Rapid, reversible response of iridescent feather color to ambient humidity

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Abstract: Colorful traits can vary in response to neural control, hormone levels, reproductive state, or abiotic factors. In birds, colorful plumage traits are generally considered static ornaments that only vary irreversibly due to abrasion, bacterial degradation, or wear. However, in this work it is shown that iridescent feather color varies rapidly and reversibly in response to changes in ambient humidity. Based on optical models and sorption experiments, these changes appear to be caused by a swelling of the outer keratin cortex following water absorption. To our knowledge, this is the first study describing dynamic color changes in any keratinous biophotonic nanostructure.

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1. Introduction

The variety and ubiquity of colorful traits in nature is astonishing, and in many species such traits mediate the survival [1–3] and reproductive success [4] of individuals. While all colorful traits rely on the selective reflectance of certain wavelengths of light, the underlying mechanisms of color production vary considerably. For example, red coloration can be produced by the deposition of pigments that differentially absorb certain wavelengths of light [5] or by photonic structures such as diffraction gratings, interferometers, and 2D/3D photonic crystals that produce colors by the coherent scattering of light [6–8]. Furthermore, many colorful traits are phenotypically plastic and the nature of such temporal variation is itself mediated by the color-producing mechanism. For example, colors that result from the concentration and arrangement of chromatophores can vary rapidly in response to neural control [9], hormone levels [10], and reproductive state [11]. Additionally, rapid color
changes have been observed in biophotonic nanostructures in response to changes in abiotic factors [12–14].

In birds, feather colors are produced either by the selective absorbance of specific wavelengths of light by feather pigments (pigment-based colors), or by coherent light scattering due to spatial periodicity arising from the nanoscale arrangement of keratin, melanin, and air (structural colors) [7]. The latter can be further classified as iridescent structural colors that are produced by thin-film interference in feather barbules, and non-iridescent structural colors that are produced by 3D spatial periodicity in feather barbs [7]. Because of the evolutionary significance of plumage coloration [4], recent studies have focused on the temporal variation of colorful plumage traits in response to various environmental factors. For example, feather color varies in response to bacterial load [15], abrasion [16], or more generally, feather wear [17]. However, such changes are often slow processes, occurring over the course of several months [17]. Additionally, changes in plumage coloration are thought to be permanent and irreversible because feathers are assemblages of keratin, lacking the blood vessels and intercellular communication mechanisms required for dynamic modulation of color [18].

However, keratin is a hydrophilic protein that readily absorbs water vapor due to the presence of charged amino acids on individual keratin molecules, leading to swelling over a range of humidities [19]. The nanoscale arrangement of keratin and melanin at the outer edge of iridescent feather barbules results in coherent scattering of light, thereby producing brilliant, iridescent colors [7]. Thus, exposure to water vapor may disrupt or alter coloration. We tested this hypothesis using iridescent blue-green feathers of tree swallows (Tachycineta bicolor), predicting that their color would change in response to variation in ambient humidity through the absorption of water molecules and swelling of keratin.

2. Materials and methods

2.1 Specimen collection

We pulled mantle feathers from tree swallows captured at their nest boxes at the Dr. Paul E. Martin Centre for Field Studies and Environmental Education in Bath, Ohio (41°9′N 81°38′W). Additionally, we pulled breast feathers from house finches (Carpodacus mexicanus) and rump feathers from eastern bluebirds (Sialia sialis) captured on the campus of Auburn University in Lee County, Alabama (32°35′N, 82°28′W). We stored all feathers in small envelopes in a climate-controlled environment until further testing.

2.2 Electron microscopy

To characterize the nanostructure responsible for producing iridescent color in these feathers, we used transmission electron microscopy (TEM) and prepared samples for TEM following Shawkey et al. [20]. Briefly, we cut three barbs from the iridescent region of two separate feathers, washed them in a solution of 0.1% Tween and 0.25 M NaOH, and fixed them in a 2:3 (v/v) solution of formic acid and ethanol. Next, we dehydrated the samples in 100% ethanol (twice for 20 minutes each time) and infiltrated them in 15, 50, 70, and 100% Epon (24 h each time). After curing the blocks at 60°C for 16 h in an oven, we trimmed them with a Leica S6 EM-Trim 2 (Leica Microsystems GmbH, Wetzlar, Germany) and cut thin 100-nm sections using a Leica UC-6 ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany). Finally, we stained the sections with uranyl acetate and lead citrate and viewed them on a Tecnai TEM (FEI, Hillsboro, OR, USA) at an operating voltage of 120 kV.

From the obtained TEM images, we used ImageJ [21] to measure the thickness of the keratin cortex and the thickness of the underlying layer of melanosomes at 10 evenly-spaced locations along the dorsal surface of five separate barbules from two different barbs (each from a different feather on the same bird). From these measurements, we calculated the mean and standard deviation of each variable for use in subsequent thin-film optical modeling.
2.3 Humidity experiments

To test the responses of feather color to changes in humidity, we taped a single iridescent tree swallow feather to a piece of black velvet and mounted it to the side of an angled plastic holder with a 1-cm opening to allow the dorsal surface of the feather to be exposed to the surrounding atmosphere. Using a block holder (Avantes Inc., Boulder, CO, USA), we then mounted the probe of a bifurcated fiber optic cable (FCR-7xx200-2-ME, Avantes Inc., Boulder, CO, USA) to the holder at a distance of approximately 10 mm from the feather surface and placed the entire apparatus within a large plastic box. The relative humidity in the chamber was controlled by varying the relative amounts of dry and moist air entering the chamber (moist air was achieved by bubbling dry nitrogen gas through a column of water, see Agnarsson et al. [22] for technical details). We monitored relative humidity using a digital hygrometer (VWR, Batavia, IL, USA).

To determine the effect of humidity on the spectral reflectance of feathers, we exposed a single tree swallow feather to the following series of conditions: 20%, 80%, and 20% RH. At each humidity level, we allowed the feather to acclimate for 10 min and measured reflectance as described above. We performed the entire experiment a total of 9 times with feathers from different individuals. As a negative control, we repeated the procedure on feathers with non-structural, carotenoid pigment-based coloration (house finch, *Carpodacus mexicanus*) [23]. We also tested feathers from eastern bluebirds (*Sialia sialis*), a species with non-iridescent structural coloration [24], to examine whether the effects were specific to iridescent structurally-colored feathers.

Next, to understand the dynamic nature of color changes, we tested the response of feathers to abrupt step changes in humidity from 0 to 80% and 80–0% RH, measuring reflectance every 2 sec for a period of 5 min after each change. We determined the response time of feathers as the time required for the feather to reach 90% of the final value (i.e. maximum hue change), both during increases and decreases in humidity. We performed the entire experiment twice on feathers from different individuals. To facilitate the rapid replacement of residual air within the humidity chamber, we decreased the volume of the air surrounding the feather by enclosing the 10 mm space between the feather surface and block holder with Parafilm (Pechiney Plastic Packaging Co., Chicago, IL, USA).

2.4 Spectrometry

We measured spectral reflectance in the range of 300–700 nm, normal to the surface of each feather, using an AvaSpec-2048 spectrometer with an attached deuterium light source (Avantes Inc., Boulder, CO, USA). For all reflectance measurements taken during humidity experiments, we calibrated the spectrometer to a white standard (Avantes Inc., Boulder, CO, USA) placed outside the humidity chamber, thereby controlling for spectrometer drift during the course of the experiment.

After taking reflectance measurement, we interpolated all spectra to 1-nm bins, smoothed spectra using local polynomial regression techniques, and calculated the wavelength of maximum reflectance ($\lambda_{\text{max}}$) and the magnitude of peak reflectance ($R_{\text{max}}$). Because the reflectance of red, pigment-based colors of our negative controls continually increased into the red region of the spectrum, making all $\lambda_{\text{max}}$ values equal to 700 nm and thus not representative of observed color, we calculated hue as the wavelength where the reflectance curve reached a maximum positive slope [25].

2.5 Thin-film optical modeling

From the dimensions of the keratin and melanin layers measured from the TEM images, we used the transfer matrix method [26] to predict spectral reflectance at baseline humidity and during humidity changes. We constructed a 3-beam model considering reflection at the air-keratin, keratin-melanin, and melanin-keratin interfaces, assuming uniform layer thicknesses
for simplicity [27]. We used published values for the complex refractive indices of keratin ($n = 1.56-0.03i$) and melanin ($n = 2.00-0.6i$) [28,29] and compared the resulting spectra to the reflectance measured from the feathers.

2.6 Statistical analysis

We performed separate repeated-measures ANOVAs with hue ($\lambda_{\text{max}}$) and magnitude of peak reflectance as response variables. We used Mauchly’s test of sphericity and, when significant ($P<0.05$), adjusted degrees of freedom using Greenhouse-Geisser epsilon values. Comparisons between pairs of treatments were made using Tukey HSD tests with a significance level of 0.05.

To determine the statistical significance between color responses of species with different color-producing mechanisms, we ranked hue changes for all species (due to unequal variances between groups) and performed an ANOVA on the ranked data. We checked all data for normality and performed all analyses in the R environment [30].

3. Results

3.1 Electron microscopy

To determine how iridescent color is produced in tree swallow feathers, we examined the morphology of barbule cross-sections using transmission electron microscopy (TEM). A thin layer of keratin with a thickness of $148.4 \pm 5.1 \text{ nm}$ [mean $\pm$ s.e.; Fig. 1(b)] overlaid an irregular layer of disc-shaped melanosomes (thickness = $172.8 \pm 27.1 \text{ nm}$), which is in good agreement with previous work done on a closely-related species [31]. Considering that our model assumes uniform flat layers of keratin and melanin, while feathers are complex structures with variations in the alignment of reflecting surfaces (i.e. barbules) as well as in the uniformity of keratin and melanin layers, the predicted $\lambda_{\text{max}}$ matched the observed values remarkably well [Fig. 1(c)]. This suggests that the color produced by tree swallow feathers is due to thin-film interference.

![Fig. 1. TEM cross-section of an iridescent T. bicolor feather barbule used to predict reflectance (b). Iridescent tree swallow feather (a) and magnified barbules (inset) showing plane of cross-section (white line perpendicular to barbules). Representative barbule cross-section (b) shows melanosomes (black) and keratin cortex (grey region surrounding melanosomes). Scale bar = 500 nm. Letters correspond to dorsal (d) and ventral (v) surfaces. Measured (solid line) and predicted (dashed line) reflectance based on thin-film optical model (c).](image)

3.2 Humidity experiments

We then tested the response of feather coloration to gradual changes in relative humidity from 20 to 80% RH. Hue increased significantly (20% RH: $497.3 \pm 3.6 \text{ nm}$, 80% RH: $513.2 \pm 4.3 \text{ nm}$; $F_{2,16} = 49.6, P<0.0001$), changing by up to 25 nm, and then reverted to near the initial value after return to baseline humidity ($500.6 \pm 3.45 \text{ nm}$). However, because the water content of the feather was slow to return to equilibrium [e.g., see Fig. 2(b)], hue remained slightly and
significantly higher after treatment. The magnitude of peak reflectance did not significantly change through the course of the experiment ($F_{2,16} = 0.60, P = 0.56$).

Feather color also varied rapidly and reversibly in response to more rapid humidity changes (Fig. 2). When exposed to abrupt increases in humidity (0–80% RH), color began to change in under 2 sec (the resolution of our measurements). The mean response time of $\lambda_{\text{max}}$ following increases in humidity was 63.8 sec ($n = 2$), and the response time after an abrupt decrease back to 0% RH was 46 sec ($n = 2$).

**Fig. 2.** Reversible color change in tree swallow feathers. Reflectance of feathers at 0% RH (solid line) and after an abrupt increase to 80% RH (dashed line) [a]. Representative response of $\lambda_{\text{max}}$ to abrupt increases (vertical dashed line, $t = 2$ sec) and decreases (vertical dashed line, $t = 304$ sec) in humidity (b). Points connected with grey line to aid in trend visualization. Inset shows plot of hue vs. time$^{1/2}$ and linear regression (solid line) for times under 70 sec.

### 3.3 Optical modeling of color change

Because it is nearly impossible to view the state of water in biological tissues with traditional TEM (due to the necessary dehydration of the sample) [32], we were unable to test our prediction of a swelling mechanism using this methodology. However, thin-film optical models allow us to identify the effects of changes in nanostructure properties (e.g., thickness, refractive index) on overall reflectance.

The outer cortex of feather barbules is comprised of keratin, a hydrophilic protein that absorbs water and swells over a wide range of humidities [19]. In addition, the reflectance of iridescent feather barbules may vary with relative thickness, refractive index, and viewing angle [33]. Using a baseline model for thin-film reflectance, we considered four possible mechanisms by which the coloration of feather barbules could change in response to increasing humidity. In model 1 we considered a 10 nm increase in the thickness of the keratin cortex (comparable to another study on the swelling of keratin fibers [34]). In model 2, since adsorbed layers of water are known to form films up to 3 nm thick on the surface of hydrophilic substances such as mica and silicon [35] or butterfly scales [12], we considered the effects of a 3 nm thick layer of water on the surface of the keratin cortex. In model 3, since many solids are porous, we modeled an increase in the refractive index of keratin from 1.56 – 1.6, which would occur if air spaces were infiltrated by water. Finally, in model 4 we considered a decrease in the refractive index from 1.56 to 1.52, a magnitude of change similar to that observed in human fingernail keratin [36].

Models 1-3 accurately predicted the direction of change in $\lambda_{\text{max}}$, however, model 1 provides the best fit to the observed data. The magnitude of hue change was minimal in model 2 [Fig. 3(c)], and a physically improbable 20-nm-thick layer of water would be required to
explain the observed hue shift. Furthermore, model 3 [Fig. 3(d), solid red line] predicted an increase in $R_{\text{max}}$, which contradicts the experimental data [Fig. 3(a)]. Although model 4 predicted a decrease in $R_{\text{max}}$ [Fig. 3(d), dashed red line], it predicted blue-shifted $\lambda_{\text{max}}$ values, opposite the observed effect.

Fig. 3. Potential mechanisms for the observed color change. Observed mean reflectance change of tree swallow feathers ($n = 9$) before (black line) and after (red line) humidity change from 20 to 80% RH (insets are images of a single feather taken at 0% and 100% RH). Model predictions before (black line) and after humidity increases (red line) for keratin swelling (b), surface adsorption (c), and increases (solid line) or decreases (dashed line) in the refractive index of keratin (d). Insets are schematic drawings of barbule cross-sections showing melanosomes (black), keratin cortex (grey), and water molecules (blue circles). Drawings not to scale, allowing for easier visualization of mechanisms.

4. Discussion

Here, we demonstrate for the first time that plumage color produced by a biophotonic nanostructure varies in response to ambient humidity. Four results suggest that this reversible color change from blue-green to yellow-green is largely the result of swelling of the keratin cortex. First, a thin-film optical model of an increase in the thickness of keratin cortex accurately predicted the behavior of color change [Fig. 3(b)]. Second, assuming that the keratin cortex expands in one dimension along the direction of thickness, and that water and keratin mix additively [37], the initial linear relationship between $\lambda_{\text{max}}$ and time$^{1/2}$ [Fig. 2(b),
inset] is consistent with expected results from Fick’s law of diffusion [19], and hence with keratin swelling (model 1). However, this does not rule out model 3, which may follow a diffusion process as well. Third, the magnitude of peak reflectance decreased with increasing humidity [Fig. 3(a)], providing support for model 1 and against model 3. Fourth, the hue of non-iridescent structurally colored feathers (eastern bluebird, Sialia sialis) also changed significantly and reversibly with humidity while a pigment-based color did not (Fig. 4). Non-iridescent structural colors are produced by periodically-arranged keratin and air channels forming a spongy layer in feather barbs [7]. Swelling of keratin in these spongy layers should increase the size of this periodicity and thereby produce longer-wavelength hues [20]. Thus, these results are consistent with our proposed mechanism (model 1) and inconsistent with surface adsorption (model 2), since in these feather types the color-producing structures are located below a relatively thick cortex that plays no role in color production [7] and would therefore be unaffected by surface adsorption. In this analysis, we did not consider all possible interactions between proposed mechanisms (which are not mutually exclusive except for models 3 and 4), and it is conceivable that some parameters may co-vary (e.g., keratin swelling would likely be accompanied by changes in refractive index).

![Graph showing species and hue change](image)

**Fig. 4.** Variation in color response of different color-producing mechanisms. Filled circles show mean hue change before and after humidity changes and error bars show ± 2 SE. Hue was calculated as $\lambda_{\text{max}}$ in bluebirds and swallows and as wavelength of maximum positive slope in house finches (see Materials & methods for details). Overall effect of species on hue change was significant (ANOVA, $F_{2,24} = 49.21$, $P<0.0001$), and all pair-wise comparisons were significant (Tukey tests, $P<0.05$).

Recently, there has been considerable interest in the effects of water vapor on the optical properties of biological structures. For example, in some beetle species, color changes reversibly due to the infiltration of water into air spaces [14], thus lowering the refractive index contrast. This mechanism differs from ours; however the direction of change in hue is comparable. In *Morpho* butterflies, scales become brighter with increasing vapor pressures, probably due to the adsorption of a thin film of water and capillary condensation into mesopores [12], which contrasts with our findings because we show that brightness does not change. Furthermore, in longhorn beetles (*Tmesisternus isabellae*), color changes occur when the periodicity of melanoprotein layers increases with swelling [13], a mechanism similar to the one we describe. Liu et al. [13] observed an increase in spatial periodicity of 175–190 nm that is comparable to our proposed increase from 148 to 158 nm. The current study adds to
our understanding of the interactions of water vapor with biological materials and also reveals a new photonic structure whose optical properties co-vary with relative humidity.

These results may provide insight into the design of new materials for color-changing sensors. Swelling biopolymers may have advantages over sensors relying on surface adsorption because they may be less prone to surface contamination and can absorb larger quantities of vapor, resulting in greater vapor sensitivity [38]. Furthermore, sensors based on the swelling of keratin thin-films may outperform existing humidity sensors, as indicated by a quicker response time [39]. Further research into the response of these structures and of keratin in general to adsorption of different materials will determine the utility of keratin-like materials for future biomimetic sensor design.

Finally, our findings may also have important implications for the understanding of sexual selection and signal reliability. It could be argued that, assuming that all individuals within a population are exposed to similar humidity, changing color may not affect signal reliability. However, inter-individual variation in the magnitude of color response to humidity changes (as seen in the high variance of response among our samples; see Fig. 4) may have two implications for signal reliability. First, if the magnitude of hue change is quality-dependent (i.e. higher-quality individuals change optimally, perhaps due to the amount of feather preen oils or density and cross-linking of keratin microfibrils [40]), signal reliability would be maintained. Second, if hue changes are quality-independent (i.e. no covariation between individual quality and the magnitude of color change), signal reliability would decline and possibly weaken female preferences for such traits [41]. This may partially explain why neither male plumage hue nor brightness significantly predicted female reproductive success or reproductive investment in this species [42]. Future studies should investigate the reliability and geographical variation of iridescent plumage ornaments that vary with environmental humidity or other abiotic factors. Environmental factors may mediate the evolution of structural plumage coloration and play a previously unforeseen role in explaining the observed variation of structural plumage colors in nature.

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