoe individuals kept in captivity at the University of Granada. We caught 13 different individuals and kept them in individual bags for taking measurements of each of the gland parameters in three consecutive rounds (i.e. three measurements per bird). We obtained highly significant repeatabilities for the three parameters (gland width $R = 0.92$, $F_{12,28} = 36.6$,

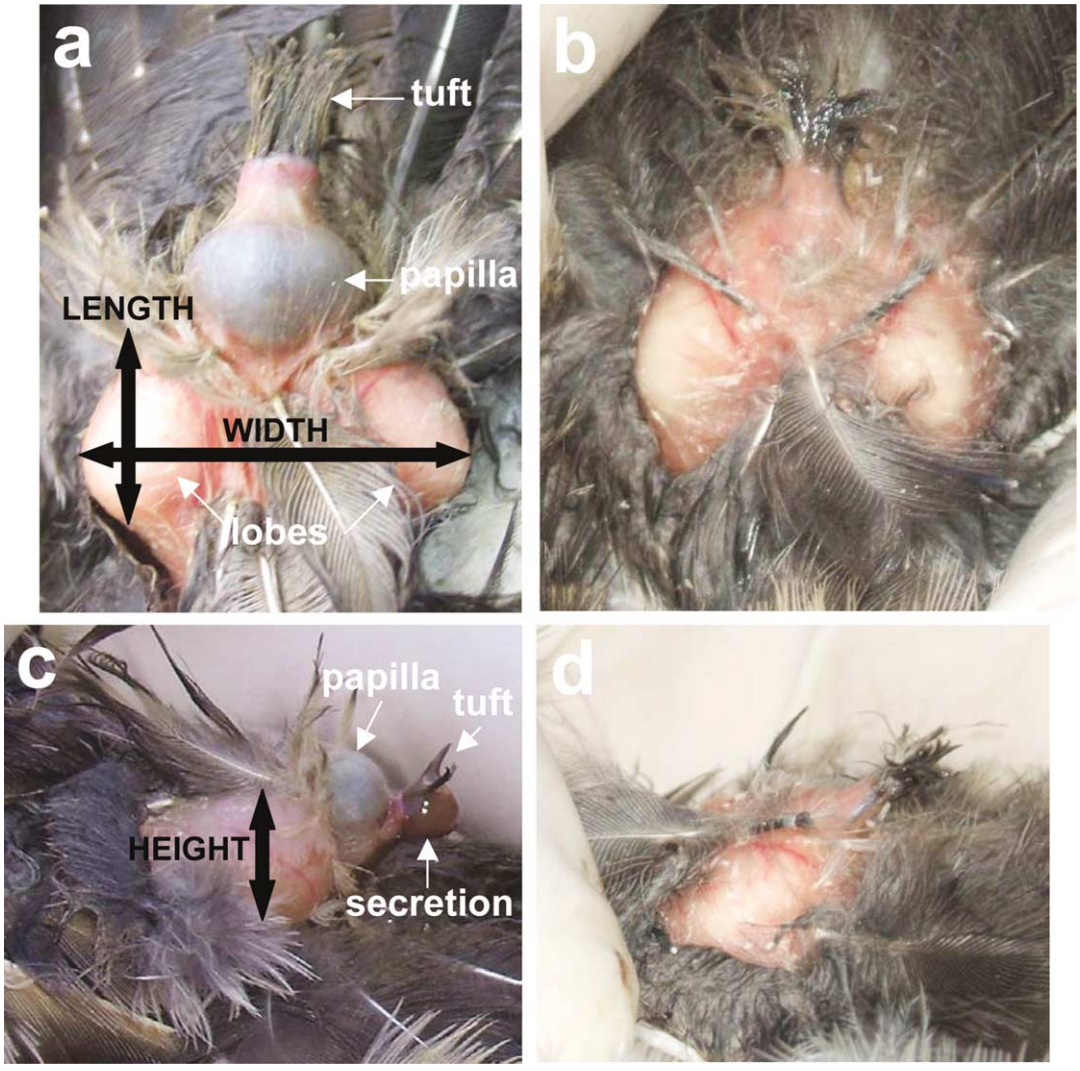

Figure 1. Pictures of the uropygial glands of a breeding female hoopoe (a, c), and a male (b, d). (a) Dorsal view of a female gland showing a papilla filled with dark secretion. (b) Dorsal view of a male gland. (c) Lateral view of a female gland showing a papilla filled with dark secretion, part of which is being exuded through the papilla aperture. (d) Lateral view of the male gland. The different parts of the gland, and the three linear measurements taken are indicated.

$P < 0.001$; gland length $R = 0.94$, $F_{12,28} = 37.8$, $P < 0.001$; gland heigth $R = 0.85$, $F_{12,28} = 18.6$, $P < 0.001$).

Secretion measurements

Given that hoopoes can exude the secretion from the gland when handled, to estimate the amount of secretion produced and its colour, we extracted all the secretion available in the papilla as soon as possible after capture, a procedure that lasted only a few min. In the few cases when part of the secretion was expelled before sampling, we estimated the volume out of the gland by filling capillary tubes. We wore latex gloves throughout the process. The extraction protocol was as follows. First, we softly washed the circlet and surrounding skin with a cotton swab soaked in ethanol to reduce the risk of contamination of the secretion with external bacteria. After evaporation of the alcohol, a sterile needle was used to open the circlet of feathers and uncover the papilla entrance where a sterile tip of a 1–10 μl micropipette (Finpipette) was introduced gently inside the papilla. While the papilla was pressed softly with a finger the automatic pipette was filled several times until the papilla was empty. The extracted secretion was transferred to a sterile Eppendorf vial. Immediately after extraction, approximately 5 μl of the secretion was introduced into a capillary tube, the ends sealed with plasticine, and kept at about 4° C in a portable icebox until colour evaluation in the laboratory within the next 10 h. The secretion colour was estimated under standardized light conditions by comparing the colour of the secretion (i.e., filled capillary tubes) with colour tables available in Küppers Colour Atlas (Küppers 2002) in the field of orange (combination of black-yellow-magenta coordinates). We recorded the values on the black, yellow, and magenta coordinates, each ranging from 0 to 99 in 10-unit steps, of the colour square best matching the secretion colour.

Sampling of bacteria within secretions

In order to check for evidence for bacteria, we observed secretions under a TEM microscope and a fluorescence microscope. Secretions were obtained from alive individuals by extracting them with a micropipette as explained above. After extraction, samples used for electronic microscope were immediately fixed in cold 2.5% glutaraldehyde, washed and post-fixed in osmium tetroxyde, dehydrated and embeded in Embed 812 resin. Ultrathin sections were stained with uranil acetate and lead citrate and examined in a C. ZEISS EM 902 electronic microscope. When used for fluorescence microscope, 2 μl of the secretion were transferred directly from the gland of living birds to a gelatinized excavated-slide, air-dried, fixed in 4% paraformaldehyde, dehydrated, stained with hoescht and examined with a blue filter.

Experimental methods

In 2004, we manipulated bacterial presence in breeding females that were randomly assigned to two treatment groups. For three consecutive days, after the papilla was emptied as explained above, the gland of experimental females was injected with 0.04 ml of amoxyciline (100 mg/ml). Then 0.02 ml of antibiotic were injected through the wall of each of the two secretor lobes, directly into the secretory tissues. In all cases a small part of the antibiotic solution poured out the papilla which assured that the papilla was full of antibiotic. Control females were treated in the same way but we injected sterile saline solution into their gland. The adult females were first captured on the third d after the first egg hatched, while the fourth capture took place seven d after the third injection (13 d after hatching). Since hatching date is the moment of maximum development of female glands, the treatment was not intended to affect growing but instead the rate of reduction after the gland reached its maximum size. We measured gland size, secretion volume, and secretion colour of females at each capture.

During the breeding seasons of 2005–2006, we performed a similar experiment in nestling hoopoes. Hatchlings were ranked according to their size. Half of them were assigned the antibiotic treatment and half the control (saline solution) treatment. Starting with the heaviest hatchling, and proceeding in order of size, every other hatchling was inoculated with the same treatment (starting treatment of inoculation or control was assigned at random). The treatments were similar to those used in females but nestling were injected every second days after the start of the experiment. Nestling glands start to have visible lobes and to produce secretion after the 4th d of life (Cramp 1998) and we started the experiment when the oldest nestling in the nest was 11 d old. All nestlings were injected 5 times, but glands were measured and the secretion extracted to estimate volume and colour only twice, before the first and two d after the last injections.

To check the effectivity of the antibiotic in killing gland bacteria, the secretions sampled from experimental and control nestlings after the treatment were plated in solid culture media. Given that the bacteria previously isolated from both woodhoopoes and hoopoes were *Enterococcus* species (Law-Brown and Meyers 2003, Martín-Platero et al. 2006, Soler et al. 2008) we used a selective growth medium for enterococci: Kenner fecal agar (KFA) supplemented with 0.01% 2-3-5 triphenyl-tetrazolium chloride. Inoculations were made in the laboratory in sterile conditions within 24 h after sampling. From sampling to inoculation, secretions were stored at 4° C. Plates were inoculated with 5 μl of secretion taken with a micropipette with sterile tips, and subsequently spread with a sterile glass Drigalsky spatule. Plates were incubated aerobically at 32° C for 72 h after which were examined for bacterial growth.

To see whether the antibiotic affected the general health of nestlings, we compared their immunocompetence, body condition and growing rate between experimental and control nestlings. Immunocompetence of nestlings was estimated by the in vivo T-cell mediated immune response to an injection of phytohemagglutinin-P (PHA-P; Cheng and Lamont 1988). We injected nestlings subcutaneously in the left wing web with 0.2 mg of PHA dissolved in 0.04 ml of physiological saline solution after removing feathers on the area and marking the point of injection with an indelible felt-tip pen. The right wing web was injected with 0.04 ml of saline solution. We measured the thickness

of each wing web at the pre-marked injection site with a digital pressuresensitive micrometer (Mitutoyo, model ID-CI012 BS; to the nearest 0.01 mm) before and 24 h after the injection. The T-cell mediated immune response or wing web index was then estimated as the change in thickness of the left minus that of the right wing web (Lochmiller et al. 1993). Measurements of each wing web on each occasion were repeated three times, and the mean value was used in subsequent analyses. Body condition at age of ringing was estimated as body mass controlled for tarsus length in the analyses. Growth rates of body mass, wing length and bill length were estimated as the ratio between the measurement at the age of 19–21 d (after treatment) and the measurement at the age of 11–14 d (before treatment).

Statistical methods

Although some non-experimental adults were captured and measured several times and/or study years, except when specifically mentioned, only information collected during the first capture was used. The changes over the breeding season in gland size, secretion volume and secretion colour were analysed by mean of quadratic regressions. For comparisons between periods and between sexes, General Linear Models (GLMs) were used. To control for the allometric effects of gland size and secretion volume, but also for among-year variations, we used residuals after these variables were controlled for tarsus length and year in our analyses. On the other hand, since the three values (i.e. coordinates) of secretion colour (black, magenta, and yellow) did not vary among years (one-way ANOVA, all $P > 0.15$), we pooled data from different years.

Repeated-measure ANOVA with brood stage (two levels, for 11–14 and 19–21 d old nestling) including only the oldest nestling in each brood, were used to detect the changes in gland properties over the nestling period. We used only a nestling per brood to avoid pseudoreplication. Further, given that size hierarchies are extreme in hoopoe broods and that younger nestlings are frequently under-nourished, only the oldest well-nourished nestling was used.

We used GLMs with nest identity (nested within year) and year as random factors, and treatment as fixed factor to test the effects of the antibiotic treatment. Several dependent variables were homoscedastic and normally distributed within treatments (gland volume, level of magenta in secretion, level of yellow in secretion, non-significant Levene and Kosmogorov-Smirnov tests). On the other hand, the volume of secretion and level of black colour in secretion had to be log-transformed, or converted in an ordinal scale from 1 to 6 (by collapsing adjacent classes in the Küppers table), respectively, to reach homoscedascity and approximately normal within-treatment distributions.

To test how bacterial elimination influenced seasonal changes in gland properties in females, we used repeated-measures Anova with the measurements compiled in consecutive captures as within-effect and treatment as between-effect. Planned comparisons of least-square means were used to test the specific effects of interest: (a) changes of gland properties between consecutive captures within treatments, and (b) interaction between the treatment and the changes between pairs of captures.

All statistical tests used were two-tailed, with the α-level established at 0.05. Points, bars and whiskers in graphs represent means, SE and SD except where indicated.

Results

Natural variation in gland properties

During the prelaying period, uropygial glands of adult males were larger than those of non-reproducing females after controlling for tarsus length and year (Table 1, Fig. 2a). Such sexual differences were due mainly to differences in height, but not in width or length of the gland (Table 1, Fig. 2b–d). Despite inter-sexual differences in volume of the uropygial gland, males and females did not differ in the amount of secretion produced in this phase (GLM with sex as fixed factor, year as random effect and tarsus length as covariable, $F(1,15) = 0.37$, $P = 0.55$).

The uropygial gland of pre-laying females increased considerably in volume ($F_{1,13} = 36.87$, $P < 0.001$, Fig. 3a), length ($F_{1,13} = 16.24$, $P = 0.001$), and height ($F_{1,13} = 14.40$, $P = 0.002$, in all cases including tarsus length as covariable in GLMs). Moreover, larger glands of pre-laying females produced larger volumes of secretion than did non-reproducing females glands (GLM with tarsus length as covariable $F_{1,13} = 6.99$, $P = 0.03$, Fig. 3b).

Once females started to reproduce, their gland changed drastically over the breeding cycle. Gland volume increased after the start of laying up to hatching, when gland size reached maximum values. After hatching time, gland size decreased drastically up to the second half of the nestling period, when gland volume returned to values similar to those of the prelaying period (Fig. 4a). Thus, variation in both gland volume and volume of secretion of females during the breeding cycle fitted quadratic regression lines (Fig. 4). Significant quadratic regressions were also detected

Table 1. Intersexual differences in size of hoopoe uropygial glands in the non-reproducing period, still with white secretions. The table shows the results of General Linear Models with year and nest (nested within year) as random factors, and sex as fixed factor.

	df	Volume		Width		Length		Height	
		F	P	F	P	F	P	F	P
Intercept	1	4.02	0.05	2.14	0.15	1.88	0.18	2.14	0.01
Tarsus	1	2.26	0.14	1.05	0.31	0.04	0.85	5.44	0.03
Year (random)	2	26.09	0.03	10.22	0.18	2.01	0.43	53.46	0.048
Sex	1	8.50	0.01	2.51	0.20	0.46	0.58	6.04	0.044
Year × sex (random)	1	0.03	0.86	0.16	0.69	0.45	0.51	0.08	0.76
Error	28								

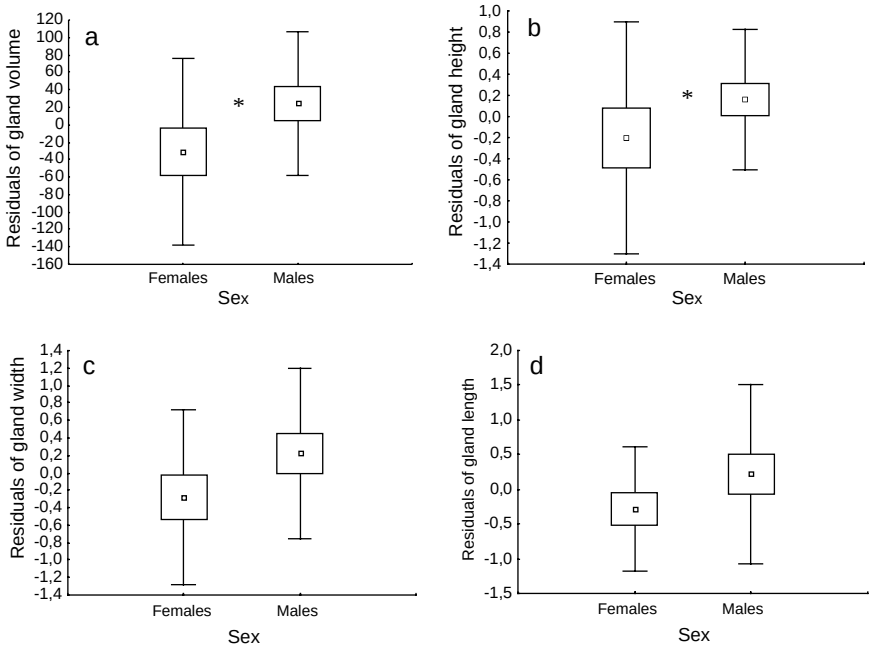

Figure 2. Intersexual differences in hoopoe gland properties in the non-reproducing period (individuals with white secretions) after controlling for year and tarsus length in GLMs. Asterisks mark significant differences at the 0.05 α-level.

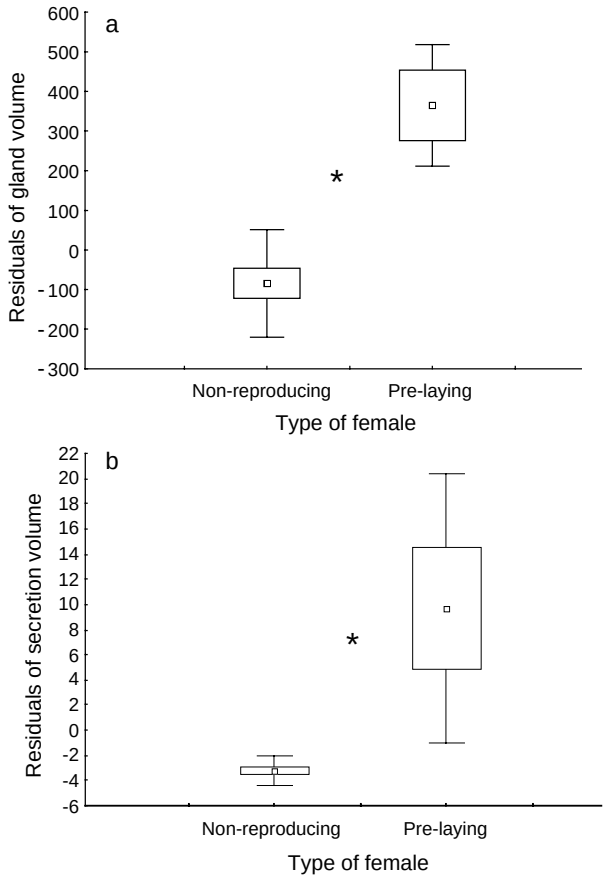

Figure 3. Differences in gland volume and volume of secretion between non-reproducing females and prelaying females after controlling for year and tarsus length in GLMs. Means, SE and SD for the dependent variables are shown. Asterisks mark significant differences at the 0.05 α-level.

for the variation of three linear measurements of gland size during breeding (result not shown). In addition, secretion volume was significantly and positively correlated with gland volume throughout the breeding season (GLM, with year (random factor) and tarsus length as covariables, beta (SE) = 0.57 (0.10), $F_{1,65} = 31.01$, $P < 0.001$), as well as with the three linear biometric measurements (GLMs, with year (random factor) and tarsus length as covariables, gland width: beta (SE) = 0.65 (0.10), $F_{1,65} = 45.36$, $P < 0.001$; gland length: beta (SE) = 0.53 (0.11), $F_{1,65} = 24.80$, $P < 0.001$; gland height: beta(SE) = 0.60 (0.10), $F_{1,65} = 32.67$, $P < 0.001$).

On average, the volume of glands in females close to hatching time (i.e. from five d before to five d after hatching date) was about eight-fold larger (mean ± SE = 1,844.7 ± 187.3 mm^3, n = 12, maximum = 3,268.3) than in non-reproducing females (239 ± 34 mm^3, n = 13). Similarly, the average amount of secretion produced by glands of females in the reproductive stage was almost 30-fold larger (43.33 ± 12.3 µl, n = 11, maximum = 137.8) than in non-reproducing females (1.53 ± 1.32 µl, n = 15). None of the seasonal changes reported for uropygial glands and secretions in females occurred in males, in which the volume of both gland and secretion remained very similar over the entire breeding season (Fig. 4b, d).

The most drastic change in females gland properties occurred in the characteristics of the secretion. While the colour of the uropygial glands of non-reproducing females was predominantly white, it became dark for the entire period that females stayed within the nest incubating or brooding. Estimated colourations of the uropygial glands, i.e. coordinates of the black-yellow-magenta axes within the field of orange (Küppers 2002), varied in different breeding phases. All estimated colour values rose during the laying period and fell markedly during the second half of the

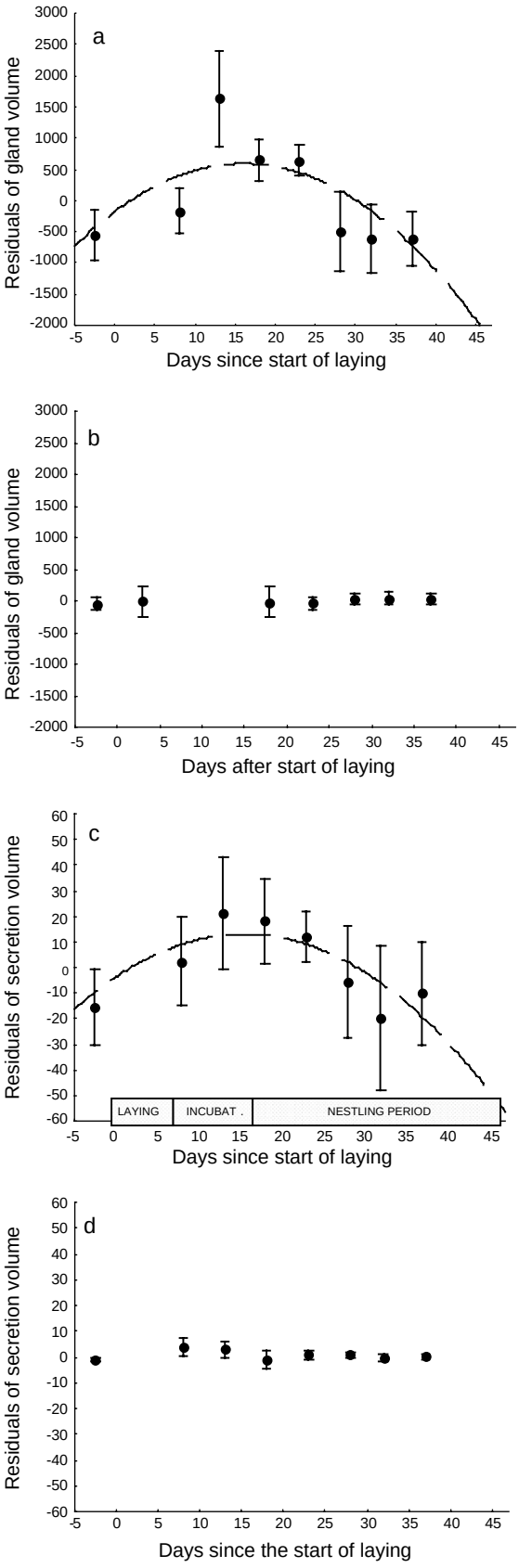

Figure 4 (*Continued*)

nestling period (Fig. 5). Again, colour characteristics of male secretions did not change during the breeding season (Fig. 5).

When using data from individual nestlings of different age but measured a single time during the nestling period, gland size explained volume of secretion produced even after statistically controlling for the effect of age, year (random factor), and nest identity (random factor nested within year). Gland length, but not gland width or height, was significantly and positively correlated with secretion volume when analysed separately (GLMs, gland length: beta (SE) = 0.38 (0.13), $F_{1,96} = 7.98$, $P = 0.006$; gland height: beta (SE) = 0.19 (0.15), $F_{1,96} = 1.59$, $P = 0.209$; gland width: beta (SE) = 0.28 (0.16), $F_{1,96} = 2.97$, $P = 0.088$).

When comparing uropygial gland secretions of 11–14 d vs. 19–21 d old broods (only control nestlings), the volume increased with age (repeated-measures ANOVA, age effect: $F_{1,13} = 6.75$, $P = 0.022$, Fig. 6a). None of the biometric parameters increased with nestling age at this stage (repeated-measures ANOVA, age effect, for gland width: $F_{1,13} = 0.22$, $P = 0.645$; gland length: $F_{1,12} = 0.15$, $P = 0.704$; gland height: $F_{1,12} = 0.073$, $P = 0.792$). Regarding the variation in colour of secretion of nestlings, we found that values on the black axis, but not those in the magenta or yellow axes, significantly increased with age (repeated-measures Anova, effect of age, for black: $F_{1,8} = 8.08$, $P = 0.022$; yellow: $F_{1,8} = 0.14$, $P = 0.715$; magenta: $F_{1,8} = 0.645$, $P = 0.445$), Fig. 6e–g).

Microscopic examination of secretions

All six examined secretions (three different breeding females and three different nestlings from two different broods) under either TEM or fluorescence microscopes contained bacteria at a high density. Fluorescence images of two secretions of females clearly showed stained bacteria homogeneously distributed in the secretion, but forming big dense aggregates in some areas (Fig. 7 A–C). In addition, TEM images of secretions from three nestlings and one female allow detailed observation of bacteria in different phases of binary division (Fig. 7 D–F).

Figure 4. Changes in adult hoopoe uropygial gland properties over the breeding cycle. Broken lines are the quadratic regressions fitting raw data. Means and 95% confidence intervals for the dependent variables are shown for periods of five consecutive days. The correspondence with the different phases of the breeding attempt (laying, incubation and nestling period) are indicated in (c). (a) Changes in female gland volume: quadratic regression, $F(3,59) = 14{,}61$: $P < 0.001$: $R^2 = 0.40$: $b_0 = -176.3 \pm 162.1$ ($t = -1.09$: $P = 0.28$); $b_1 = 95.7 \pm 16.6$ ($t = 5.76$: $P < 0.001$); $b_2 = -2.98 \pm 0.47$ ($t = -6.28$: $P < 0.001$)). (b) Changes in male gland volume: quadratic regression, $F_{3,40} = 1.17$: $P = 0.33$. (c) Changes in the amount of secretion produced by female glands: quadratic regression, $F_{3,68} = 6.26$: $P < 0.001$: $R^2 = 0.20$: $b_0 = -3.8 \pm 5.2$ ($t = -0.73$: $P = 0.47$); $b_1 = 2.2 \pm 0.5$ ($t = 4.02$: $P < 0.001$); $b_2 = -0.07 \pm 0.02$ ($t = -4.07$: $P < 0.001$)). (d) Changes in the amount of secretion produced by male glands: quadratic regression, $F_{3,48} = 1.92$: $P = 0.139$.

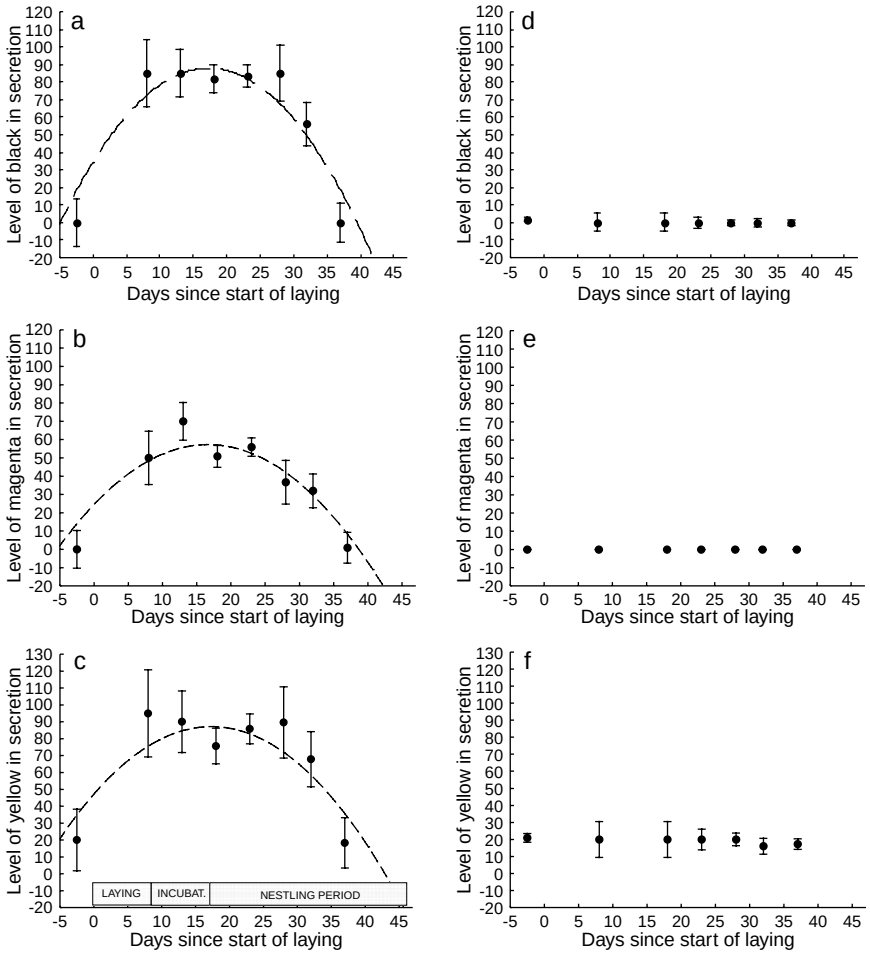

Figure 5. Changes in the colour of female and male secretions over the breeding cycle. Broken lines are the quadratic regressions fitting raw data. Means and 95% confidence intervals for the dependent variables are shown for periods of five consecutive days. The correspondence with the different phases of the breeding attempt (laying, incubation and nestling period) are indicated in (c). (a) Changes in the level of black colour in female secretion: quadratic regression, $F_{3,50} = 395.2$: $P < 0.001$: $R^2 = 0.82$: $b_0 = 34.58 \pm 5.25$ ($t = 6.6$: $P < 0.001$); $b_1 = 6.15 \pm 0.47$ ($t = 13.1$: $P < 0.001$); $b_2 = -0.18 \pm 0.01$ ($t = -14.4$: $P < 0.001$)). (b) Changes in the level of magenta colour in female secretion: quadratic regression, $F_{3,50} = 273.4$: $P < 0.001$: $R^2 = 0.77$: $b_0 = 24.54 \pm 4.08$ ($t = 6.0$: $P < 0.001$); $b_1 = 3.93 \pm 0.37$ ($t = 10.8$: $P < 0.001$); $b_2 = -0.118 \pm 0.01$ ($t = -12.7$: $P < 0.001$)). (c) Changes in the level of yellow colour in female secretion: quadratic regression, $F_{3,50} = 235.3$: $P < 0.001$: $R^2 = 0.59$: $b_0 = 47.15 \pm 6.97$ ($t = 6.8$: $P < 0.001$); $b_1 = 4.61 \pm 0.62$ ($t = 7.39$: $P < 0.001$); $b_2 = -0.133 \pm 0.02$ ($t = -8.39$: $P < 0.001$)). d) Changes in the level of black colour in male secretion: quadratic regression, $F_{3,44} = 3.1$: $P < 0.037$: $R^2 = 0.12$: $b_0 = 1.24 \pm 0.53$ ($t = 2.4$: $P < 0.024$); $b_1 = -0.09 \pm 0.07$ ($t = -1.4$: $P = 0.166$); $b_2 = 0.002 \pm 0.002$ ($t = 0.78$: $P = 0.44$)). e) Changes in the level of magenta colour in male secretion: there is no variation, all values are zero. d) Changes in the level of yellow colour in male secretion: quadratic regression, $F_{3,44} = 234.3$: $P < 0.001$: $R^2 = 0.1$: $b_0 = 21.18 \pm 1.12$ ($t = 18.93$: $P < 0.001$); $b_1 = 0.03 \pm 0.14$ ($t = 0.24$: $P = 0.81$); $b_2 = -0.004 \pm 0.004$ ($t = -0.86$: $P = 0.40$)).

Do bacteria mediate variations in properties of hoopoe uropygial gland and its secretion?

The experimental injection of antibiotic successfully reduced Enteroccocci from the uropygial gland of nestlings since prevalence of bacteria (i.e. the probability of bacterial growth from secretions in selective medium for *Enterococcus*) was higher for control than for experimental siblings (percentage of nestlings with Enterococci growth: Control nestlings 75% ($n = 28$), experimental nestlings 21.7% ($n = 23$), Fisher exact test, $P < 0.001$).

In accordance with the hypothetical role of bacteria explaining changes in uropygial glands of hoopoes, we found that the antibiotic treatment affected the volume of secretion stored in the papilla of the gland of nestlings, but did not affect the volume of the uropygial gland (Table 2, Fig. 8) or any linear measurements of gland size (all $P > 0.05$). Nestlings treated with antibiotic produced a lower amount of secretion (Fig. 8a) which, in comparison with secretions of control nestlings, was paler (i.e. less black, Table 3, Fig. 8b) and more reddish (Table 3, Fig. 8c). On the other hand, the antibiotic injections did not affect the estimated colour values of the nestling secretions in the yellow axis (Table 2, Fig. 8d).

The effects of the experiment on secretion properties were not due to a generalized effect of the antibiotic on nestlings since experimental and control nestlings did not differ in body mass (GLM with year, nest (nested within year) as random factors and tarsus-length as covariable: treatment $F_{1,60} = 0.042$, $P = 0.839$), immune response (GLM with year and nest (nested within year) as random factors: treatment $F_{1,38} = 0.151$, $P = 0.7$), or growing rates

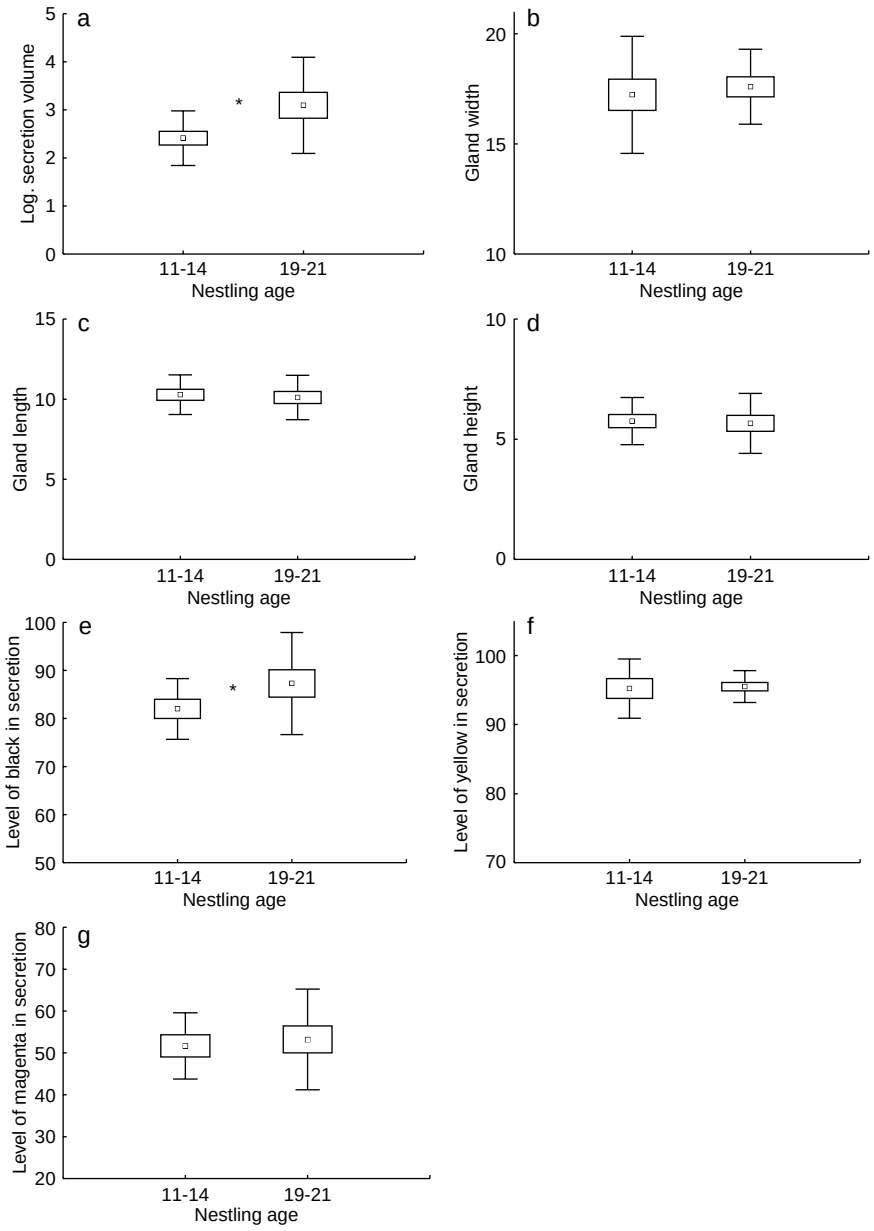

Figure 6. Within individual differences in gland properties between the early and late nestling period. Asterisks mark significant differences at the 0.05 α-level. Means, SE and SD for the dependent variables are shown.

either estimated for body mass (GLM with year and nest (nested within year) as random factors: treatment $F_{1, 61} = 0.708$, $P = 0.406$), wing length or bill length (only data for 2005, GLM with nest as random factor: wing length, treatment $F_{1, 27} = 0.276$, $P = 0.605$; bill length, treatment $F_{1, 27} = 0.016$, $P = 0.9$).

In a repeated-measures design, with three measurements per adult female we detected a significant reduction in most of the considered parameters of the uropygial gland and its secretion among consecutive measurements, regardless of the experimental treatment (Fig. 9, repeated-measures ANOVAs, planned comparisons of least-square means, $F_{1,12/13}$, all $P < 0.01$). The experimental injection of the antibiotic did not affect gland volume, gland width, gland length or volume of secretion, but had an effect on gland height and colour of the secretion for two of the three measurement times (i.e., reproduction phases; Fig. 9). Females treated with antibiotic showed a sharper reduction of gland height than did control females (repeated-measures ANOVA, interaction between experimental treatment and phase of reproduction, planned comparisons of least-square means between the second and third measurements: $F_{1,13} = 3.78$, $P = 0.074$; between first and third measurements: $F_{1,13} = 4.75$, $P = 0.048$; Fig. 9d). The effect of the antibiotic on the level of black coloration of the secretion over the three measurements did not reach significance (Fig. 9f, repeated-measures ANOVA, interactions between the antibiotic treatment and the reproduction phase,

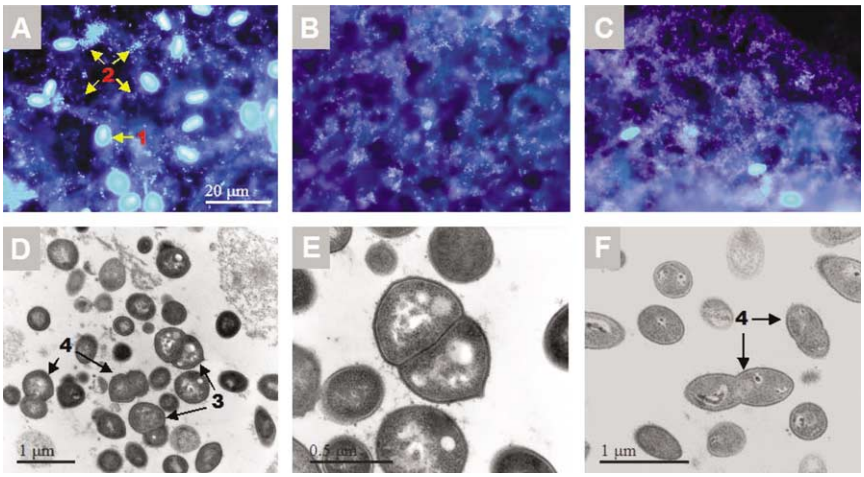

Figure 7. Microscopic images showing bacteria within the uropygial gland secretions of hoopoes. The secretions were fixed immediatly after extraction from inside the ampulla of the gland of alive birds with sterile micropipete tips after washing the surface of the gland with ethanol, therefore bacteria are in the same developmental stage that were within the bird. A, B, and C are fluorescece microscope pictures of smears of secretions of females after staining with Hoescht, and show the natural density and distribution of bacteria found in hoopoes. Black areas are sections of the smear without secretion. B and C are two samples of secretion of the gland of the same individual, the secretion in B was in the most external part of the ampulla, and the secretion in C was in the most internal part of the ampulla, close to the joining with gland lobes. A is from the internal part of the ampulla of a different female. D, E and F are TEM microscope pictures of bacteria found in secretions showing individual bacteria in different developmental stages. These three last images do not reflect the natural density and distribution of bacteria within the gland, since sections were obtained from pellets after spinning a mix of the secretion with fixing solution. D and F are from two different nestlings from two different broods. E shows a higher magnification of bacteria in picture D. (1) Eucariotic nucleus of hoopoe cell, (2) Bacteria, (3) Pair of bacteria joined as result of a binary division, and (4) Bacteria starting a binary division (see the incomplete cell wall).

planned comparisons of least-square means between first and second measurements $F_{1,12} = 4.58$, $P = 0.053$; between first and third measurements: $F_{1,12} = 4.31$, $P = 0.060$). On the other hand, the colour values of secretions for the magenta channel increased after two days of treatment in experimental but decreased in control females (Fig. 9g, repeated-measures ANOVA, planned comparisons of least-square means between first and second measurements, $F_{1,12} = 8.18$, $P = 0.014$). The variation in yellow colour followed a similar trend, but was not significant (repeated-measures ANOVA, planned comparisons of least-square means between first and second measurements, $F_{1,12} = 1.47$, $P = 0.25$; Fig. 9h). Finally, variation in yellow and magenta colour decreased from the second to the third measurements at the same rate in secretions of both control and experimental females (Fig. 9g–h, repeated-measures Anovas, planned comparisons of least-square means between second and third measurements, magenta: $F_{1,12} = 0.16$, $P = 0.699$; yellow: $F(1,12) = 0.08$, $P = 0.78$).

Discussion

We have quantified changes in uropygial gland size and secretion colour of female hoopoes and shown that start before egg laying, maximum gland size is reached around the hatching date of nestlings, and it decreases in size after the eighth day of the nestling period to values similar to those of the prelaying period.

Over the reproductive phases, the amount and properties (colour and odour) of secretions produced by female glands showed a similar pattern of change to that of the uropygial gland. Although it is well established that gland size in many species varies between sexes, among individuals of a particular sex, and seasonally within individuals (Jacob and Ziswiler 1982), to our knowledge the most extreme changes cited are cases of doubling size (referred in Jacob and Ziswiler 1982, e.g. *Corvus frugileus*, Kennedy 1971). This is far from the changes detected in the uropygial gland of female hoopoes, which around hatching time was on

Table 2. Effect of the antibiotic treatment on the volume of secretion produced by nestlings (log secretion volume), and the volume of their gland (gland volume). The table shows the results of GLMs with year and nest (nested within year) as random factors, and treatment as fixed factor.

	Log (secretion volume)			Gland volume		
	df	F	P	df	F	P
Intercept	1	39.3	0.10	1	8.0	0.22
Year	1	8.4	0.007	1	46.0	<0.001
Nest (year)	26	1.8	0.082	25	3.6	<0.001
Treatment	1	22.7	<0.001	1	1.7	0.201
Nest (year) × treatment	23	1.34	0.184	24	0.8	0.760
Error	56			61		

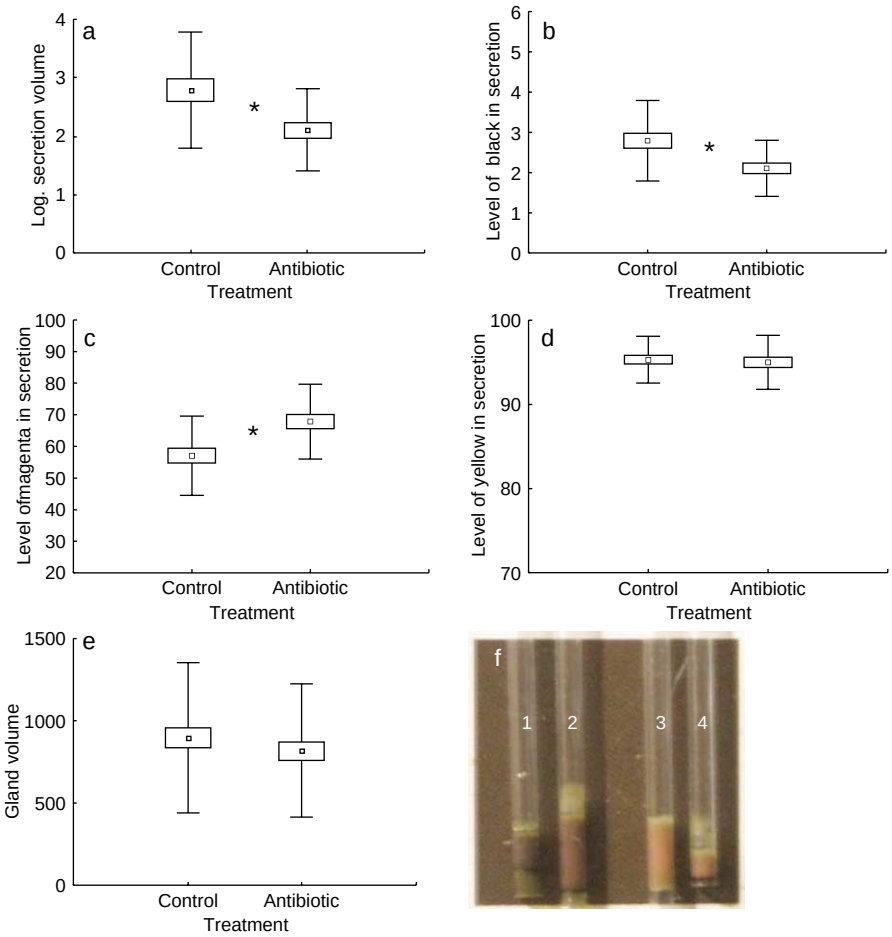

Figure 8. Effects of the antibiotic treatment on properties of nestlings glands. Analyses of secretion and gland volumes include data from 2005 and 2006; those of colour of secretions are only from 2006. Means, SE and SD for the dependent variables are shown. (a) Volume of secretion (log transformed), (b) Black colour of secretions (in an ordinal scale), (c) Magenta colour of secretions, (d) Yellow colour of secretions, (e) Gland volume, and (f) Picture of capillary tubes filled with four secretions of the chicks from one experimental nest, 1 and 2 control nestling, 3 and 4 antibiotic-treated nestling.

average 8-fold larger, and secreted 30 times the volume produced by gland of hoopoes before laying. Such magnitude of seasonal changes in uropygial-gland size and secretion has no parallel in any other bird species. Further, gland size of different hoopoe females (all measurements of gland size) and nestlings (only gland length) was correlated with the volume of secretion produced, which suggests that the gland-size increase involves a proliferation of secretory tissues responsible for producing secretions.

Uropygial secretions of nesting females differ from those produced by non-nesting females, not only in the amount produced, but also in some conspicuous properties such as colour and odour. Secretions of nesting females were much darker and odorous, implying a change in chemical composition. Indeed, brown secretions incorporate several volatile compounds to the wax esters and fatty acids present in white secretions (Martín-Vivaldi et al. unpubl. data). Several studies have found that the composition of uropygial secretions in ducks and shorebirds changes from monoester to diester waxes before laying, and switches back again to monoesters after egg hatching, but no change was reported in gland size (Kolattukudy et al. 1987, Reneerkens

Table 3. Effect of the antibiotic treatment on the colour (within the three-axis system in Küppers 2002: black, magenta and yellow) of the secretion produced by nestlings in 2006. The table shows the results of GLMs with nest as a random factor and treatment as a fixed factor.

	Black			Magenta			Yellow		
	df	F	P	df	F	P	df	F	P
Intercept	1	524.5	<0.001	1	2067.6	<0.001	1	25663.0	<0.001
Nest	11	1.71	0.194	11	0.43	0.910	11	9.72	<0.001
Treatment	1	17.80	0.001	1	8.03	0.014	1	0.00	0.953
Nest × Treatment	11	1.44	0.202	11	1.26	0.289	11	0.22	0.994
Error	33			33			33		

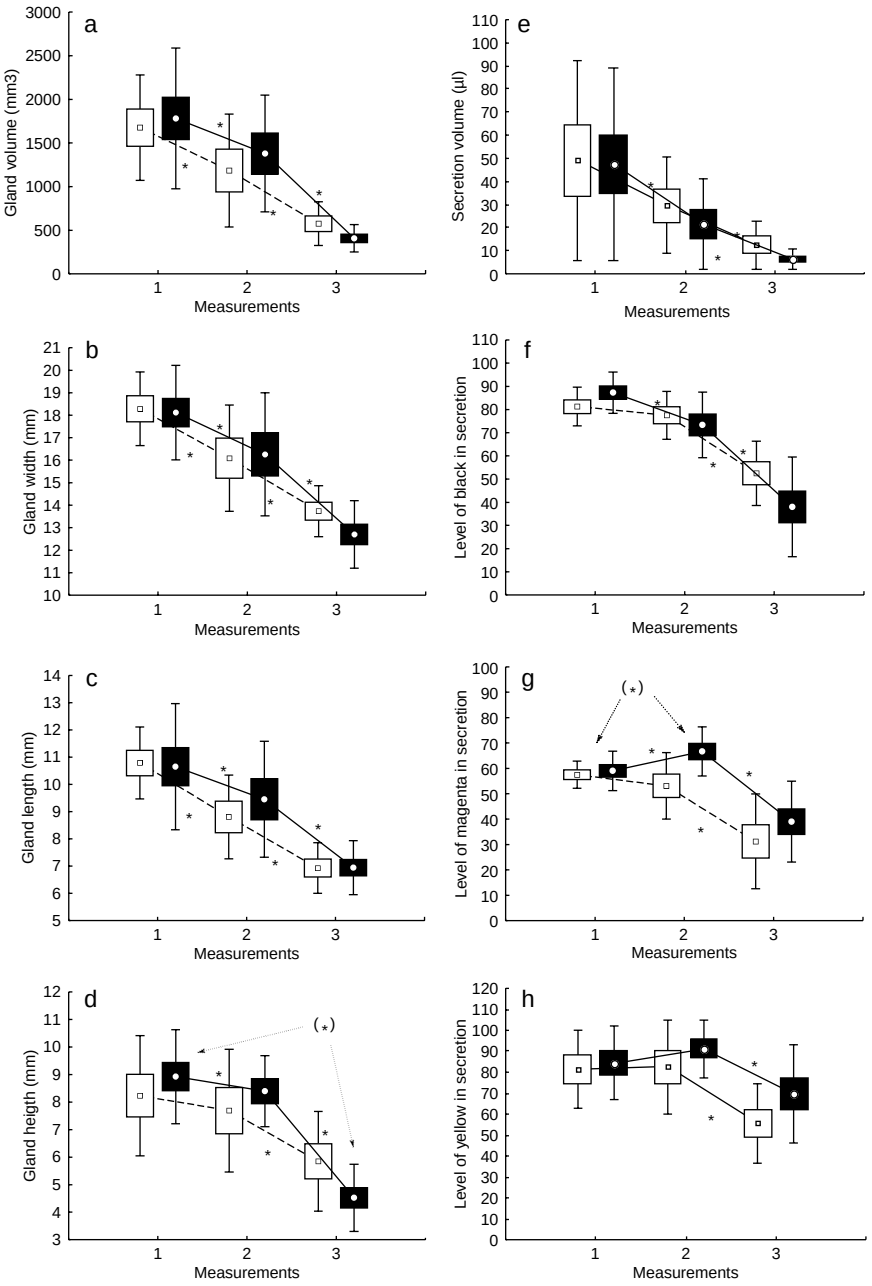

Figure 9. Effects of the antibiotic treatment on properties of female glands after hatching date, therefore a period where the values for all variables decrease naturally (see Figs. 3 and 4). Graphs show the results of repeated-measures Anovas with three consecutive measurements per individual (1 = before inj., 2 = after two inj., 3 = seven d after the third injection) as the within effect, and antibiotic treatment (antibiotic versus saline solution) as the between effect. Asterisks indicate significant planned comparisons of least-square means: * indicates significant differences between consecutive measurements within a particular treatment (antibiotic or saline solution); (*) indicates a significant interaction between the antibiotic treatment and the within effect. Points, bars and whiskers are means, SE and SD. Black bars and circles are values for antibiotic-injected females and white bars and squares values for saline solution-injected females.

et al. 2002, 2006). It has been suggested that these changes in composition of the uropygial gland secretion are to produce a female sexual pheromone in the case of mallards (Jacob et al. 1979, Kolattukudy and Rogers 1987, Kolattukudy et al. 1987), or olfactory crypsis in incubating open-nesting shorebirds (Reneerkens et al. 2005). This latter explanation does not apply to female hoopoes because their secretions are rather more odorous during the nesting phase (Cramp 1998).

The very particular trait of brown secretion of hoopoes, but also of secretions of the closed related green woodhoopoes is that it contains bacteria (Law-Brown and Meyers 2003, Soler et al. 2008). With our microscopic study we have confirmed here that in the case of the European hoopoe, bacteria are highly prevalent and are present at a very high density in brown secretions. Further, TEM images of secretions indicate active binary division of bacteria, which strongly suggest that they were reproducing

within the gland. Thus, hoopoes producing brown secretions host within their uropygial glands a community of symbiotic bacteria. Our experimental injection of antibiotics in nestling glands successfully reduced or eliminated *Enterococcus* bacteria within the secretion. We have not checked for its effect in other groups of bacteria, but the significant reduction of enteroccoci serves to justify that the experiment effectively reduced the bacterial load of the gland. In any case, because we cannot rule out the possible presence of other non-enterococci bacteria in the glands, our experimental broad-spectrum antibiotic treatment probably have cleared most bacteria in the secretion and, therefore, the detected effects cannot be exclusively assigned to Enterococci. Furthermore, since we did not detect any effect of the antibiotic on estimates of overall health and growing rate in the nestlings, we assume that the results cannot be explained by indirect effects of the antibiotic on the gland mediated by its effects in other body parts.

Independently of functionality of the uropygial gland secretion of hoopoes and its changes, we found here evidences suggesting a role of bacteria driving some of the detected natural changes. The uropygial glands of both females and nestlings experimentally injected with antibiotics produced secretions that differed in colour from those of control individuals, which suggests that some of the compounds present in the dark secretions of hoopoes are mediated or directly synthesised by the bacteria. However, experimental individuals produced lighter and redder, but not white secretions as those produced by males or non-breeding females. Working with green woodhoopoes, a close relative of hoopoes with very similar gland and secretion characteristics, Law-Brown (2001, cited in Law-Brown et al. 2003) found similar results. Briefly, working in aviaries they observed that the treatment of the gland of three adults of this species with the antibiotic enrofloxacin also changed the colour of secretions that also became less viscous and less malodorous than that of three control birds. We also found that experimental hoopoes produced less pungent and less viscous secretions than did control individuals (pers. obs.) but, because of the difficulty in quantifying these aspects under field conditions, we were not able to perform statistical comparisons. Therefore, for the two bird species known to harbour bacteria within their uropygial gland and produce malodorous secretions, experimental evidence relates bacteria to such secretion properties.

The second effect of the experimental elimination of bacteria living in the uropygial gland of hoopoe was that it affected the volume of secretion available in the papilla of the gland of nestlings. This result apparently is not the consequence of a reduced glandular tissue, since, contrary to the result found in females, we detected no effect of the antibiotic on gland-size measurements in nestlings. In nestlings, we injected the antibiotic when the gland had already developed, as shown by the absence of differences in gland biometric measurements between the start and end of the experiment in control individuals. On the other hand, females were treated in a phase of marked change in gland size. That difference in the experimental protocol could explain the absence of an effect on gland size in nestlings despite it was detected in females. Nevertheless, given that the experimental nestlings produced less viscous secretions, a possible alternative explanation for the lesser volume of secretion in experimental nestling may be that their more fluid secretion exits the papilla more easily than the viscous secretion of control nestlings.

The extraordinary growth of the gland in females was strictly associated with the period that they stay within the nest, which extends from laying to the eighth day of the nestling period. Because, from other bird species, the antimicrobial activity of different components of secretions is known (Jacob and Ziswiler 1982, Jacob et al. 1997, Shawkey et al. 2003), it is possible that females used such abundant secretion to cover the eggs and protect them against pathogens (as suggested by Reneerkens et al. 2006). Several reasons suggest that the change in hoopoe uropygial secretion during reproduction might function as barrier against pathogenic infection of offspring (both eggs and hatchlings). First, one bacteria strain that was isolated from hoopoe glands produces bacteriocins active against a variety of potential pathogens (Martín-Platero et al. 2006) and, given the high density of bacteria found in secretions in the present study (see Fig. 7 A–D), such substance may be relevant in the secretion. Second, the very similar brown and malodorous secretion of woodhoopoes includes several compounds with antimicrobial activity against pathogenic bacteria (Burger et al. 2004). Third, whole European hoopoe brown secretions have been shown to inhibite in vitro growing of the pathogen *Bacillus licheniformis* (Soler et al. 2008). Fourth, during the first days of incubation the colour of hoopoe eggs changes drastically from pale blue to a brown colour similar to that of the secretions (Fig. 10), which suggests that eggshells may became impregnated with female secretion, directly (given that thin dark-brown stains recalling bill prints are abundant on the eggshell, Fig. 10), or indirectly by preening of their belly feathers that are in contact with eggs. Given that bacterial pathogens are a major cause of hatching failure in wild birds (Cook et al. 2003, 2005a,b), and current evidences suggest antimicrobial properties of uropygial secretions in general and of incubating hoopoes in particular, covering the eggshells with the secretion could reduce the risk of bacterial infection of the embryo and therefore would be of selective advantage.

A further possible functional explanation of changes in the uropygial gland and secretions of hoopoes may be to deter predators, as has been suggested previously both for hoopoes (Cramp 1998) and woodhoopoes (Burger et al. 2004). In agreement with this function, the antipredator behaviour of nestling hoopoes consists on ejecting faeces against predators while maintaining a drop of the uropygial secretion on the tuft of the gland (see Fig. 1c; Cramp 1998). In any case, the hypothetical antipredatory and antimicrobial functions of uropygial secretions of hoopoes are not mutually exclusive, and the presence of antimicrobial compounds in secretions of nestlings that they use for preening might confer advantages preventing infections. Thus, both functions would explain the enlargement of nestling glands during feather development.

Given the common antipredator behaviour of nestlings of hoopoes and woodhoopoes, consisting in the forceful expulsion of faeces against possible predators (Cramp 1998, Ligon 2001) the association of both bird species with bacteria may be related to the implications of this behaviour if for example the need of avalability of faeces for defence

Figure 10. A hoopoe clutch during laying showing the difference in egg colour between the last (pale blue) and previously laid eggs (beige). Notice the dark stains on the surface of beige eggs, probably resulting from the female bill impregnated with uropygial secretion.

difficultes nest sanitation, and thus imposes selective forces for antimicrobial defence within the nest. This would explain the fact that in Upupiformes the special secretion is linked to the stay of birds within the nest (hoopoes) and communal roosting (woodhoopoes) holes where a lot of faeces and food remains accumulate. Nevertheless, the other way around is also possible, because the existence of this antipathogen mechanism might allow development of the anti-predator defense without increasing probability of diseases (i.e., infection).

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