

Short Communication

Cosmetic plumage coloration by iron oxides does not confer protection against feather wear

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The active acquisition of plumage cosmetic coloration by iron oxides has been described in several bird species (notably the Bearded Vulture *Gypaetus barbatus*, but also in cranes, pelicans and other vulture species) but its function remains controversial. We tested the hypothesis that iron oxide acquired through mud bathing protects feathers from wear. We experimentally stained 13 breast feathers collected from two White Storks *Ciconia ciconia* with mud from a ferruginous pond regularly used by Bearded Vultures, while another 13 feathers (controls) were treated with water. All feathers were exposed to a controlled physical abrasion treatment. We found no differences in wear between control and stained feathers, which is counter to predictions of the wear protection hypothesis for the acquisition of this cosmetic plumage coloration.

Keywords: adventitious coloration, Bearded Vulture, *Gypaetus barbatus*, mud bathing, plumage protection.

Plumage coloration may play a variety of roles, including signalling, concealment and protection (Hill & McGraw 2006a). In most cases, plumage coloration is achieved either by the nanostructure of the feather or by the deposition of certain pigments (e.g. melanins, carotenoids, psitacofulvins) during feather growth (Hill & McGraw 2006b). However, some bird species can also acquire or modify their plumage colour by applying

certain endogenous or exogenous substances (cosmetic colorations) or as a result of a rather passive exposure to environmental factors (adventitious colorations) (Montgomerie 2006, Delhey *et al.* 2007).

The presence of cosmetic colorations has been described in at least 28 species belonging to 13 bird families (Delhey *et al.* 2007). Substances used by birds include soil, uropygial and skin secretions, or iron oxides (reviewed by Delhey *et al.* 2007; see also Piault *et al.* 2008, Amat *et al.* 2011, Soler *et al.* 2014, van Overveld *et al.* 2017). Iron oxides are often acquired by repeated bathing in mud ponds rich in this compound (Fe₂O₃). This cosmetic coloration has been described in the Bearded Vulture *Gypaetus barbatus*, Egyptian Vulture *Neophron percnopterus*, Sandhill Crane *Antigone canadensis*, Common Crane *Grus grus* and Great White Pelican *Pelecanus onocrotalus* (reviewed by Delhey *et al.* 2007). Similar stains have been described in other pelican species but their ferruginous origin has not been ascertained (Cramp & Simmons 1977). At least in the first three mentioned species, the acquisition of this cosmetic coloration has been shown to be deliberate: individuals meticulously distribute the iron oxide throughout feathers when visiting mud ponds rich in iron oxide (Nesbitt 1975, Frey & Roth-Callies 1994, van Overveld *et al.* 2017, Duchateau & Tellechea 2019).

Different hypotheses have been proposed to explain this behaviour and the potential function of iron oxide cosmetic coloration, especially addressing the case of the Bearded Vulture, which is the species where it has received more attention. These include:

- 1 signalling individual dominance status (Negro *et al.* 1999),
- 2 providing antiparasitic and antibacterial plumage protection (Brown & Bruton 1991, Frey & Roth-Callies 1994, Arlettaz *et al.* 2002),
- 3 providing antioxidants to developing embryos via transfer of iron oxides through the eggshell (Arlettaz *et al.* 2002),
- 4 providing a physical protection against feather abrasion (Brown & Bruton 1991).

The first three hypothesis have been empirically addressed to some extent (Frey & Roth-Callies 1994, van Overveld *et al.* 2017, Margalida *et al.* 2019). The fourth hypothesis, however, has not been experimentally tested. The species for which the use of iron oxides has been reported so far belong to different taxa and exploit different habitats and trophic niches. However, they all share the common feature of being large and long-living species. For these species, complete replacement of the feathers on each plumage tract usually takes several years (Zuberogoitia *et al.* 2008, Sesé-Franco 2019). Consequently, the use of cosmetic substances that may increase plumage strength and resistance to abrasion

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would be selectively advantageous, making this a plausible hypothesis.

In this study we tested the hypothesis that iron oxide cosmetic coloration acquired via mud bathing by several bird species has evolved as an adaptive strategy to protect feathers from wear. To do so, we exposed unpigmented White Stork *Ciconia ciconia* feathers to mud from natural bathing areas used by Bearded Vultures, and compared their resistance to abrasion with that of control (non-stained) feathers. Our prediction was that the feathers stained with iron oxides would have a higher resistance to wear than would the non-stained ones.

MATERIAL AND METHODS

Feather staining and colour quantification

For the experiment, 26 White Stork feathers were obtained from two dead individuals admitted at the 'El Chaparrillo' Wildlife Recovery Centre (Ciudad Real, Spain). Pairs of adjacent breast feathers were collected from the same bird and randomly allocated to the control or stained group. Feathers from the stained group were immersed for 3 min in mud from a pond frequently used by Bearded Vultures from Astún (Huesca, Spain) (J.A.G., pers. obs.). Once removed from the mud bath, feathers were dried and gently shaken to remove mud remains. Control feathers were handled in the same way but were treated with water instead of mud.

To verify the effectiveness of mud treatment to stain the feathers, digital images of every single feather were collected using a CanoScan 9000F Mark II (Canon, Japan) scanner. Images were collected against a black cardboard, including an X-Rite Mini card (ColorChecker; USA) as a reference. We normalized and linearized all feather images using the software SpotEgg (Gómez & Liñán-Cembrano 2017). To estimate colour intensity, we quantified feather saturation levels using Photoshop CS4. Colour was estimated in this way at three time points: before staining, after staining – but before starting the wear treatment-, and at the end of the experiment.

To verify that our experimental staining treatment resulted in a pigmentation comparable to what could be naturally acquired by birds, we used the Bearded Vulture as a reference. Thus, we relied on 53 feather samples from the neck, breast and belly collected from 11 different adult wild Bearded Vultures captured between 2011 and 2016 at different locations in the central Pyrenees (Aragón, Spain) as part of an official surveillance programme for this species. These feathers were scanned and their colour was quantified as described above.

Experimental abrasion treatment

We exposed control and stained feathers to a standard abrasion treatment by repeatedly passing them, from the shaft to the vane, through a narrow slit (30 mm wide by 1 mm high) lined with 1000-grit size sandpaper. Feathers were repeatedly exposed to this abrasion treatment, and we evaluated the degree of wear before and after staining treatment, and after they had passed through the slit 5, 15, 35, 50 and 70 times. This method was designed to mimic the physical effect of repeated friction with a rough surface (see Fig. S1).

Feather wear was analysed following Sæther *et al.* (1994) with minor modifications. Wear was scored only in 0.5-cm vane margins of the terminal 1.4 cm of each feather. This area was divided into four quadrants (terminal and sub-terminal portions of the left and right vane), which were delimited with a permanent marker to allow constant scoring areas throughout the experiment. At each sampling time, we scored the degree of wear at three different structural levels (barbs, barbules and hooklets) using a binocular zoom stereo microscope (Nikon SMZ800; Japan). A score of 0 to 3 was given for each level and quadrant depending on the degree of wear observed: 0 indicated no wear, and scores of 1, 2 and 3 indicated 10%, 10% and 50%, with more than 50% of structures of the corresponding level being damaged, respectively. Scores of the four quadrants of each feather and structural level were summed to obtain the degree of wear at each sampling point. This feather wear scoring method showed a high repeatability in a subset of samples scored in triplicate ($r = 0.92$, $F_{6,14} = 35.4$, $P < 0.001$).

Statistical analyses

We used R 3.5.0 for all analyses. Feather identity was entered in the models as a random effect using the lme4 statistical package (Bates *et al.* 2015). To assess the effect of stain treatment on feather coloration, stain treatment (control or stained) and sampling time (before, after staining and at the end of exposure to wear treatment), together with their interaction, were entered as fixed factors. To assess the effect of iron oxides on feather resistance to abrasion, we set up a second model with the same structure of random effects and feather wear score as a response variable. We entered stain treatment, sampling time and structural level (barbs, barbules or hooklets) as fixed terms, together with all two- and three-level interactions. We report results from the full model, as backward stepwise reduction did not change the results. Both feather saturation levels and wear score were log-transformed for the analyses when required to fulfil the requirements of normality and homoscedasticity of model residuals (i.e. in all cases for feather saturation, and in the model testing for initial

differences among groups in wear score). Values reported are means \pm sd.

RESULTS

Before starting the experiment, feathers assigned to each staining treatment did not differ either on initial colour saturation (treatment: $F_{1, 24} = 0.25$, $P = 0.62$) (Fig. 1a) or in wear score at any structural level (structural level \times treatment: $F_{2, 72} = 0.58$, $P = 0.56$; treatment: $F_{1, 72} = 1.27$, $P = 0.26$).

Treatment with mud rich in iron oxide created the expected differences in feather colour among groups (time \times treatment: $F_{1, 24} = 373.2$, $P < 0.001$). Although the colour saturation of stained feathers faded slightly during wear treatment (from 63.3 ± 6.5 to 58.7 ± 5.5 , $F_{1, 12} = 53.0$, $P < 0.001$), the striking colour difference between experimental groups remained until the end of the study (treatment: $F_{1, 24} = 301.8$, $P < 0.001$). Colour saturation of stained feathers was within the range of naturally stained feathers collected from wild Bearded Vultures (Fig. 1a,b).

Exposure to feather abrasion treatment successfully induced a gradual increase in feather wear at all three structural levels of analysis (barbs, barbules and hooklets; Fig. 2). However, stain treatment did not affect the rate of feather wear at any of these levels (Table 1). Non-significant trends of the interaction between structural level and exposure to abrasion reflected slightly different responses of feather structures to abrasion (more linear increases in barb wear vs. rather curvilinear increases in barbules and hooklets as a result of abrasion). Similarly, the marginal effect of the interaction between stain treatment and structural level reflected that control feathers tended to show on average (irrespective of exposure to abrasion) slightly lower wear levels than did stained feathers (control: 3.0 ± 2.81 ; stained: 3.6 ± 2.73 ; Fig. 2c). The effects of the interaction between stain treatment and exposure to abrasion on feather wear remain unchanged if the triple interaction was removed from the model ($F_{6, 492} = 301.8$, $P = 0.09$).

DISCUSSION

Our experimental results indicate that iron oxide staining does not confer a significant protection to feathers against physical abrasion. Although we applied a gradual abrasion treatment and evaluated feather wear at different structural levels (barbs, barbules and hooklets), the wear scores of stained and control feathers did not differ. This is opposed to the hypothesis that the behaviour of mud bathing in ferruginous ponds displayed by several vulture, crane and pelican species has evolved as a protective strategy against feather wear (Brown & Bruton 1991).

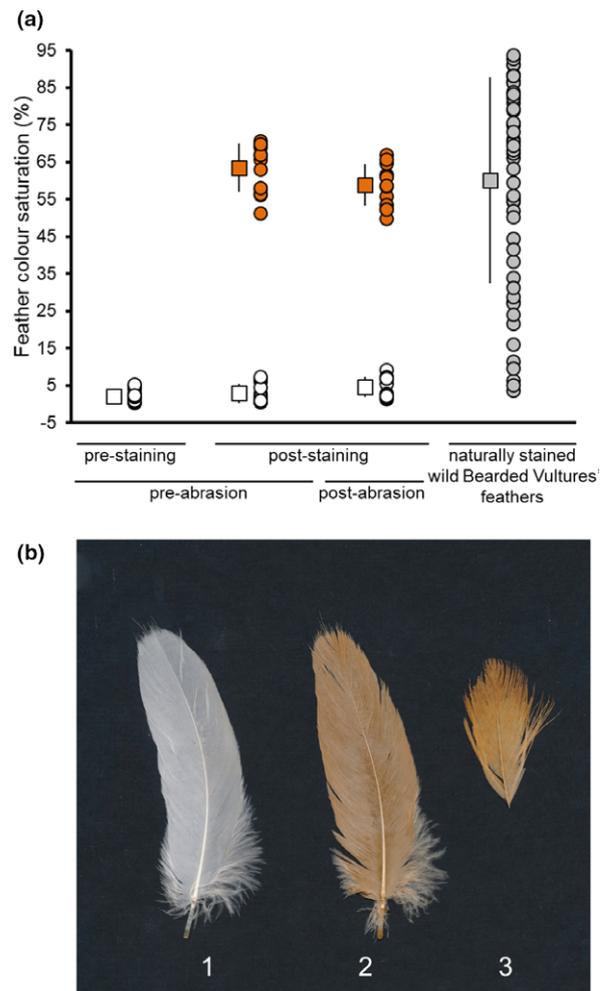


Figure 1. (a) Effect of stain treatments with mud rich in iron oxides (orange symbols) or water (open symbols) on the saturation of White Stork feathers throughout the experiment. Colour measurements were performed before the experiment started (pre-staining) and twice after staining treatment – before applying the abrasion treatment and at the very end of it. Colour saturation values of naturally stained ventral feathers from wild Bearded Vultures are also displayed (grey symbols) for comparison with experimental feathers. Squares indicate means \pm sd. Note that in the pre-staining values, mud-treated feathers are not visible because they are overlaid by control dots. Similarly, sd bars are not visible in that sampling point because they are too small. (b) Illustrative image of control (1) and stained (2) White Stork feathers, together with a naturally pigmented Bearded Vulture belly feather tip (3) to allow comparison of how our treatment matched the natural cosmetic coloration achieved by this species. [Colour figure can be viewed at wileyonlinelibrary.com]

In this experiment we used White Stork feathers under the assumption that their resistance to wear – and the effect of iron oxides on this – would be comparable

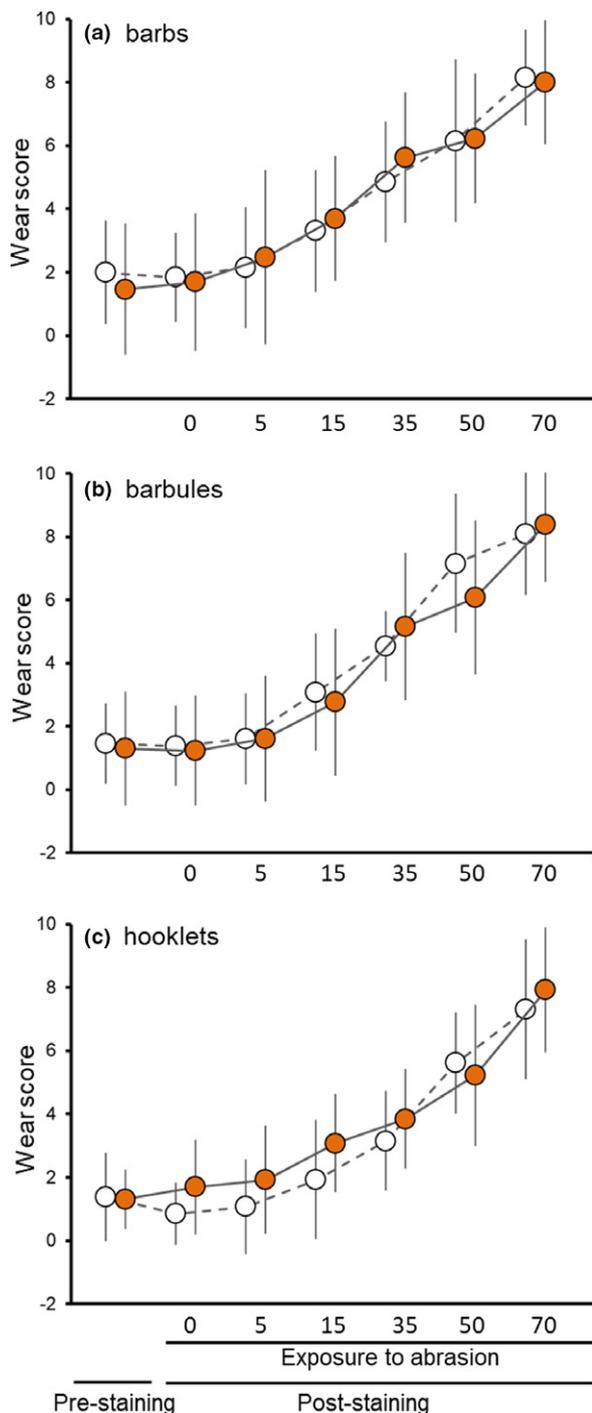


Figure 2. Changes in wear score of White Stork feathers as a result of exposure to different degrees of abrasion according to staining treatment with mud rich in iron oxide at the structural level of (a) barbs, (b) barbules and (c) hooklets. Open symbols and dashed lines correspond to control feathers, whereas orange symbols and solid lines correspond to stained feathers. Values are means \pm sd. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 1. General linear mixed model testing the effect of stain treatment (control vs. stained with mud rich in iron oxide) and exposure to abrasion (number of passes on a sandpaper) on feather wear measured at three different structural levels (barbs, barbules and hooklets). Feather identity was entered as a random effect affecting the intercept of the model. We report the full model, as backward stepwise reduction does not change the results.

Effect	F	df	P
Stain treatment (Trt)	0.107	1, 24	0.74
Exposure to abrasion (Abr)	291.5	6, 480	<0.001
Structural level (Lvl)	17.9	2, 480	<0.001
Trt \times Abr	1.82	6, 640	0.09
Trt \times Lvl	2.79	2, 480	0.06
Abr \times Lvl	1.70	12, 480	0.06
Trt \times Abr \times Lvl	0.54	12, 480	0.88

to that of other species where this type of cosmetic coloration has been described. White Stork feathers showed remarkable susceptibility to iron oxide staining when immersed in ferruginous mud for a few minutes, and indeed resulted in a final colour comparable to that achieved by wild Bearded Vultures (Fig. 1). It seems therefore that this initial assumption does not compromise the conclusions of this experimental study. Finally, it is remarkable that our result matches observations reported by Frey and Roth-Callies (1994), who found no significant differences in wear between naturally stained and non-stained feathers of Bearded Vultures.

Previous studies have tested other hypotheses aimed at explaining the function of this type of cosmetic coloration, mostly focused on Bearded Vultures. The antiparasitic and antibacterial functions have been experimentally tested and rejected by Frey and Roth-Callies (1994) and Margalida *et al.* (2019), who demonstrated no protective effect of iron oxides against mallophaga and feather-degrading bacteria, respectively. In turn, the hypothesis of a protective antioxidant effect for the developing embryo via transfer of iron oxides through the eggshell (Arlettaz *et al.* 2002) has not been formally tested to date. However, there is no evidence that clutches incubated by intensely coloured individuals show higher hatching or fledging success compared with that of paler birds (Margalida *et al.* 2019). In addition, this hypothesis would require the transfer of iron oxides through the eggshell, or through the chick's skin, and it has yet to be shown that such transfer is physiologically possible. This leaves the hypothesis of a territorial-status signalling role as the most plausible hypothesis for the occurrence of cosmetic coloration in this species (Negro *et al.* 1999).

In conclusion, current empirical evidence does not support any of the protective functions (against ectoparasites, bacteria, oxidative stress of feather wear) proposed for cosmetic plumage colorations based on iron oxides. Rather, the seasonal, sexual and age-related patterns of expression of this kind of cosmetic plumage

coloration in different species points towards a visual function, either camouflage or sexual signalling (reviewed by Delhey *et al.* 2007). In those cases where a sexual signalling role has been proposed, empirical supportive evidence is mostly observational, which leaves the signalling function still hypothetical (Negro *et al.* 1999, van Overveld *et al.* 2017). Experimental studies are therefore required in these species to confirm that the cosmetic use of iron oxides in the plumage plays an actual signalling role (i.e. influence conspecifics' behaviour) and, if so, what information is conveyed by this coloration. These studies are essential to understand the function and evolution of this type of cosmetic plumage coloration in birds.

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AUTHOR CONTRIBUTION

Raquel Crespo-Ginés: Data curation (equal); Methodology (equal); Writing-original draft (equal). **Juan Antonio Gil:** Data curation (supporting); Methodology (supporting); Resources (supporting). **Lorenzo Pérez-Rodríguez:** Conceptualization (lead); Funding acquisition (lead); Investigation (equal); Project administration (lead); Supervision (lead); Validation (lead); Writing-original draft (equal).

Data Availability Statement

The data that support the findings of this study are openly available as online Supporting Information.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Illustrative image of control unmanipulated feathers (left) and after passing 70 times through the slit covered with sandpaper (right).

Appendix S1. Data set 'data_colour' corresponding to the colour scores of the feathers depicted in Figure 1(a).

Appendix S2. Data set 'data_wear' corresponding to the wear scores of the feathers across the experiment.

Appendix S3. Explanation of the variables included in Appendices S2 and S1.