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Structural color change following hydration and dehydration of iridescent mourning dove (*Zenaida macroura*) feathers

Matthew D. Shawkey[a,*], Liliana D'Alba[a], Joel Wozny[a], Chad Eliason[a], Jennifer A.H. Koop[b], Li Jia[c]

[a] Department of Biology and Integrated Bioscience Program, University of Akron, Akron, OH 44325-3908, USA
[b] Department of Biology, University of Utah, Salt Lake City, UT 84112, USA
[c] Department of Polymer Science, University of Akron, Akron, OH 44325, USA

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ABSTRACT

Dynamic changes in integumentary color occur in cases as diverse as the neurologically controlled iridiphores of cephalopod skin and the humidity-responsive cuticles of longhorn beetles. By contrast, feather colors are generally assumed to be relatively static, changing by small amounts only over periods of months. However, this assumption has rarely been tested even though structural colors of feathers are produced by ordered nanostructures that are analogous to those in the aforementioned dynamic systems. Feathers are neither innervated nor vascularized and therefore any color change must be caused by external stimuli. Thus, we here explore how feathers of iridescent mourning doves *Zenaida macroura* respond to a simple stimulus: addition and evaporation of water. After three rounds of experimental wetting and subsequent evaporation, iridescent feather color changed hue, became more chromatic and increased in overall reflectance by almost 50%. To understand the mechanistic basis of this change, we used electron microscopy to examine macro- and nanostructures before and after treatment. Transmission electron microscopy and transfer matrix thin-film models revealed that color is produced by thin-film interference from a single (~335 nm) layer of keratin around the edge of feather barbules, beneath which lies a layer of air and melanosomes. After treatment, the most striking morphological difference was a twisting of colored barbules that exposed more of their surface area for reflection, explaining the observed increase in brightness. These results suggest that some plumage colors may be more malleable than previously thought, leading to new avenues for research on dynamic plumage color.

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1. Introduction

One important function of the metazoan integument is the display of color for crypsis or communication. While in many cases this color is static, changing only with age or environmental damage, in others it changes dynamically in response to background coloration (e.g., the skin of cephalopods; Hanlon, 2007), breeding condition (e.g., the skin of the toad *Bufo luetkenii*; Doucet and Mennill, 2009), excitation state (e.g., wattles of wild turkeys *Meleagris gallopavo*; Eaton, 1992), or in response to abiotic factors (e.g., to humidity in the beetle *Tmesisternus isabellae*; Liu et al., 2009).

The colors of feathers, while extremely diverse and frequently vivid, are thought to be largely static (Andersson and Prager, 2006). Feathers are composed of dead keratin tissue with no innervation or vascularization, and therefore cannot be under direct control of the animal or linked to other physiological processes. However, many of them contain highly organized tissues, or biophotonic nanostructures, that produce color not through pigments but through coherent light scattering (Prum, 2006). Because the refractive index, nanoscale size and arrangement of these nanostructures determine the colors they produce, they may be subject to change by external factors. For example, the replacement of air (refractive index (RI) = 1.00) by water molecules (RI = 1.33) in the biophotonic nanostructures of *Morpho* butterfly scales under high vapor pressure changes their refractive index contrast and hence color (Potyrailo et al., 2007). A few studies have shown that structural plumage colors can substantially change over longer periods of time from days to weeks (Montgomerie et al., 2001; Örnborg et al., 2002; Barreira et al., 2007; Shawkey et al., 2007; Delhey et al., 2010), and while in some cases "cosmetics" like colored preen oil may effect rapid changes (reviewed in Montgomerie, 2006), whether nanostructures can rapidly respond to simple environmental variables is largely unknown (but see Eliason and Shawkey, 2010).

Non-iridescent structural feather colors are typically created by matrices of keratin and air forming a single medullary layer (termed a "spongy layer") within feather *barbs*, while iridescent colors are typically created by stacks of melanin granules within a keratin

* Corresponding author.
E-mail address: shawkey@uakron.edu (M.D. Shawkey).

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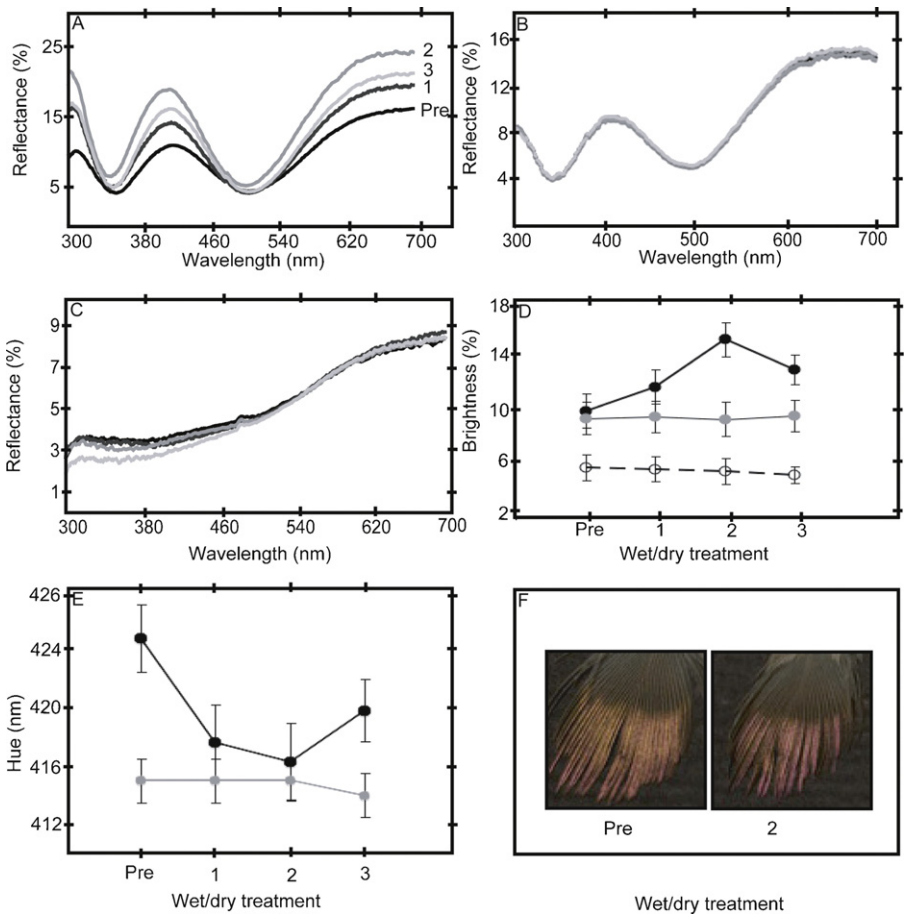

Fig. 1. Effects of experimental rounds of wetting and drying and sham treatments on color of mourning dove feathers. (A–C) Average reflectance spectra ($n = 12$) before wet/dry treatment (Pre) and after each of the three wet/dry treatments (1–3) for (A) iridescent feathers treated with water (STACK1), (B) iridescent feathers treated with dry shams (STACKCONTROL), and (C) non-iridescent brown feathers treated with water (NONIRID). (D and E) Means (±1 SE; $n = 12$) showing change in brightness (D) and hue (E) with each wetting and drying treatment. Black solid lines represent data from iridescent feathers treated with water, grey lines represent data from sham-treated iridescent feathers and the dashed line represents non-iridescent feathers treated with water. Reflectance of non-iridescent feathers continually increased into the red region of the spectrum, making all hue values equal to 700 nm. Thus, hue and chroma (which is calculated based on hue) as we measured them were not meaningful and were not included in the analyses. (F) Picture of individual iridescent feathers before wet/dry treatment and after the 2nd wet/dry treatment, when brightness was highest.

substrate in feather *barbules* (Prum, 2006). One of the simplest such structures is a single layer of melanin granules or air surrounded by an even keratin cortex that can produce a broad range of colors by thin-film interference (Prum, 2006). The color of these structures is largely determined by the thickness of the keratin cortex (Brink and van der Berg, 2004; Doucet et al., 2006; Shawkey et al., 2006; Yin et al., 2006; Nakamura et al., 2007). Since by definition iridescent colors change with the angle of incidence, the angle of barbules relative to incident light also affects color (Yoshioka and Kinoshita, 2002; Nakamura et al., 2007; Yoshioka et al., 2007). Because avian keratin is sensitive to hydration (Bonser, 2002), we hypothesized that wetting and drying of feathers may shrink or swell the keratin matrix of the barbules, changing their color through (i) alteration of the thickness of the outer keratin layer and/or (ii) twisting of barbules.

To test these hypotheses, we used iridescent neck feathers from mourning doves (*Zenaida macroura* Linnaeus 1758). These feathers appear pink/violet to the human eye and contrast with the brown feathers comprising the majority of the species' plumage (Otis et al., 2008). We experimentally tested the response of reflected color (as measured by a UV–vis spectrometer) of these feathers to repeated rounds of wetting and drying. To understand the mechanistic basis of any changes, we used scanning and transmission electron microscopy (SEM and TEM) and thin-film optical modeling to first determine the morphological and physical basis of color production and then identify changes in this morphology after treatment.

2. Materials and methods

We pulled adjacent violet (iridescent) and brown (non-iridescent) captive-grown neck feathers from 24 different birds in an indoor captive population of mourning doves at the University of Utah. Because they were housed in indoor cages for the entire period of growth and afterwards, these feathers had only been exposed to small amounts of water. All feathers were stored in a climate-controlled room in opaque paper bags until analysis.

2.1. Spectrometry

We taped feathers in stacks of five directly on top of one another to gloss-free black construction paper. We recorded spectral data from the colored distal portion of these feather stacks using an AvaSpec 2048 spectrometer (range 250–880 nm; Avantes Inc., Broomfield, CO, USA) in a dark room. We initially collected color data at normal incidence (0° incident light/0° measurement) using a bifurcated micron fiber optic probe held by a probe holder (RPH-1; Avantes) with matte black interior that excluded ambient light. This geometry was used for all color measurements in the wetting experiments.

To investigate the influence of measurement geometry on reflectance, and the ability of thin-film modeling to predict this changing reflectance (see Section 2.4 below), we re-measured the iridescent feathers of a single individual using separate fiber optic cables for transmitting incident and reflected light. Using an angled fiber holder (AFH-15; Avantes), we measured the reflectance of feathers at matching incident and reflected light angles of 15°, 30°, 45°, 60° and 75°. In all cases, the reading area was illuminated by a pulsed UV–vis unpolarized light source (AvaLight-XE; Avantes), and all data were generated relative to a white standard (WS-2; Avantes). We used AvaSoft software (Avantes) to record measurements from five haphazardly chosen points on each sample. Each measurement was the average of 20 recordings taken in rapid succession.

From the resulting reflectance spectra, we calculated color variables for each sample using CLR version 1.03 (Montgomerie, 2008) and macros in Excel. Brightness, the mean reflectance of each 1 nm wavelength interval from 300 to 700 nm, is a measure of overall reflectance of the feathers (Montgomerie, 2008). Reflectance spectra of iridescent feathers had one peak near 400 nm and a plateau beginning at around 650 nm, so we calculated hue (wavelength of peak reflectance) as wavelength of maximum reflectance of the lower-wavelength peak because it was more clearly defined in all spectral curves. We calculated chroma, an index of color purity, as the reflectance within a 50 nm interval of wavelengths (hue ± 25 nm) divided by total reflectance (brightness) (Montgomerie, 2008). Reflectance of non-iridescent feathers continually increased into the red region of the spectrum, making all hue values equal to 700 nm (see Fig. 1C). Thus, hue and chroma (which is calculated based on hue) as we measured them were not meaningful and were not included in analyses.

2.2. Experimental wetting and drying

To determine whether repeated exposure to water affects color and nanostructure of iridescent feathers, we wetted feathers and allowed them to dry. Using forceps, we gently removed feathers from the construction paper and tape and placed each one in a separate 15 ml plastic tube (Falcon, San Jose, CA, USA) containing 10 ml of distilled water. Feathers were immersed in the water for approximately 5 min. Then, we removed the feathers, placed them in separate open Petri dishes and allowed them to dry overnight (approximately 14 h) before re-taping them in the same order in the stack and measuring them as above. This entire process of wetting, drying and measuring color was performed a total of three times.

To ensure that our results were repeatable, we repeated the entire experiment using different iridescent feathers from the same birds. To examine changes in gross barbule morphology and how these might relate to color change (see Section 2.3), we then repeated the same experimental procedure above using single feathers, rather than stacks of feathers, from the same birds. We used single feathers both because of the simplicity of relating color to structure of one feather and because we reasoned that any additional information gained from using multiple feathers did not justify the time and cost of analyzing them with the scanning electron microscope (see Section 2.3). We then repeated this experiment, without measuring color, to ensure that our morphological results were repeatable. As controls against the effects of removing and re-taping feathers, we performed the experiment without water on 10 stacks of iridescent feathers, and then on 5 single feathers. For comparison with a non-structurally colored feather, we performed this experiment once on stacks of non-iridescent brown mourning dove feathers.

In summary, we performed this experimental procedure a total of seven times (each one referred to henceforth by the codes in parentheses): on stacks of iridescent feathers to identify color and nanostructural changes (STACK1), on stacks of iridescent feathers to verify color changes (STACK2), on stacks of iridescent feathers without using water as a negative control (STACKCONTROL), on single iridescent feathers to identify color and macrostructural changes (SINGLE1), on single feathers to verify macrostructural changes (SINGLE2), on single feathers without water as a negative control (SINGLECONTROL), and on non-iridescent feathers (NONIRID).

2.3. Morphological basis of color change

To identify changes in nanostructure potentially contributing to color change, we sampled barbules from experiment STACK1. We cut 5 barbs containing iridescent barbules from random locations on the experimental feathers before treatment and after the second treatment, and imaged them using transmission electron microscopy (TEM) following the methods of Shawkey et al. (2006). Identical methodology was used for the microscopy of feathers both before and after experimental treatment. Briefly, we trimmed iridescent barbs and barbules, dehydrated them using 100% ethanol (20 min) twice, infiltrated them with 15, 50, 70 and 100% Epon (24 h each step; Electron Microscopy Sciences, Hatfield, PA, USA) and cut thin sections on a UC-6 ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany). We viewed unstained thin sections on a Tecnai 12 TEM (60 kV; FEI Company, Hillsboro, OR, USA). We used ImageJ (available for download at http://rsb.info.nih.gov/nih-image/index.html) to measure the thickness of the keratin cortex and the thickness of the outer air layer at six evenly spaced points surrounding each barbule. These variables are likely critical to color production because they form the layers used in thin-film interference (Nakamura et al., 2007; Yoshioka et al., 2007). In some barbules, melanin was found in contact with the cortex while in others it was found further away. One study (Doucet et al., 2006) has shown that a higher percentage of melanin around the edge of barbules increases chroma so we used a threshold procedure in ImageJ to measure the proportion of melanin in each barb, and the proportion of that melanin in the outer layer.

To identify changes in gross barbule morphology potentially contributing to color change, we examined whole feathers from experiments SINGLE1 and SINGLE2 before treatment and after the second treatment using scanning electron microscopy (SEM). We mounted individual feathers to aluminum stubs with carbon tape and obtained images using a JEOL JSM-7301F field emission SEM (5 kV; JEOL Ltd., Tokyo, Japan). We only obtained images of barbs from the iridescent portion of the feather and, to control for variation in barbule morphology along the barb ramus, only took pictures of barbules located near the midpoint of each barb. After obtaining images, we removed the feathers from the stubs using forceps and stored them in capped 1.7 ml microcentrifuge tubes until further analysis. We measured between 5 and 19 barbules (depending on the number of visible barbules) from the same three barbs on each feather both pre- and post-treatment. Using ImageJ software, we measured the length, exposed area, and angle from the barb ramus (which may affect both overall reflectance and hue; see Greenewalt et al., 1960; Osorio and Ham, 2002) for each barbule. The exposed area was defined as the area of the barbule not obscured by neighboring barbules on the same barb. Thus, if a barbule was obscured by barbules on an adjacent barb, we did not record the exposed area. We also noted the presence of dirt or amorphous materials that could potentially affect color.

2.4. Thin-film optical modeling

To determine how iridescent color is produced in these feathers, we used standard transfer matrix thin-film optical models (Jellison, 1993) implemented in the statistical program R (R Development

Core Team, 2009) using the *slmodels* command in the *thinfilms* package (Maia et al., 2009). We used previously published, empirically estimated refractive indices of air ($n = 1.00$) and keratin ($n = 1.56$) (Land, 1972; Brink and van der Berg, 2004), the estimated lower limit extinction coefficient for keratin ($k = 0.03$) (Brink and van der Berg, 2004), and angles of incidence and reflectance matching those of our measured spectra in all of our calculations. We created a set of thin-film reflectance models, using all possible two- and three-beam combinations for the upper surface of the barbule. These models have been outlined numerous times (Brink and van der Berg, 2004; Doucet et al., 2006; Shawkey et al., 2006; Maia et al., 2009). Model 1 included all three interfaces of materials of different refractive indices (air/keratin, keratin/air layer, air layer/keratin) and the thicknesses of the keratin and air layers. Model 2 included only the outer two interfaces (air/keratin, keratin/air) and the thickness of the keratin layer. Model 3 included only the air/keratin and air layer/keratin interfaces, and the thickness of the air layer. Model 4 included only the inner two interfaces (keratin/air layer, air layer/keratin) and the thickness of the melanin layer. We visually compared the spectra produced by these models to measured reflectance spectra from the feathers. For the model with the best predictive ability, we compared hues generated by the model to measured hues of individual birds.

2.5. Statistical analyses

To determine if our experimental wetting and drying treatments significantly affected color, we used separate repeated measures ANOVA (Zar, 1996) to compare color variables among treatments. We used Tukey's post-hoc tests to compare changes between individual wetting and drying treatments. To identify the nanostructural basis of any color changes, we first identified the variables that significantly contributed to variation in color using linear mixed models. In separate analyses, hue, chroma and brightness were dependent variables while mean cortex and air layer thickness and percentage of melanin in the barbule both overall and in the outer layer of melanin were covariates, treatment was a fixed factor and sample ID was a random factor. In each case, we tested for interactions of morphological variables with treatment. To identify the gross morphological basis of any color changes, we used the same models with hue, chroma and brightness as independent variables and barbule angle and surface area as independent variables. We then tested for changes in these morphological variables with treatment using paired *t*-tests. All analyses were performed in PASW Statistics 18.00 (SPSS Inc., Chicago, IL, USA).

3. Results

At normal incidence, reflectance spectra of iridescent feathers were characterized by decreasing reflectance at short wavelengths and by a single peak whose wavelength (~420 nm, see Table 1) we classify as hue and a short plateau at longer wavelengths (Fig. 1). In experiments STACK1 and STACK2, the color of iridescent feathers significantly changed following experimental rounds of wetting and drying (Table 1, Fig. 1A, D–F). In experiment STACK1, hue significantly decreased (Table 1, Fig. 1A and E), while brightness and chroma significantly increased (Table 1, Fig. 1A and D). In experiment STACK2, hue significantly increased, and brightness and chroma also significantly increased (Table 1). In experiment SINGLE1, hue did not change, but brightness and chroma again significantly increased (Table 1).

Although many individual changes were not statistically significant, all experiment groups showed a qualitatively similar pattern of color change in one direction for the first two wet/dry treatments followed by change in the opposite direction after the third treatment (Table 1). Color of feathers in the control group STACKCONTROL did not significantly change throughout the course of the experiment (all $p > 0.5$, Table 1, Fig. 1B). Brightness and spectral patterns of non-iridescent feathers in the experiment NONIRID remained constant throughout (Table 1, Fig. 1C). Because changes in iridescent feathers were visible to human eyes (see Fig. 1F), we assumed that they are visible to birds (which have superior vision to humans; e.g. Cuthill et al., 1999) as well.

Table 1
Color variables (mean ± 1 S.E.M.) measured from iridescent and non-iridescent mourning dove barbules before treatment and after each of three experimental rounds of wetting and drying. Reflectance of non-iridescent feathers continually increased into the red region of the spectrum, making all hue values equal to 700 nm. Thus, hue and chroma (which is calculated based on hue) as we measured them were not meaningful and were not included in analyses. *F* and *p* values from repeated measures ANOVA are presented. Letters next to means indicate those that were not significantly different ($p < 0.05$) in Tukey's post-hoc tests. $n = 12$ in all cases.

Pre/post treatment	Hue	Chroma	Brightness
Iridescence experiment STACK1			
Pre	422.7 ± 2.3	13.4 ± 0.7^{a}	9.8 ± 1.0
Post 1	415.6 ± 2.6^{a}	$14.4 \pm 0.7^{a,b}$	11.6 ± 1.0^{a}
Post 2	414.3 ± 2.6^{a}	15.4 ± 0.3^{b}	15.1 ± 1.0^{b}
Post 3	417.8 ± 2.1^{a}	15.3 ± 0.6^{b}	$12.9 \pm 0.6^{a,b}$
$F_{3,9}$, p	7.8, 0.007	6.2, 0.01	8.2, 0.006
Iridescence experiment STACK2			
Pre	417.1 ± 3.0	13.3 ± 0.3^{a}	17.4 ± 1.2
Post 1	420.8 ± 3.4^{a}	$14.3 \pm 0.3^{a,b}$	22.2 ± 2.1^{a}
Post 2	422.0 ± 2.5^{a}	14.7 ± 0.3^{b}	22.8 ± 1.9^{a}
Post 3	420.7 ± 2.8^{a}	$14.4 \pm 0.4^{a,b}$	20.8 ± 2.0^{a}
$F_{3,9}$, p	4.8, 0.03	6.2, 0.01	8.2, 0.006
Negative control STACKCONTROL			
Pre	413.2 ± 1.5^{a}	13.1 ± 0.2^{a}	9.2 ± 1.2^{a}
Post 1	413.1 ± 1.4^{a}	13.3 ± 0.2^{a}	9.4 ± 1.2^{a}
Post 2	413.1 ± 1.5^{a}	13.4 ± 0.3^{a}	9.2 ± 1.3^{a}
Post 3	412.0 ± 1.5^{a}	13.4 ± 0.4^{a}	9.4 ± 1.4^{a}
$F_{3,9}$, p	1.0, 0.50	0.20, 0.90	0.30, 0.71
Iridescence experiment SINGLE1			
Pre	427.4 ± 5.2^{a}	12.7 ± 0.4	13.1 ± 0.5
Post 1	426.0 ± 5.6^{a}	13.6 ± 0.3^{a}	17.6 ± 1.2^{a}
Post 2	428.4 ± 5.2^{a}	13.8 ± 0.3^{a}	17.2 ± 1.2^{a}
Post 3	427.5 ± 2.6^{a}	13.9 ± 0.4^{a}	16.8 ± 1.4^{a}
$F_{3,9}$, p	0.08, 0.78	3.1, 0.04	10.13, <0.001
Non-iridescence experiment NONIRID			
Pre	n/a	n/a	5.49 ± 1.0^{a}
Post 1	n/a	n/a	5.39 ± 1.0^{a}
Post 2	n/a	n/a	5.23 ± 1.0^{a}
Post 3	n/a	n/a	4.96 ± 0.6^{a}
$F_{3,9}$, p	n/a	n/a	0.33, 0.79

3.1. Morphology

The iridescent pink/violet color of mourning dove feathers (Fig. 2A) is produced by flattened and elongated barbules that are twisted at the base so that the flat surface lies in plane with the barb (Fig. 2B). The nanostructure of iridescent mourning dove feathers consists of a thick (~330 nm, see Table 2), regular keratin cortex above a thicker (~500 nm but highly variable, see Table 2) layer of air with a variable number of melanosomes in contact with the

Table 2
Nanostructural variables (mean ± 1 S.E.M.) measured from iridescent mourning dove barbules before treatment and after two experimental rounds of wetting and drying. Melanin/barbule is the percentage of the total cross-sectional barbule surface area composed of melanin. Melanin in outer layer is the percentage of the total cross-sectional surface area of melanin that is in contact with the outer keratin cortex. *t* and *p* values from paired *t*-tests are presented. $n = 12$ in all cases.

	Pre-treatment	Post-treatment	t	p
Cortex thickness (nm)	343.5 ± 9.9	326.6 ± 1.4	−1.7	0.12
Air layer thickness (nm)	462.5 ± 47.3	646.2 ± 65.9	3.4	<0.01
Melanin/barbule (%)	17.3 ± 3.0	17.7 ± 2.1	0.22	0.83
Melanin in outer layer (%)	25.3 ± 4.9	36.9 ± 5.2	3.11	0.01

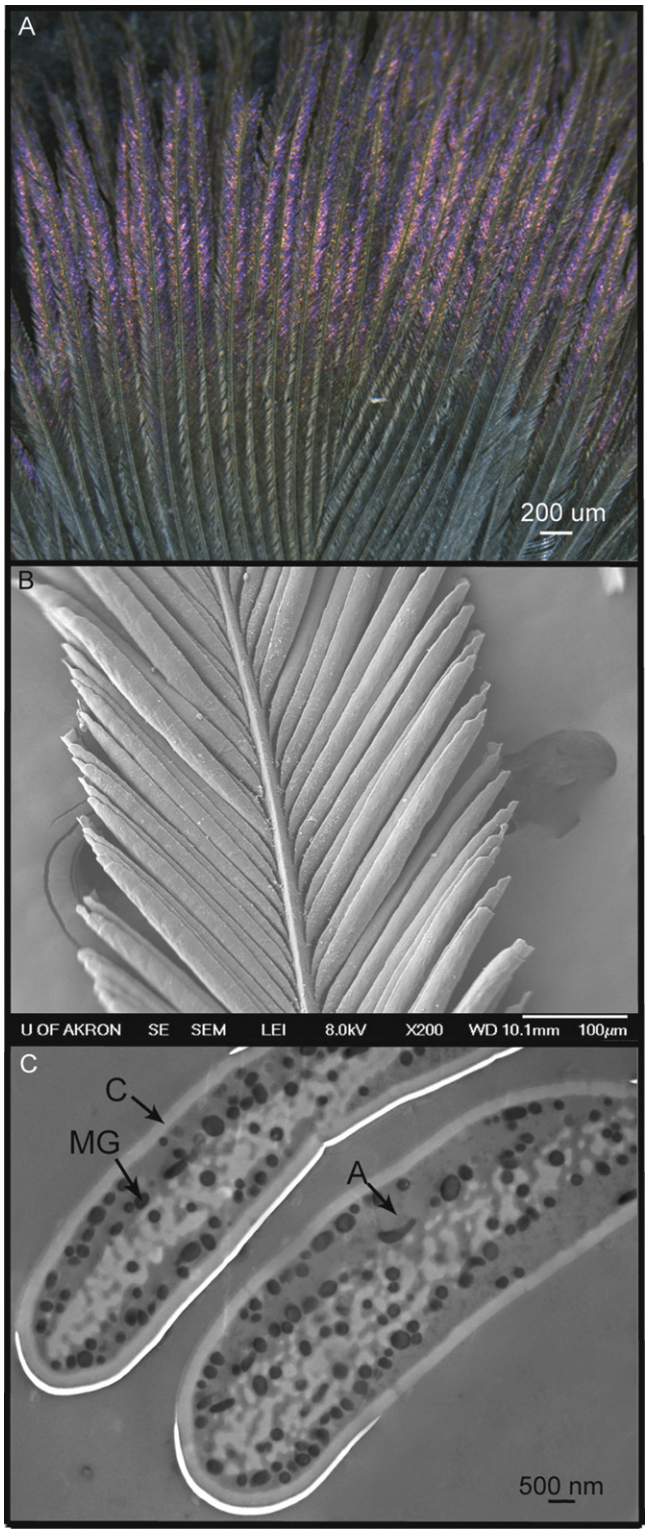

Fig. 2. (A) Optical microscope image of an iridescent mourning dove neck feather. The distal portion of the feather is iridescent pink but the basal portion is matte grey. The iridescent color is seen in the barbules, but not the barbs. (B) Scanning electron micrograph of a barb from an iridescent feather, showing flattened barbules that are twisted at their base such that they are in plane with the barb. (C) Transmission electron micrograph of a cross section of an iridescent barbule. Abbreviations: A = air, C = keratin cortex, MG = melanin granules. Air is dark in this image because the section was not counter-stained.

outer cortex and below it (Fig. 2C). This basic structure is similar to that of iridescent barbules from closely related rock doves (*Columba livia*; Nakamura et al., 2007) and another dove in the same genus (*Zenaida asiatica*; Durrer, 1977).

3.2. Spectrometry and optical modeling

Thin-film model 2, which only considers scattering at the air/keratin and keratin/air interfaces, predicted spectral curves closely matching measured curves (Fig. 3A). While the fit was not perfect, feathers are complex structures that vary in the alignment of reflecting surfaces (i.e., barbules) as well as in the uniformity of layers, and the predicted hues matched the observed values well (Fig. 3B). Predicted spectra from models 1, 3 and 4 bore some similarity to measured spectra, but were less accurate than model 2 (Fig. 3C). Thus, thickness of the outer keratin layer seems the most critical factor to the production of spectral patterns. Measured and predicted hues of feathers were highly and significantly correlated (Fig. 3B; $n = 24$, $r = 0.61$, $p = 0.001$), and this held true both before treatment ($n = 12$, $r = 0.58$, $p = 0.04$) and after the second wet/dry treatment ($n = 12$, $r = 0.68$, $p = 0.02$). The mean difference between measured and predicted hues was 5.5 ± 5.8 nm, and ranged from 0 to 70 nm.

As expected, the color of iridescent feathers changed with the angle of light incidence. Reflectance spectra shifted towards the UV with increasing obliqueness of incident light and, above 30°, the plateau observed at longer wavelengths became a distinct secondary peak (Fig. 4). These changes were qualitatively predicted by model 2 (Fig. 4).

3.3. Nanostructural predictors of color

In experiment STACK1, there were no significant interactions of morphological variables with treatment in any analysis (all $p > 0.09$), so we removed those interaction terms from final analyses. Thickness of the outer cortex significantly positively predicted hue of iridescent feathers (linear mixed model $F_{1,14.73} = 5.52$, $p = 0.03$; Fig. 5A), although when considered separately this was only true after treatment (before treatment: Pearson $r = 0.37$, $p = 0.12$; after treatment: $r = 0.69$, $p = 0.01$; $n = 12$ in both cases). No variable significantly predicted chroma (all $p > 0.11$). The percentage of melanin in the outer layer significantly positively predicted brightness ($F_{1,14.87} = 4.97$, $p = 0.04$; Fig. 5B), although when considered separately this was not true (before treatment: Pearson $r = 0.24$, $p = 0.48$; after treatment: $r = 0.35$, $p = 0.20$; $n = 12$ in both cases). No other nanostructural variables significantly predicted any color variable (all $p > 0.20$).

3.4. Macrostructural predictors of color

In experiment SINGLE1, there were no significant interactions of morphological variables with treatment (all $p > 0.18$), so we removed those interaction terms from final analyses. Surface area of exposed barbules positively predicted brightness ($F_{1,17.99} = 9.72$, $p < 0.01$; Fig. 6A), although when considered separately this was only true after treatment (before treatment: Pearson $r = 0.20$, $p = 0.52$; after treatment: $r = 0.70$, $p = 0.02$; $n = 12$ in both cases). Angle of barbule was not correlated with any color variable (all $p > 0.10$).

3.5. Changes in structure with treatment

In experiment STACK1, the thickness of the air layers in barbules significantly increased following treatment (Fig. 5C, Table 2), as did the percentage of melanin in the outer layer (Fig. 5D, Table 2).

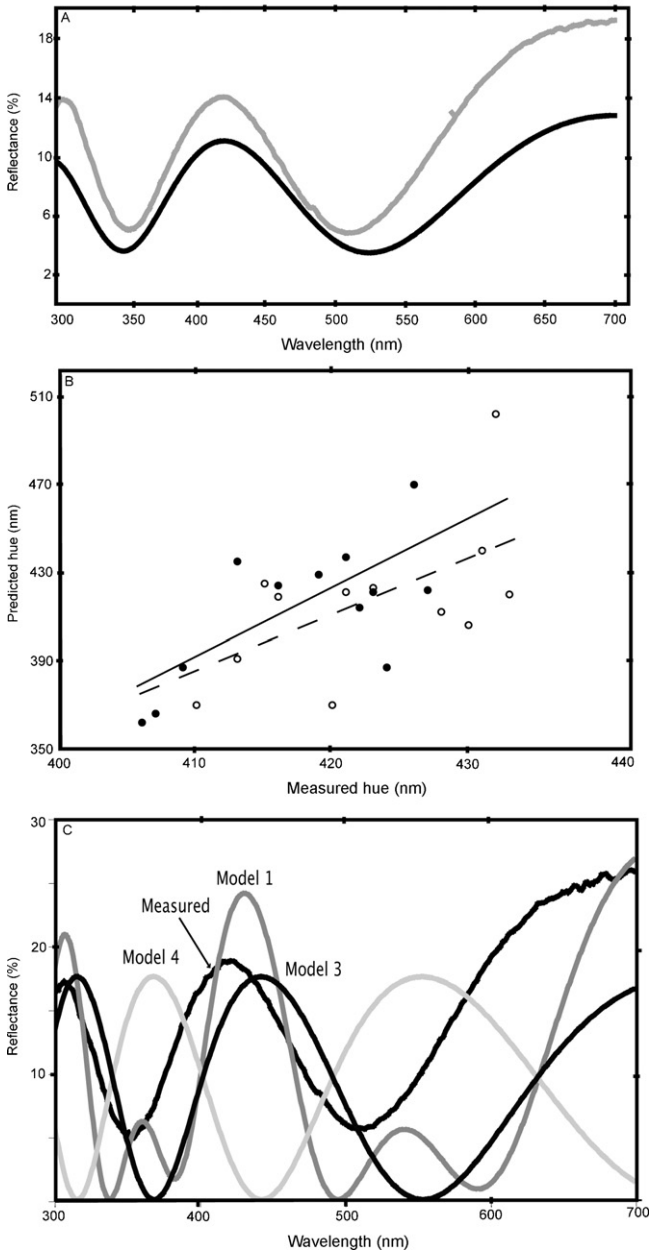

Fig. 3. (A) Mean reflectance at normal incidence of 12 iridescent mourning dove feathers before experimental treatment either empirically measured with a UV–vis spectrometer (light line) or predicted using thin-film optical modeling based on scattering of light at the air/keratin and keratin/air interfaces in barbules (dark line). (B) Scatterplot showing the relationship between 12 iridescent feathers as empirically measured by a spectrometer and as predicted by thin-film optical modeling based on nanostructure of barbules. Solid points are hues before treatment and hollow points are hues after two rounds of wet/dry treatment. All data are from experiment STACK1. Regression lines for data both before (solid line) and after (dashed line) treatment are presented; in both cases the relationship is significant (Pearson correlational test, $p < 0.05$). (C) Reflectance spectra of feathers of a single iridescent mourning dove (*Zenaida macroura*) measured by a spectrometer (black line) and predicted by three models of thin-film reflectance using data from iridescent barbules of a mourning dove feather. Model 1, which incorporates scattering at the air/keratin, keratin/air layer and air layer/keratin interfaces, and models 3 and 4, which only consider scattering at more basal interfaces, bear less resemblance to measured spectra than does model 2 (see Fig. 4).

In experiments SINGLE1 and SINGLE2, the surface area of barbules increased on average by 500 nm^2 (1st replicate: paired $t_{10} = 4.95$, $p = 0.001$; 2nd replicate: paired $t_{10} = 2.31$, $p = 0.04$; Fig. 6B) and the angle relative to the barb increased by 10 degrees (experiment SINGLE1: paired $t_{10} = 11.29$, $p < 0.001$; experiment SINGLE2: paired $t_{10} = 6.85$, $p < 0.001$; Fig. 6C). These changes are easily seen in the SEM images in Fig. 6D and E. By contrast, morphological variables of sham-treated barbules in experiment SINGLECONTROL did not significantly change (angle: paired $t_4 = 0.80$, $p = 0.46$; area: paired $t_4 = 0.76$, $p = 0.48$).

We observed almost no dirt on feathers either before or after treatment in experiments SINGLE1 and SINGLE2 (see Figs. 2B and 6D and E for examples), suggesting that observed color changes were not caused by cleaning.

4. Discussion

In this study we show that iridescent plumage color changes in response to water. Wetting and drying iridescent feathers noticeably and significantly increased their overall reflectance and chroma and changed their hue. The effects on achromatic color were stronger and more repeatable than those on chromatic color (hue).

The color-producing nanostructure of these feathers consists of a single thick layer of keratin cortex over a layer of air and melanosomes in barbules. The close match of measured and predicted spectra shows that light scattering at the air/keratin and keratin/air interfaces is sufficient to produce this color at all measured angles of incidence and thus that the thickness of the air layer is not critical to variation in color pattern. Rather, as we demonstrate with both empirical and theoretical data, variation in thickness of the cortex layer is the primary determinant of both the reflectance spectrum in general and the hue in particular. Air is needed as an interface for light scattering, but the thickness of the air layer did not have significant effects on the color produced.

Several other recent studies have shown similar results for diverse avian species ranging from bowerbirds (family *Ptilonorynchidae*) to ibis (family *Threskiornithidae*), with colors ranging from purple to green (Brink and van der Berg, 2004; Doucet et al., 2006; Shawkey et al., 2006; Maia et al., 2009). In the related rock dove *Columba livia* two-tone (green and purple) iridescent color is similarly produced solely by the keratin cortex over a layer of air (Yin et al., 2006; Nakamura et al., 2007; Yoshioka et al., 2007). In every case the thickness of the outer cortex was critical to hue while the thickness of the underlying layer was not. It is worth noting that all of these studies used the wavelength of peak reflectance as their index of hue; however, other indices may give different results (Montgomerie, 2006). Our results add to a growing body of evidence that thick films are widespread in birds and may be responsible for a wide variety of iridescent colors. Furthermore, the broad range of hues produced by highly similar structures shows that they are malleable, and capable of producing large differences in color with minimal changes in morphology (i.e., thinning or thickening of the cortex).

Because it is closely associated with hue, and hue significantly increased following treatment, we expected to find a significant increase in cortex thickness following treatment as well. We did not, perhaps because of the relatively small change in hue and the large variation in cortex thickness. Variation in cortex thickness of 100 nm has been noted even in a single sample of rock dove feathers (Nakamura et al., 2007) with a mean thickness around 650 nm. Within mourning doves, hue varied within a range of 26 nm while individual cortex thickness measurements varied within a range of 140 nm (around a mean of ~330 nm). This disconnection is almost certainly due to measurement error (expected when measuring

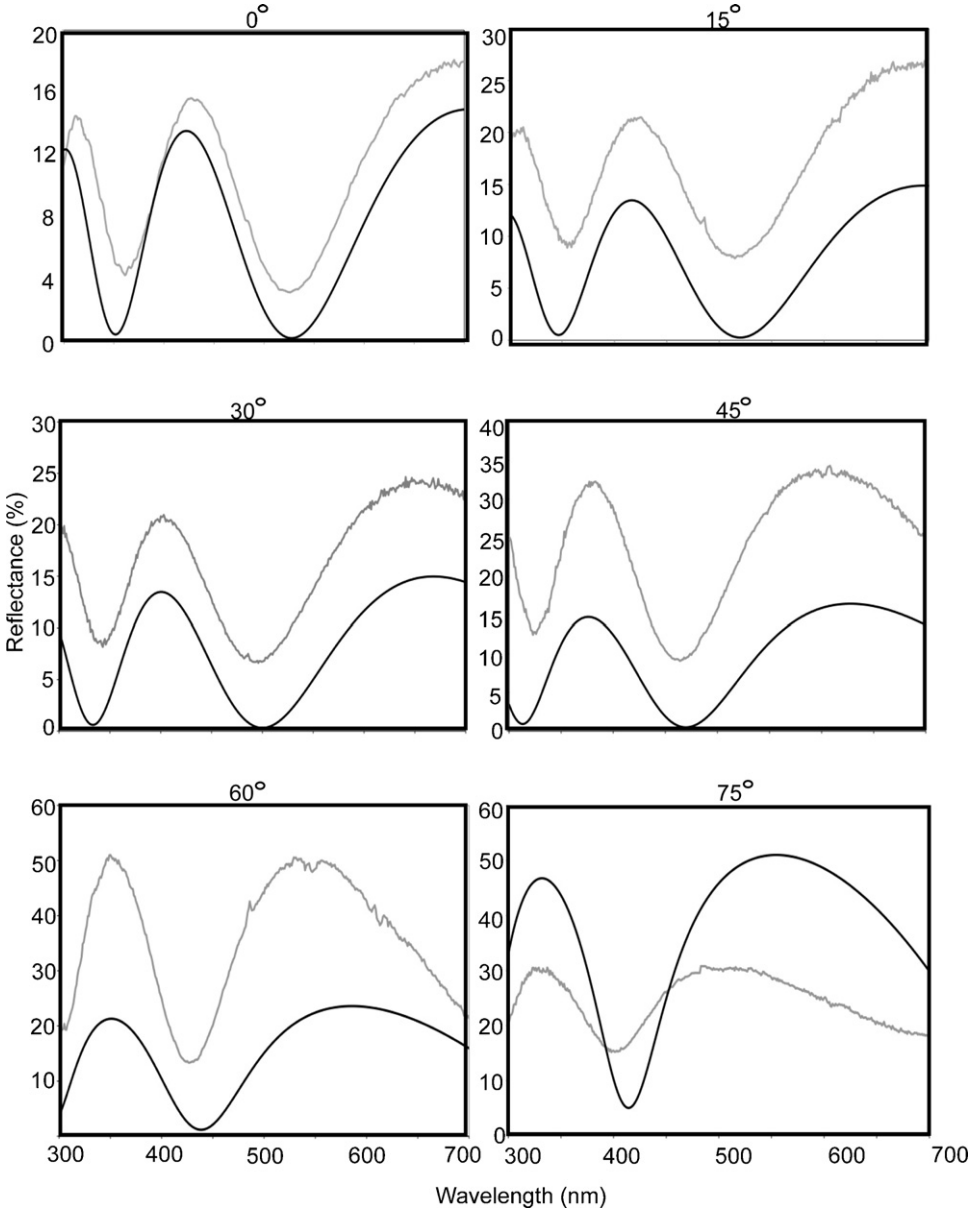

Fig. 4. Reflectance spectra of iridescent mourning dove feathers from a single individual before treatment either measured with a UV–vis spectrometer (light line) or predicted using thin-film optical modeling based on nanostructure of barbules (model 2, dark line). Each panel shows the same sample measured at the angle of incident light and measurement listed above the panel.

a small number of barbules), as according to theory every 1 nm change in cortex thickness should result in a 1 nm change in hue. This error may also explain the lack of observed change in the cortex following treatment despite the significant, but small (10 nm) change in hue. Alternatively, hydration may change the refractive index of keratin as well as layer thickness. In our other experiments, hue changed stochastically, shifting towards the UV in some cases, and towards the red or not at all in others. If water affects cortex thickness, it does not appear to do so in a consistent manner and we may have to measure much larger numbers of barbules to detect any change.

Although cortex thickness determines hue, achromatic variables appear to be more strongly affected by other nano-and macrostructural features. Interestingly, feathers with higher percentages of melanin in the outer layer had higher brightness. This may be because melanosomes in contact with the cortex break up the smooth interface between keratin and air, leading to increased incoherent scattering and hence higher reflection at all wavelengths. Gaps and irregularities in layers can affect the chroma of the reflected color by reducing the layer's average refractive index and/or by interrupting the thin-film reflector, and have been shown to affect intra-specific and inter-specific patterns of iridescence (Doucet et al., 2006; Shawkey et al., 2006; Lee et al., 2009). The percentage of melanosomes in contact with the cortex increased following treatment, concurrent with an increase in the size of the air layer. Water could enter this air layer during hydration and thereby expand it. During subsequent dehydration, melanosomes may be pulled to the edge of the cortex by capillary action similar to that involved in coffee ring formation (Deegan et al., 1997; Deegan, 2000). That a similar process may occur during keratin polymerization in the final stages of iridescent feather development is an intriguing idea that we are currently investigating.

Exposed surface area of barbules predicted their brightness, perhaps because increases in the area available for reflection increased the total amount of reflection. Interestingly, when considered independently before and after treatment, this relationship (as well as

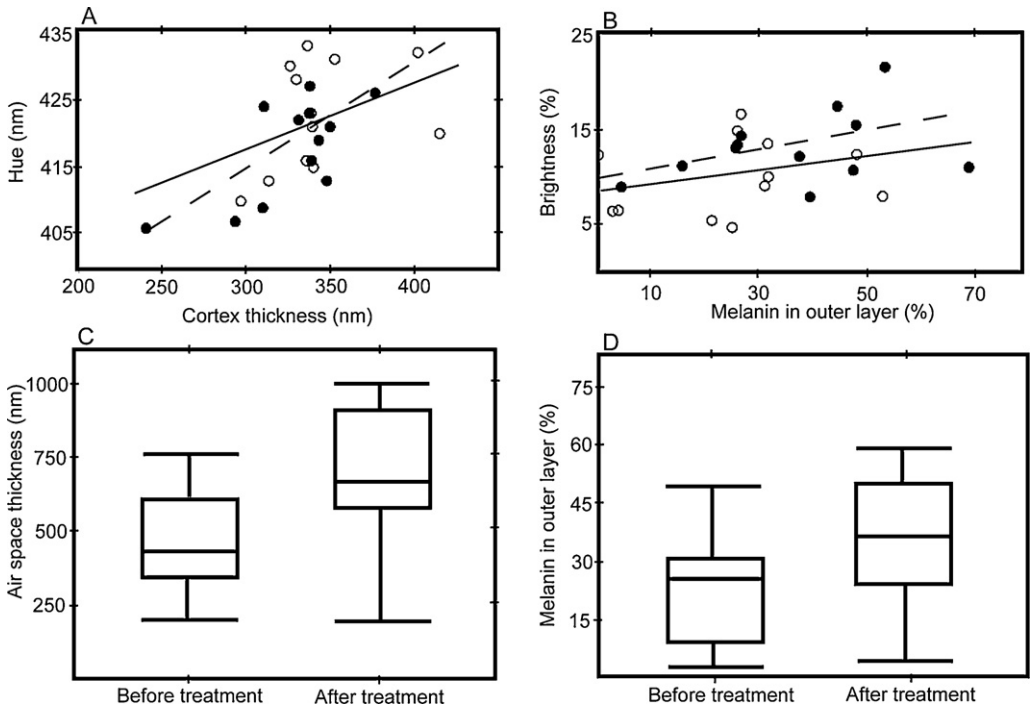

Fig. 5. (A) Scatterplot showing the relationship between 12 iridescent feathers as empirically measured by a spectrometer and thickness of the outer keratin cortex of barbules. (B) Scatterplot showing the relationship between brightness and percentage of total melanin touching the cortex of barbules. In both cases, all data are from experiment STACK1; solid points are values before treatment and hollow points are values after two rounds of wet/dry treatment. Regression lines for data both before (solid line) and after (dashed line) treatment are presented; in both cases the relationship is significant when all data are considered together (linear mixed model, $p < 0.05$). However, when considered separately, the relationship is only significant for (A) after treatment and is not significant either before or after treatment for (B) (Pearson correlational test, $p < 0.05$ for significance). (C and D) Boxplots comparing air space thickness (C) and % total melanin in the outer layer of iridescent barbules (D) before and after 2 rounds of wetting and drying. Both comparisons are from experiment STACK1 and are both significant (paired *t*-test; $p < 0.05$).

that between cortex thickness and hue) was only significant after treatment. These results may be explained by the more uniform orientation of barbules after treatment, which could decrease the amount of reflection "noise" caused by varying barbule orientation. Kemp et al. (2006) showed that UV brightness in *Morpho* butterflies is related to the density of color producing ridges, and that this density can be affected by perturbations during development. By contrast, our experimental work showed that the surface area of structures affecting brightness in iridescent feathers can be altered after development. As the barbules spread out and changed orientation relative to the barb, the size of the surface area for reflection increased. These changes in microstructure paralleled the increase in reflectance, suggesting that the morphological change caused the color change.

This observed increase of barbule surface area may occur as a result of the loosening of the keratin structure through hydration (Bonser, 2002). Filshie and Rogers (1962) showed that the structure of feather keratin consists of longitudinal β-keratin fibers embedded within an amorphous matrix. As water is absorbed by keratin, the matrix swells (Feughelman and Nordon, 1962), resulting in decreased torsional rigidity (Watt, 1980). This swelling, in addition to the attraction of water molecules to the hydrophilic groups of keratin molecules (reviewed in Watt, 1980), may result in the disruption of secondary bonds (i.e., hydrogen bonds) between adjacent molecules (Feughelman and Nordon, 1962). Furthermore, in the hydrated state, the globular keratin proteins that comprise the feather matrix are freer to move (Bertram and Gosline, 1987) and, upon drying, the reformation of hydrogen bonds between adjacent molecules may "fix" them in place, possibly explaining the observed shift in barbule alignment after re-drying the feathers. As its keratin structure swells during hydration and then contracts during drying, the barbule may twist at its point of attachment, pulling the flat plane of the barbule perpendicular to the normal, thereby increasing exposed surface area. Separation of barbules from one another by the same process may further increase the exposed surface area.

Our results may have implications for the potential signaling functions of colorful iridescent plumage. Typically these colors are assumed to be static following development, but our results show that they are in fact highly malleable, changing significantly following a minimal treatment. By contrast, color of non-iridescent brown feathers did not change. One interpretation of these data is that melanin-based colors are a reliable signal while iridescent colors are not. If iridescent feather colors can change easily, and females cannot distinguish between a naturally bright male and one that becomes bright, then the color signal reliability is compromised and females should lose interest in it over evolutionary time (Andersson, 1994). Alternatively, bright color could be maintained through a null process in which traits are arbitrarily preferred by females (Prum, 2010). However, considerable variation in iridescent color remained, even after treatment (see Table 1), suggesting that wetting does not homogenize color and that enough variation exists for females to make meaningful choices. Indeed, color variables were significantly correlated before and after treatment (Pearson correlation: hue: $r = 0.89$, $p < 0.001$; brightness; $r = 0.77$, $p = 0.003$, chroma: $r = 0.81$, $p = 0.001$), and color after two washes appeared relatively stable, suggesting that naturally bright and dull males remained distinguishable. Furthermore, it is unclear whether actively maintained feathers on live birds get wet or change color to the same degree, and whether or not these changes occur in the components of color that birds pay attention to when making mate choice decisions. Nevertheless, it may be interesting to test whether males with iridescent plumage vary in how often they intentionally wet their feathers through bathing and how this affects variation in color. Whether the amount or quality of preen oil affects the wetting of feathers and hence color change will also be an interesting avenue for future research. Future studies should also examine

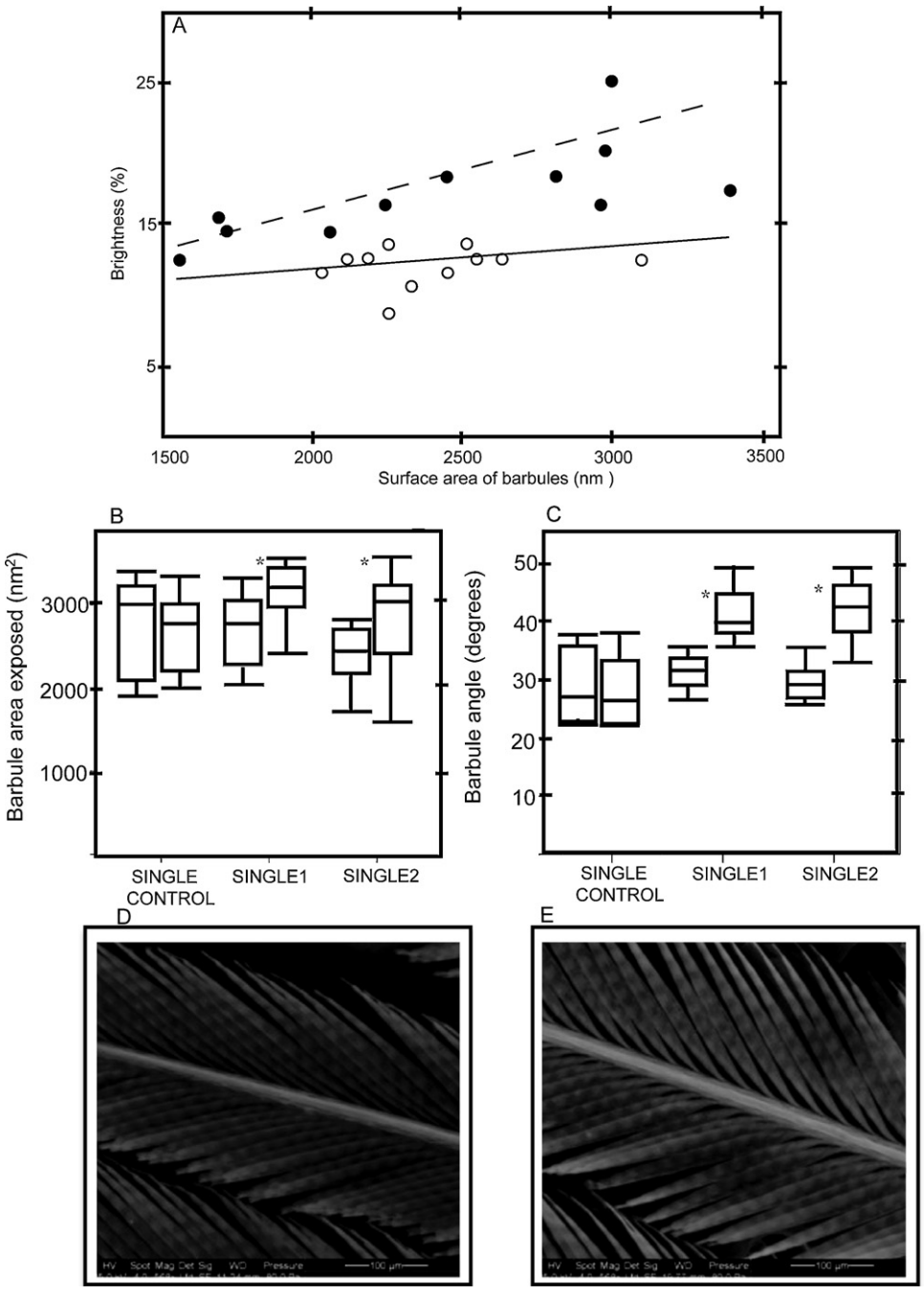

Fig. 6. (A) Scatterplot showing the relationship between exposed surface area of barbules and brightness of 12 iridescent mourning dove feathers. All data are from experiments SINGLE1. Solid points are values before treatment and hollow points are values after two rounds of wet/dry treatment. Regression lines for data both before (solid line) and after (dashed line) treatment are presented; the relationship is significant when all data are considered together (linear mixed model, $p < 0.05$). However, when considered separately, the relationship is only significant after treatment (Pearson correlational test, $p < 0.05$ for significance). (B–E) Effects of two replicated experimental rounds of wetting and drying on the morphology of iridescent barbules. All data are from experiments SINGLE1, SINGLE2 and SINGLECONTROL. (B) Exposed surface area of barbules before and after two rounds of experimental wetting and drying. Controls were not wetted/dried. Asterisks indicate significant differences (paired *t*-test; $p < 0.05$). (C) Barbule angle relative to barb before and after two rounds of experimental wetting and drying. Controls were not wetted/dried. Asterisks indicate significant differences (paired *t*-test; $p < 0.05$). (D and E) Scanning electron micrographs of barb and barbules of an iridescent feather before (D) and after (E) treatment, illustrating the twisting of barbules to reveal more surface area.

other types of structural color as well as carotenoid-based color to determine the generality of this effect.

Finally, nanostructures that change in response to simple environmental variation should have numerous applications. Recent research has shown that *Morpho* butterfly scales change color in comparable ways (i.e., increased brightness) in response to water, ethanol and methanol vapor (Potyrailo et al., 2007). Our research suggests a similar response to liquid water that may be based on changes in the molecular structure of β-keratin. Future research should work to identify the nature of these changes. This may enable us to better understand the mechanisms for these color changes and allow mimicry of them in artificial structures for applications in color-changing sensors (Potyrailo et al., 2007). This work thus represents a significant step forward in several areas of inquiry and should stimulate considerable research in evolutionary biology as well as biomimetic materials research and design.

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