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Experimental support for the makeup hypothesis in nestling tawny owls (*Strix aluco*)

Romain Piault,[a] Julien Gasparini,[a] Pierre Bize,[b] Mariane Paulet,[a] Kevin J. McGraw,[c] and Alexandre Roulin[a]
[a]Department of Ecology and Evolution, Biophore, University of Lausanne, 1015 Lausanne, Switzerland, [b]Division of Environmental and Evolutionary Biology, IBLS, Graham Kerr Building, Glasgow University, Glasgow G12 8QQ, UK, and [c]School of Life Sciences, Arizona State University, Tempe, AZ 85287-4501, USA

Body condition can affect coloration of traits used in sexual selection and parent–offspring communication by inducing rapid internal changes in pigment concentration or aggregation, thickness of collagen arrays, or blood flux. The recent "makeup hypothesis" proposes an alternative honesty-reinforcing mechanism, with behaviorally mediated deposition of substances on body surfaces ("cosmetics") generating covariation between body condition and coloration. In birds, the uropygial gland wax is actively spread on feathers using the bill and changes in its deposition rate may cause rapid changes in bill and plumage coloration. Using tawny owl nestlings, we tested 3 predictions of the makeup hypothesis, namely that 1) quantity of preen wax deposited accounts for variation in bill coloration, 2) an immune stimulation (induced by injection of a lipopolysaccharide [LPS]) impairs uropygial gland wax production, and 3) different intensities of immune stimulations (strong vs. weak stimulations induced by injections of either LPS or phytohemagglutinin [PHA], respectively) and high versus low food availabilities result in different bill colorations. We found that 1) preen wax reduced bill brightness, 2) a challenge with LPS impaired uropygial gland development, and 3) nestlings challenged with LPS had a brighter bill than PHA-injected nestlings, whereas diet manipulation had no significant effect. Altogether, these results suggest that a strong immune challenge may decrease preen wax deposition rate on the bill of nestling birds, at least by impairing gland wax production, which causes a change in bill coloration. Our study therefore highlights that cosmetic colors might signal short-term variation in immunological status. *Key words:* bill coloration, immune status, makeup hypothesis, preening behavior, preen wax, uropygial gland. *[Behav Ecol 19:703–709 (2008)]*

Throughout the animal kingdom, coloration is thought to play an important role in visual communication by signaling aspects of body condition (e.g., sexual activity, hunger level, and state of energy/fat stores) in contexts ranging from courtship and mating to parent–offspring communication. For instance, female Old-World monkeys reveal their estrus by developing a prominent reddening and swelling of the skin that surrounds the perineum around the time of ovulation (Pagel 1994), and canary nestlings (*Serinus canaria*) signal their state of need by displaying redder mouths when they are hungry (Kilner 1997). Because body condition is a dynamic trait that can vary over short periods of time, its signaling implies the existence of biological structures such as skin, tongue, retina, and bill that can rapidly change in coloration. Color changes can occur inside the signaling structure and involve the synthesis, deposition or intracellular movements of pigments (Blount et al. 2003; Faivre et al. 2003; Logan et al. 2006), the rearrangement of light-scattering nanostructures (Prum and Torres 2004), and/or variation in blood flow and composition (Kilner 1997; Negro et al. 2006). Rapid color changes can also result from deposition of substances (usually referred to as cosmetics) on external body surfaces. These substances can be produced by the organisms themselves, as in the hippopotamus (*Hyppopotamus amphibius*), whose subdermal glands produce a viscous sweat that when spread on the skin turns red, then brown under sunlight (Saikawa et al. 2004). Alternatively, such substances can be derived from substrates such as in bearded vultures (*Gypaetus barbatus*) that yellow their plumage by bathing in soils stained with iron oxides (Negro et al. 1999). By contrast to internal mechanisms that have been well described and that underlie most studied covariations between body condition and coloration, external deposition of cosmetics has been overlooked until recently. The renewed interest in cosmetic colorations (Negro et al. 1999; Arlettaz et al. 2002; Reneerkens and Korsten 2004; Montgomerie 2006; Delhey et al. 2007) came from the papers by Piersma et al. (1999) and Negro et al. (1999), who, under the makeup hypothesis, proposed that the deposition of cosmetics might provide an alternative honesty-reinforcing mechanism linking body condition and coloration. However, whether deposition of cosmetics can generate such covariations is still unknown due to lack of experimental tests.

Birds are suitable study organisms to test the makeup hypothesis for at least 2 reasons. First, in most species, individuals have an uropygial gland on their rump whose secretion, the preen wax, has been proposed to function as a cosmetic (Piersma et al. 1999). These secretions, collected and spread on feathers using the bill, can be colored (i.e., differentially absorbing or reflecting light of a certain wavelength range) and thus readily stain plumage such as in several (8 out of 54) hornbill species (the Asiatic genera *Buceros*, *Aceros*, *Penelopides*, and *Rhinoplax*; Kemp 2001). When transparent, preen wax might also cause a change in the appearance of bill and feathers by glossing them (i.e., increasing achromatic brightness, Delhey et al. 2007; but see Reneerkens and Korsten 2004). Second, major environmental factors known to affect body condition, such as parasites and food availability, have been found to affect both preen wax production and intensity of preening behavior, 2 components that determine wax

Address correspondence to R. Piault. E-mail: romain.piault@unil.ch.
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deposition rate on bill and feathers. Malarial infection decreases time devoted to preening behavior in Apapane (*Himatione sanguinea*) juveniles (Yorinks and Atkinson 2000), and disease or inappropriate diet result in malfunction of the uropygial gland (and thus alter the production of preen wax) in captive parrots (Hochleitner et al. 1996). Altogether, these results suggest that changes in bill and feather coloration due to the deposition of preen wax might reveal body condition because major environmental factors known to affect body condition can also influence wax production and/or preening behavior. In the present paper, we focus on bill coloration and evaluate some predictions of the makeup hypothesis in wild nestling tawny owls (*Strix aluco*), a species presenting a bill free from carotenoids, pigments known to mediate infection-induced changes in bill coloration (Faivre et al. 2003). Whether bill coloration in nestling (or adult) tawny owls functions as a signal of body condition is unknown. However, because the mechanism of color change proposed by the makeup hypothesis is derived from preening (Delhey et al. 2007), a common behavior to all bird species, covariation between bill coloration and body condition due to the deposition of preen wax is expected even in a species in which bill coloration may not be used as a signal.

In a series of 3 experiments, we tested the following 3 predictions of the makeup hypothesis, namely that 1) quantity of preen wax deposited on the bill affects its coloration, 2) environmental factors modulate the production of preen wax, and 3) bill coloration is associated with body condition. To test the first prediction, we measured bill coloration not only before and after having cleaned bills with water but also after having applied manually preen wax extracted from the uropygial gland of individuals to bills. For the makeup hypothesis to be valid, bill coloration should be different between cleaned and preen wax–coated bills. In a test of the second prediction, we examined whether disease challenge, an environmental factor known to affect individual immune status (i.e., intensity of stimulation of the immune system, which is an aspect of body condition), can modulate preen wax production. To this end, we created 2 groups of offspring differing in their immune status by injecting them either with an endotoxin lipopolysaccharide (LPS), which mimics a bacterial infection, or with a control phosphate buffer saline (PBS) solution. We then measured the uropygial gland growth over 3 days, which serves as a proxy for preen wax production capacity (Jacob and Ziswiler 1982), with the prediction that LPS stimulation of the immune system impairs uropygial gland growth. Finally, using another set of nestlings, we tested whether bill coloration can mirror immune and nutritional status (i.e., well fed as opposed to food restricted). For this purpose, we induced a strong (i.e., a cutaneous inflammatory response against LPS; Bonneaud et al. 2003; Cheng et al. 2004) or a weak stimulation of the immune system (i.e., a cutaneous inflammatory response against the plant protein phytohemagglutinin (PHA); Merino et al. 1999; Martin et al. 2003) and then fed LPS- and PHA-injected nestlings either ad libitum or with a restricted diet during 6 days. PHA is usually used to measure cutaneous immunity without imposing high stress levels in nestling birds (Merino et al. 1999; Martin et al. 2006), whereas LPS is currently used as an immune stressor to evaluate the cost of immune function activation in the absence of pathogens (Bonneaud et al. 2003; Cheng et al. 2004), all of which suggesting that an injection of LPS results in a stronger immune stimulation than an injection of PHA. Seven days after having measured cutaneous inflammatory responses to LPS or PHA, we measured bill reflectance, with the expectation that LPS- and PHA-injected nestlings, as well as well-fed and food-restricted nestlings, would differ in bill coloration.

METHODS

The study was carried out in 2005 and 2006 using a population of tawny owls located in western Switzerland, where 365 nest-boxes have been installed in a study area of 911 km^2 in woodland patches mostly surrounded by fields. Adults of this species prey on a large number of animals, including mammals, birds, and frogs, and have therefore little access to food resources rich in carotenoids such as fruits, vegetables, and caterpillars (Dimitrios 2006). Reproduction takes place between January and May, with brood sizes ranging from 1 to 7 nestlings. Only the female incubates the clutch, and half way through the rearing period, she starts to help her partner collect food for the offspring. Nestlings leave the nest-box before being able to fly, at an age of 25–30 days (A. Roulin, personal observation). We visited nest-boxes to record clutch size (mean $\pm$ SD, 2005: 4.1 $\pm$ 1 eggs, range: 2–6; 2006: 2.9 $\pm$ 0.8 eggs, 2–5), hatching date (2005: 4th April $\pm$ 12 days, 8th March–3rd May; 2006: 26th April $\pm$ 12 days, 6th April–25th May), and brood size at hatching (2005: 3.53 $\pm$ 1.14 nestlings, 1–6; 2006: 2.32 $\pm$ 0.84 nestlings, 1–4). We marked nestlings with a numbered aluminum ring to recognize them individually.

Measurement of bill coloration and carotenoid content

We measured spectral reflection on the right and left sides of bills in the typical range of bird-visible wavelengths (320–700 nm) using an Ocean Optics S2000 spectrometer and a PX-2 pulsed xenon lamp (Ocean Optics Inc., Duiven, The Netherlands). Reflectance is expressed as the proportion of reflectance from a white standard disk (WS-2). To summarize reflectance data, each spectrum was reduced to the median of 10-nm bandwidths. Variation in UVa range (320–400 nm) may not be detectable by tawny owls, as no UV-sensitive photoreceptor class has been found using microspectrophotometry (Bowmaker and Martin 1978). However, because we had no a priori reason to think that preen wax affects reflectance in a particular cone output (because in owl preen wax is transparent) and because we aimed to test the validity of the makeup hypothesis in birds in general, we divided bill reflectance data in 4 regions that correspond to the sensitivity of the 4 avian cones found in most species. Thus, we computed mean reflectance values (or brightness) between 320 and 400 nm (UVa light), 400 and 500 nm (blue), 500 and 600 nm (yellow), and 600 and 700 nm (red) for both the left and right sides of bills (Montgomerie 2006). We then calculated a mean value for each spectral region and each nestling. Brightness is a measure of the total amount of light reflected by the bill in the spectrum region considered (Endler 1990; Andersson 1999). A high brightness value stands for a brilliant surface, whereas a lower brightness value indicates a duller surface.

To ensure that variation in bill reflectance observed in our experiments was not caused by variation in carotenoid pigment concentration in bill tissues, we tested for the presence of these pigments in the bill of 3 tawny owl nestlings of similar age as those used in experiments and found dead in nests. Carotenoid extractions and analyzes were performed on >3 mg of excised, keratinized bill tissue following the methods described in McGraw et al. (2006).

Tests of predictions 1 and 2

In 2006, we used 18 nests with a total of thirty-two 25-day-old nestlings (mean $\pm$ SD: 25.1 $\pm$ 1.8 days) to assess experimentally whether preen wax affects bill coloration and whether nestling immune status affects uropygial gland growth. For each nestling, a drop of blood was collected from the brachial

vein and their sex determined from blood cell DNA using the method described in Py et al. (2006).

Experiment 1: effect of preen wax on bill coloration

We successfully recorded bill coloration in 28 individuals. This was done just before the injection of LPS or PBS and 3 days later when we came back to measure uropygial gland volume (see below). For each nestling, M.P. took a first spectrum from the left and right bill sides. Then, she washed bills by rubbing them with a piece of cotton soaked with water to reduce the amount of deposited preening secretions and to clean them from dust and dirt that may glue to them and took a second measurement. For each individual, she then harvested preen wax by squeezing gently the gland between the index and the thumb and collected wax drops using a piece of cotton. She immediately spreads preen wax evenly on bills with the cotton swab used to collect it (preen waxes were not mixed between individuals) and then recorded bill spectra a third time.

To test for an effect of preen wax on bill coloration, we entered the 4 spectral regions (from 320 to 400 nm, 400 to 500 nm, 500 to 600 nm, and 600 to 700 nm) as dependent variables in a mixed-model analysis of variance (ANOVA) for repeated measures. Fixed factors were "visit" (before vs. 3 days after injection) and bill state (before water cleaning, after water cleaning, and after preen wax coating). Because we measured several nestlings per nest, we included nest as a random factor to avoid pseudoreplication. Nestling identity nested within the immune treatment was added to the model as a random factor because each nestling got the same immune treatment (either LPS or PBS; see below for injection procedure). We added the immune challenge (LPS vs. PBS) as a factor to test whether this treatment altered bill brightness. Nonsignificant interactions were removed in a stepwise fashion, starting with the least significant one (all P values ≥ 0.10).

Experiment 2: effect of immune challenge on uropygial gland volume

The uropygial gland consists of 2 lobes that contain the secretive active tissue and a duct system (Jacob and Ziswiler 1982). We measured gland length (top of the papilla to the basis of the 2 lobes at the skin level; L), depth (distance between the gland dorsal and ventral surface; D), and width (span across the 2 lobes; W) to the nearest 0.1 mm using calipers (Roulin 2007). In nestling tawny owls, the uropygial gland is cone shaped, and thus we estimated uropygial gland volume with the formula $\pi/12 \times L \times D \times W$; this method is accurate because volume is strongly associated with gland mass (Roulin 2007), a good proxy of the amount of preen wax, as capsules where this wax is produced occupy 68% of the uropygial gland (Sandilands et al. 2004). We injected nestlings subcutaneously in the wing web either with LPS (endotoxin LPS from the cell wall of *Escherichia coli*, serotype 055:B5, Sigma, L2880, Switzerland, 20 μg diluted in 0.02 ml; $n = 19$) or with an equal volume of PBS ($n = 13$). The LPS dose we injected is 12.5 to 225 times lower than those previously used in poultry to activate the immune system (e.g., 250 μg in 0.1 ml, Parmentier et al. 1998; 4500 μg in 0.2 ml, Cheng et al. 2004). Prior to injection, nestlings from the 2 experimental groups did not differ significantly in body mass, tarsus length, wing length, age, or gland volume (Student's t-tests, all P values > 0.24). Males and females were randomly distributed between LPS- and PBS-injected nestlings (chi-square test, $\chi^2 = 0.25$, $P = 0.62$). The cutaneous inflammatory response to LPS and PBS was measured as the skin thickness change at the injection site 4 h after the injection (±15 min). The 4-h delay was chosen following Parmentier et al. (1998) in which LPS or PBS was injected in the wing web of hens of 3 different lines (selected for high or low antibody responses plus a random bred control line) and the web thickness measured before injection and after 4 and 24 h. Because the response to LPS was significantly higher than the response to PBS in the 3 lines 4 h but not 24 h after injection, we measured web swelling 4 h after injection. Three days later, we measured nestling body mass, tarsus and wing length, as well as the uropygial gland length, depth, and width; we could not take these measurements at a later age because most nestlings had already left their nest. Although we injected 38 nestlings, we had a sample size of 32 individuals (19 LPS-injected and 13 PBS-injected individuals) for the statistical analyses because 6 chicks left their nest before we could measure uropygial gland volume a second time (1 LPS- and 5 PBS-injected individuals).

We tested whether an immune challenge toward LPS affected the growth of the uropygial gland by entering the increase in gland volume between the time when nestlings were injected with LPS (or PBS) and 3 days later as the dependent variable in a mixed model ANOVA. Nest was entered as a random factor and immune challenge (LPS vs. PBS) as a fixed factor.

Experiment 3: effect of immune and nutritional status on bill coloration

In 2005, we investigated whether bill coloration reflects immune status (i.e., high vs. low level of immune system activation) by injecting subcutaneously in the wing web 45 unrelated owlets (mean age ± SD: 22.8 ± 2.5 days) either with LPS (serotype 055:B5; Sigma, 20 μg diluted in 0.02 ml) or with PHA (the plant protein PHA; Sigma, 10 μg diluted in 0.02 ml) in the field. The cutaneous inflammatory response against these mitogens was given by the skin thickness change at the injection site 4 h after an injection of LPS (±19 min) and 24 h after an injection of PHA (±2h12min; Parmentier et al. 1998; Smits et al. 1999). Inflammatory responses to both mitogens encompass increased blood supply and cellular infiltration into the damaged tissue as well as the activation of a wide array of cells, including heterophils and B and T lymphocytes (Janeway and Travers 1999; Martin et al. 2006). However, both mitogens also cause specific responses from the injected individuals. LPS mimics a bacterial infection, inducing the release of cytokines and the production of specific antibodies after the inflammatory response. For this reason, LPS is frequently used as a reliable indicator of animal's capability to adapt to immune stressors and as a means of evaluating the cost of immune function activation in the absence of pathogens (Bonneaud et al. 2003; Cheng et al. 2004). In contrast, PHA induces a local infiltration and division of T lymphocytes (Martin et al. 2006) and is used as a reliable indicator of host viability (Saino et al. 1997) without imposing high stress levels in nestling birds (Merino et al. 1999). Altogether, these results suggest that injections of LPS and PHA result in different intensities of immune system activation, strong (high level of stimulation) and weak (low level of stimulation), respectively. A corollary is that LPS was shown to negatively affect body mass, whereas PHA did not, although it increased basal metabolic rate in captive house sparrows (*Passer domesticus*; Bonneaud et al. 2003; Martin et al. 2003). We therefore checked that our immune treatments resulted in 2 groups of nestlings differing in their immune status by testing the impact of LPS and PHA injections on nestling body mass change during 6 days. For each nestling, a drop of blood was collected from the brachial vein for molecular sex determination (as above).

On the same day that we measured cutaneous inflammatory responses to LPS and PHA, we brought these nestlings in the

laboratory in the afternoon (day 0) and kept them at 24 °C and 14:10 light:dark cycle singly in a similar nest-box as the one in which they were reared under natural conditions. To minimize variation in hunger level due to the fact that nestlings consumed different amounts of food during the last 24 h in natural conditions, we offered them laboratory mice ad libitum until the next morning (08:00 h) when food treatments started (day 1) with nestlings being fed either ad libitum (i.e., they could eat as many mice as they wanted) or with a restricted diet (each nestling could eat 2 mice per day). Under natural conditions, nestlings consume between 2 and 4 small mammals per day (Galeotti 2001; main prey: yellow-necked field mice [*Apodemus flavicollis*], body mass range: 22–45 g; bank voles [*Clethrionomys glareolus*], body mass range: 15–25 g). Nestling body mass, tarsus length, and wing length were measured to the nearest 0.1 g, 0.1 mm, and 1 mm, respectively, on day 1 at 08:00 h. Thirteen of the 26 PHA-injected nestlings were assigned to the ad libitum treatment and 13 to the food-restricted treatment. Similarly, 10 LPS-injected nestlings were assigned to the ad libitum treatment and 9 to the restricted treatment. Nestling body mass, tarsus length, wing length, and age did not differ between the 4 treatments at the start of the experiment (2-way ANOVA with food treatment and immune challenge as fixed factors plus their interaction, all P values > 0.17). Males and females were randomly distributed among the 4 treatments (nominal logistic regression with nestling sex as the dependent variable, food and immune treatments as fixed factors plus their interaction, all $P > 0.25$). From days 1 to 6, we added fresh laboratory mice in nest-boxes and removed noneaten items at both 08:00 and 18:00 h every day. Owls could consume mice when they wanted because at that age they can swallow entire items. During these 6 days, 23 ad libitum fed owls consumed on average 53.8 ± 7.1 g (mean ± SD) mice per day (range: 38.7–64.0 g), whereas 22 food-restricted individuals that were offered only 2 mice per 24 h ate on average 30.6 ± 0.6 g per day (range: 29.7–31.8 g). On day 6 at 18:00 h, R.P. took bill spectra in the laboratory; in the morning on day 7, body mass and tarsus and wing length were measured and the nestlings were returned to their original nest in the wild. During the 6 days spent in the laboratory, nestlings fed ad libitum gained body mass (mean body mass change: 19.56 ± 4.85 g) whereas food-restricted nestlings lost body mass (mean body mass change: −21.05 ± 3.66 g), indicating that our food treatments effectively created 2 groups of nestlings differing in nutritional status. Nestlings challenged with LPS tended to lose more body mass than nestlings injected with PHA when food restricted, whereas no significant difference was found in body mass gain between LPS- and PHA-injected nestlings in the ad libitum group (Piault, Bize, Gasparini, Juilland, and Roulin, in preparation). This result confirms that we created 2 groups of nestlings (LPS vs. PHA) differing by the intensity of stimulation of their immune system.

We tested whether immune (LPS vs. PHA) and food treatments (ad libitum vs. restricted diet) affected bill brightness using a mixed-model ANOVA for repeated measures with brightness calculated for the 4 spectral regions (from 320 to 400 nm, 400 to 500 nm, 500 to 600 nm, and 600 to 700 nm) as the dependent variables (Bize et al. 2006) and with spectral region, type of immune challenge, and food treatment as 3 fixed explanatory variables. We nested nestling identity within immune and feeding treatments because each chick had the same immune and food treatment and as a random factor to avoid pseudoreplication (e.g., within the same chick, mean reflectance between 320 and 400 nm was not statistically independent of the mean reflectance between 400 and 500 nm). We initially entered nestling age and body mass into the model to test for effect of degree of maturation and body condition on bill brightness. However, these covariates were not significant, and thus, we removed them from the final model presented in the results.

Ethical note

Seventeen out of 80 (21.3%) nestlings brought in the laboratory were recaptured as adults in 2006 and 2007 versus 30 out of 205 (14.6%) that stayed in their nest were recaptured. Thirty-one out of 155 (20%) nestlings challenged with LPS were recaptured in 2006 and 2007 versus 16 out 130 (12.3%) nestlings injected with PHA were recaptured. Recapture rate did not significantly differ between nestlings taken in laboratory to those left in their nest (logistic regression with recapture [coded as 1 or 0] as the dependent variable and use in the laboratory experiment or not [coded as 1 or 0; $\chi^2 = 1.89$, degrees of freedom {df} = 1, $P = 0.17$] and immune treatments [$\chi^2 = 3.23$, df = 1, $P = 0.07$] as fixed factors. Their interaction was removed from the model as not significant [$P = 0.92$]), suggesting that time in laboratory did not affect nestling survival. LPS-challenged nestlings tended to be captured as adults more frequently than their PHA-injected counterparts, suggesting that although an injection of LPS results in a stronger immune stimulation than an injection of PHA, this did not lower nestling survival. The experiments were approved by the veterinary services of Canton de Vaud (licence no. 1508), and birds were ringed under the legal authorization of the Swiss Agency for the Environment, Forests and Landscape.

Statistical procedure

Statistical analyses were performed using JMP IN 6.0.0. Throughout the paper, we report means ± standard error and use 2-tailed statistical tests and a significance level of 0.05. In all models, residuals were normally distributed, and variances were homogenous between treatments.

RESULTS

Bill carotenoid content

The gray/horn–colored beaks of tawny owl nestlings contained no detectable carotenoids (detection limit = 5 μg pigment per gram tissue).

Experiment 1: effect of preen wax on bill brightness

Bill brightness was the highest after preen wax and dirt had been removed with water (12.92 ± 0.62%) and the lowest after we had coated bills with preen wax collected fresh from the nestling's uropygial gland (11.86 ± 0.62%; $F_{1,638} = 9.03$, $P = 0.0001$; Figure 1). This effect was independent of the spectral region considered (no significant interaction between bill state and spectral region; $F_{1,632} = 1.18$, $P = 0.32$; Figure 1 reports the effect of bill state on mean bill brightness between 320 and 700 nm, with least square means extracted from the model). In the same model, we did not find any difference in bill brightness between LPS- and PBS-injected nestlings ($F_{1,13.96} = 0.05$, $P = 0.83$). Bill brightness was higher just before we injected LPS or PBS than 3 days later ($F_{1,638} = 27.92$, $P < 0.0001$), a finding that was independent of bill state (before water cleaning, after water cleaning, and after preen wax coating), as shown by the nonsignificant interaction between visit and bill state ($F_{1,637} = 1.64$, $P = 0.20$).

Experiment 2: effect of immune challenge on uropygial gland volume

Change in wing web swelling was greater in LPS- than in PBS-injected nestlings measured 4 h after the injection (mixed-model

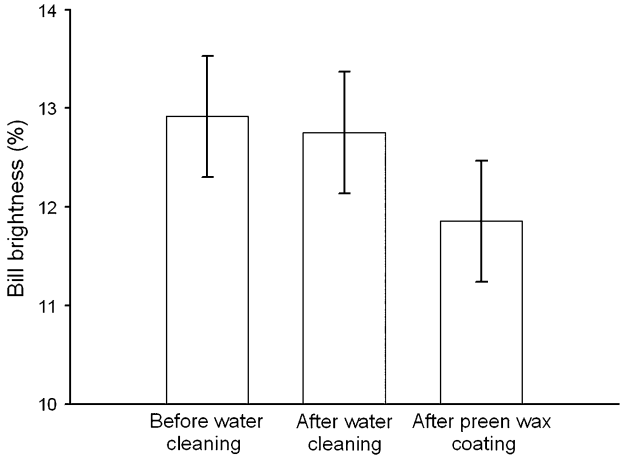

Figure 1
Least square means (±standard error) bill brightness before and after bills have been cleaned with water and after being coated with preen wax extracted from nestling uropygial gland. Data were collected in 2006.

ANOVA with change in skin thickness as the dependent variable, nest as a random factor, and immune challenge [i.e., LPS vs. PBS] as a fixed factor: $F_{1,17.94} = 13.91$, $P = 0.002$; mean change in thickness: 0.42 ± 0.05 and 0.17 ± 0.06 mm for LPS- and PBS-injected nestlings, respectively). There was no significant effect of the immune treatment on nestling body mass change over the 3 days (mixed-model ANOVA with body mass change as the dependent variable, nest as random factor, and immune challenge [LPS vs. PBS] as a fixed factor: $F_{1,16.96} = 1.46$, $P = 0.24$).

Nestlings challenged with LPS showed a lower increase in the volume of their uropygial gland 3 days after injection than offspring injected with PBS (mixed-model ANOVA with nest as a random factor, immune challenge as a factor: $F_{1,17.31} = 9.29$, $P = 0.0072$; Figure 2).

Experiment 3: effect of immune challenge and nutritional status on bill brightness

As measured by the change in wing web swelling at the site of injection, the immune challenges were effective. Nestlings mounted a significant cutaneous inflammatory response against PHA, as measured by skin swelling 24 h after the injection (Student's *t*-test comparing change in skin thickness with zero, $t = 6.84$, df = 25, $P < 0.0001$; mean change in thickness: 0.37 ± 0.05 mm) and against LPS measured 4 h after the injection (Wilcoxon signed-rank test comparing change in skin thickness with zero: $n = 19$, $Z = 66.5$, $P = 0.006$; mean change in thickness: 0.15 ± 0.04 mm). LPS-injected nestlings in 2006 mounted a higher cutaneous response than did LPS-injected nestlings in 2005 (analysis of covariance with change in skin thickness as the dependent variable; year [2005 vs. 2006] as a fixed factor: $F_{1,33} = 23.34$, $P < 0.0001$; and body mass at injection as a covariate: $F_{1,33} = 3.68$, $P = 0.06$).

Independent of food treatment, nestlings challenged with LPS displayed a brighter bill than those injected with PHA (immune challenge: $F_{1,42} = 4.07$, $P = 0.05$; food treatment: $F_{1,42} = 0.05$, $P = 0.82$). Mean bill brightness across all spectral regions for LPS- and PHA-injected offspring were 31.06 ± 1.57% and 27.48 ± 0.97%, respectively. The food treatment did not mediate the effect of the immune challenge, as shown by the nonsignificant interaction between these 2 factors ($F_{1,41} = 0.78$, $P = 0.38$). The difference in bill coloration between LPS- and PHA-injected nestlings was of the same magnitude in the different parts of the spectrum (interaction between immune challenge and spectral region: $F_{3,129} = 1.92$, $P = 0.13$; Figure 3 reports the effect of the immune challenge effect on mean bill brightness between 320 and 700 nm, with least square means extracted from the model).

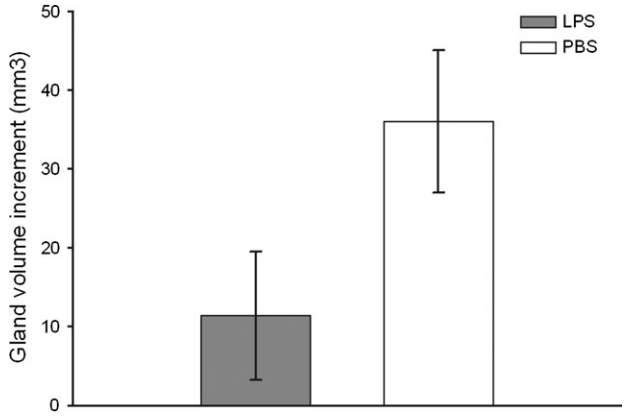

Figure 2
Least square means gland volume increment (±standard error) of the uropygial gland over 3 days after nestlings were immune challenged with LPS or PBS. Data were collected in 2006.

DISCUSSION

In 2006, we experimentally found in tawny owl nestlings that manual bill cleanings increased bill brightness and that application of preen wax to the bill reduced bill brightness throughout the UVa-VIS spectrum. In addition, an immune system challenge (LPS injection) impaired the growth of the uropygial gland. In 2005, we found that nestlings induced to mount a stronger immune response had a brighter bill (again throughout the spectrum), indicating that immune system responsiveness can be rapidly mirrored in bill coloration even in absence of carotenoids.

Although in 2005, we had no control group (nestlings injected with a PBS) and did not record bill brightness before injection of both mitogens, we believe that PHA did not affect bill coloration because it results in a local immune response (Merino et al. 1999). Altogether, our results hence suggest

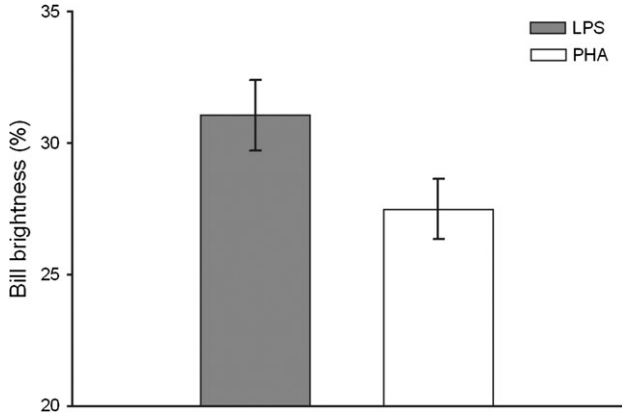

Figure 3
Least square means (±standard error) bill brightness in nestling tawny owls injected 7 days before with LPS ($n = 26$) or with PHA ($n = 19$). Data were collected in 2005.

that brighter bills of nestlings challenged with LPS in 2005 are the results of a reduced deposition of preen wax on the bill during 6 days as compared with PHA-injected offspring. Two nonmutually exclusive mechanisms can account for this reduction. First, LPS-injected nestlings may have devoted less time to preening activities as compared with their PHA-injected counterparts, as LPS is known to decrease physical activity (Cheng et al. 2004), a trait we did not quantify during our study. Second, LPS may have affected uropygial gland production of wax. This interpretation is in line with our finding that an injection of LPS resulted in a reduced growth of the uropygial gland, a trait we used as a proxy for gland capacity to produce wax. This effect may result from a direct effect of LPS on preen wax production or from an indirect effect with LPS-injected nestlings stimulating their uropygial gland less frequently, which may cause a lower preen wax production (to the best of our knowledge the later mechanism has not yet been studied).

Although the preen wax coating in experiment 1 reduced bill brightness by only 1%, we found a difference of about 4% in bill brightness between LPS and PHA nestlings, the interpretation of our results still hold. Differential accumulation of preen wax on the bill in LPS- and PHA-challenged offspring over 6 days can explain why bill brightness differed by an amount of more than 1% in 2005. Furthermore, although the differences in brightness between treatments are small both in 2005 and 2006, their significance suggests that the mechanism of color change proposed by the makeup hypothesis can occur. Studies on sensory capacities of tawny owls found that absolute eye sensitivity to brightness in this species is higher than that of human by an average of 2.5 fold (Martin 1977). Although we did not perform measurements of light intensity in our nest-boxes, tawny owl adults may hence perceive variation in bill brightness of their nestlings at night dusk and dawn (but probably not in the middle of the night when the nest cavity is dark). Thus, one possibility is that parents adjust their daily feeding rates according to nestlings' bill coloration at their first and last night visits to the nest cavity. However, the Strigiformes have relatively small uropygial glands (Jacob and Ziswiler 1982), which suggests that their capacity to produce wax may be limited, and hence, this may explain these low variations in bill brightness. Species that have relatively large uropygial glands such as the Passeriformes (Jacob and Ziswiler 1982) may therefore be better candidates to study the signaling function of wax deposition.

Interestingly, food restriction did not significantly affect bill brightness; 2 mutually nonexclusive hypotheses can explain this finding. First, low availability of food may not have negatively affected preening behavior. This hypothesis has already been confirmed in domestic fowls (*Gallus gallus*), in which an ad libitum or restricted diet did not alter preening activities (Savory and Maros 1993; Zulkifli et al. 2006). Second, low food availability (at least during a short period of time) may not have resulted in lower production of preen wax, but to our knowledge this hypothesis still remains to be addressed. In conjunction with the finding that malnutrition results in malfunction of the uropygial gland in captive parrots (Hochleitner et al. 1996), our results and those found in domestic fowls indicate that food quality rather than food quantity may affect wax production.

We found that preen wax substantially decreased bill brightness, and this result contrasts with that obtained by Reneerkens and Korsten (2004), who showed that wax deposition did not affect plumage reflectance in red knots (*Calidris canutus*). However, in their study, the authors did not standardize the amount of preen wax deposited on plumage by coating cleaned feathers with fresh wax. It would hence be worth investigating whether preen wax also decreases brightness in feathers after this manipulation. Contrary to predictions in the recent literature, which were not based on empirical data (Delhey et al. 2007), we found that preen wax application substantially decreased rather than increased brightness. In accordance, Surmacki and Nowakowski (2007) found that feathers of Great tits (*Parus major*) washed with a chloroform/methanol mixture to remove soil and preen waxes expressed brighter coloration than control feathers. During preening activities, the film of preen wax deposited on feathers and bill is likely to be thin and thus should dry rapidly. Relative dryness of wax may increase its absorbance and lower its reflectance capacity. Furthermore, our study shows that transparent preen wax did not differentially affect bill reflectance at UVa wavelengths (320–400 nm), as first proposed by Piersma et al. (1999). Future studies testing the makeup hypothesis should hence consider the entire UVa-visible spectrum for birds when working with species associated with transparent preen waxes.

Although nestlings challenged with LPS in 2006 had a higher cutaneous inflammatory response to this immunostimulant than those injected in 2005, we did not find any effect of the immune challenge in 2006 on bill coloration. This may be due to the fact that we measured bill coloration only 3 days after injection (but 7 days in 2005), and hence, differences in the amount of preen wax deposited on bills between LPS- and PBS-injected nestlings may require more time to be detected. Bill coloration may therefore reflect current immune status not before 3 days after the onset of an infection. Furthermore, in 2006, in contrast to 2005, the immune treatment did not affect significantly nestling body condition, and bill brightness recorded in 2006 was lower than in 2005, which may further explain why we found no difference between LPS- and PBS-injected individuals. Different levels of bill brightness between years may result from different environmental conditions between 2005 and 2006. As suggested by food stores found in nests, these 2 years were characterized by high (2005) and low (2006) availabilities of yellow-necked field mice and bank voles, the main prey species of tawny owls in our population. Parents fed their young with more alternative preys, such as birds, in 2006. Prey availability and quality could have affected nestling bill reflectance development and consequently lowered bill brightness.

For at least 4 reasons, our findings have important implications in the context of honest signaling including mate choice and parent–offspring interactions. Because LPS mimics a bacterial infection without the direct negative effects of pathogens, pathogens may hence induce a reallocation of resources from wax production to the immune system in the host to control pathogen infection. This indicates that producing wax is costly for individuals, and this cost might guarantee the honesty of bill brightness as a signaling trait. Second, any signaling trait may be constrained to reflect specific aspects of body condition probably because the mechanism underlying change in coloration is not sensitive to all environmental factors. For instance, our results suggest that bill brightness cannot reveal nestling hunger level because preening-mediated change in bill coloration did not appear to be sensitive to short-term food deprivation. Third, bill coloration could be used as a signal of current immune status even in species with no carotenoid-based coloration. Fourth, we found that a pronounced activation of the immune system with LPS increased bill brightness, and thus, a conspicuous (more reflective) coloration may not always reveal a better but sometimes a poorer condition in some bird species.

Here, we have to point out however that our study provides only partial support for the makeup hypothesis because we did not firmly demonstrate that a strong immune challenge affects the deposition of preen wax on bill. To do so, bills of

LPS- and PHA-injected nestlings should have been washed and a third measurement of bill brightness taken by the end of the third experiment. If true, the makeup hypothesis predicts that LPS- and PHA-challenged nestlings should not differ in bill brightness after bill cleaning. Here, we can thus not discard alternative hypotheses that can explain variation in bill brightness recorded during the third experiment. For instance, differential deposition and accumulation of melanin pigments within bill tissues is one possibility, although, to our knowledge, no study has still shown the presence of melanin in the bill of owls and no study has shown that immune challenges can affect the production of melanin bill pigments in birds. A second possibility is that LPS may have affected the microstructure of the bill, resulting in differential light reflection. But here again no study has yet addressed this issue.

In conclusion, although our study does not provide a direct causal link between an immune challenge and preen wax deposition rate, we nonetheless show in a bird species that immune status can be rapidly mirrored in bill coloration even in the absence of carotenoids. Given that most birds have an uropygial gland (Jacob and Ziswiler 1982), bill coloration can potentially signal immune status in most species. In addition, we propose, and provide evidence, that deposition of preen wax on the bill is a potential mediator of quick color changes. By affecting a bird's preening activity and/or uropygial gland growth, pathogens may limit deposition of gland secretions on bill and plumage. Because preen wax substantially decreases bill brightness, healthier individuals display less bright bills.

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