The fossil record of insect color illuminated by maturation experiments

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ABSTRACT

Structural coloration underpins communication strategies in many extant insects but its evolution is poorly understood. This stems, in part, from limited data on how color alters during fossilization. We resolve this by using elevated pressures and temperatures to simulate the effects of burial on structurally colored cuticles of modern beetles. Our experiments show that the color generated by multilayer reflectors changes due to alteration of the refractive index and periodicity of the cuticle layers. Three-dimensional photonic crystals are equally resistant to degradation and thus their absence in fossil insects is not a function of limited preservation potential but implies that these color-producing nanostructures evolved recently. Structural colors alter directly to black above a threshold temperature in experiments, identifying burial temperature as the primary control on their preservation in fossils. Color-producing nanostructures can, however, survive in experimentally treated and fossil cuticles that now are black. An extensive cryptic record is thus available in fossil insects to illuminate the evolution of structural color.

INTRODUCTION

The integumentary colors of extant organisms are generated by pigments or biophotonic nanostructures that scatter incident light via ordered or quasi-ordered variations in refractive index (Vukusic and Sambles, 2003). The latter generate highly conspicuous visual signals used in species and sex recognition, mate choice, and predator avoidance (Doucet and Meadows, 2009); they are the focus of intense research due to their biomimetic potential and the insights they offer into animal behavior (Doucet and Meadows, 2009). The gamut of structural coloration is especially diverse in insects (Kinoshita and Yoshioka, 2005) where striking visual effects are generated by multilayer reflectors and three-dimensional (3-D) photonic crystals that produce phenomena such as metallic, polarized, and UV reflection, iridescence, and/or opalescence (Doucet and Meadows, 2009; Parker, 2005). Indirect evidence from phylogenetic data indicates that insect structural color has multiple independent origins driven primarily by sexual selection, and possibly linked to diurnality, color vision, and certain light environments (Doucet and Meadows, 2009; Seago et al., 2009), but these models require testing. Fossils are the only direct source of evidence for the evolution of color, but the original colors in Cenozoic insects have altered in ways that are not fully resolved (McNamara et al., 2012a). All biophotonic nanostructures in fossil insects are multilayer reflectors (McNamara et al., 2011, 2012a); 3-D photonic crystals occur in many groups of extant insects (e.g., Saranathan et al., 2010; Colomer et al., 2012; Wilts et al., 2012) but have not been recorded in fossils. Colored fossil insects tend to be either abundant or absent in any individual fossil biota (McNamara et al., 2012b), suggesting the presence of a taphonomic "on/off" switch. It is not clear to what extent these patterns in the fossil record of structural color reflect real biological signals or artifacts induced during decay or burial. Here we use a novel experimental approach that integrates high pressure–high temperature autoclave experiments with decay experiments to reveal the effects of decay, pressure, and temperature on different biophotonic nanostructures. Our results reveal the controls on structural color preservation in insects, and confirm the potential of fossils to illuminate the evolutionary history of insect color.

METHODS

Beetles are the most abundant structurally colored fossil insects (Doucet and Meadows, 2009). Our study therefore focused on the extant leaf beetle *Chrysochroa raja* (Coleoptera: Chrysomelidae) (Figs. 1–3; Figs. DR1–DR4 in the GSA Data Repository[1]) and the weevil *Pachyrrhynchus reticulatus* (Coleoptera: Curculionidae) (Fig. 4; Fig. DR5); the color-producing nanostructure is a multilayer reflector (Noyes et al., 2007) and a 3-D photonic crystal (V. Saranathan, 2012, personal commun.), respectively. The cuticle of *C. raja* is brightly reflective and iridescent; the elytra are predominantly green (peak wavelength [λ_{max}] ≈ 550 nm) with longitudinal dorso-lateral red-orange bands (Fig. 1A, inset). The multilayer reflector is located in the epicuticle (Fig. 1F). The cuticle of *P. reticulatus* is black with yellow-orange iridescent bands (Figs. 4A and 4B) that comprise assemblies of scales; each scale exhibits many microdomains that differ in color (Fig. 4B). This is due to varying orientations of photonic crystals with a single-network diamond (Fd3m) nanostructure within the scale lumen (Fig. 4E).

Specimens for decay were placed in individual glass jars with 200 ml natural lake water and 5 ml sediment (Dart Lake, Herkimer County, New York, USA) and degraded in the dark at 25 ± 0.3 °C and 50 ± 0.5% humidity for 18 mo.

Fragments of fresh and decayed elytron for maturation were wrapped in aluminum foil and inserted into an autoclave pressurized using Ar gas at: 200 °C, 1 bar; 200 °C, 117 bar; 200 °C, 250 bar; 200 °C, 500 bar; 270 °C, 500 bar; and 25 °C, 500 bar, each for 24 h. One-hour experiments were run at 200 °C, 250 bar, and at 250 °C, 250 bar. Conditions fluctuated within ±3 °C and ±0.35 bar.

Samples of elytron were prepared for electron microscopy as in McNamara et al. (2012a) and examined using a FEI XL-30 field emission gun (FEG) environmental scanning electron microscope (ESEM) at an accelerating voltage of 15kV, and a Zeiss EM900 transmission electron microscope (TEM) at 80kV with an objective aperture of 90 μm diameter.

Reflectance spectra were obtained from elytra using the method described in McNamara et al. (2012a). Predicted reflection spectra were obtained by analyzing variation in the refractive index of biophotonic nanostructures using two-dimensional Fourier analysis of digital TEM micrographs (Prum and Torres, 2003).Values for the high- and low-refractive index layers in the multilayer reflector of the cuticle of *C. raja* are 1.68 and 1.55 (Noyes et al., 2007).

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[1]GSA Data Repository item 2013127, Figures DR1–DR6 and Tables DR1–DR3, is available online at www.geosociety.org/pubs/ft2013.htm, or on request from editing@geosociety.org or Documents Secretary, GSA, P.O. Box 9140, Boulder, CO 80301, USA.

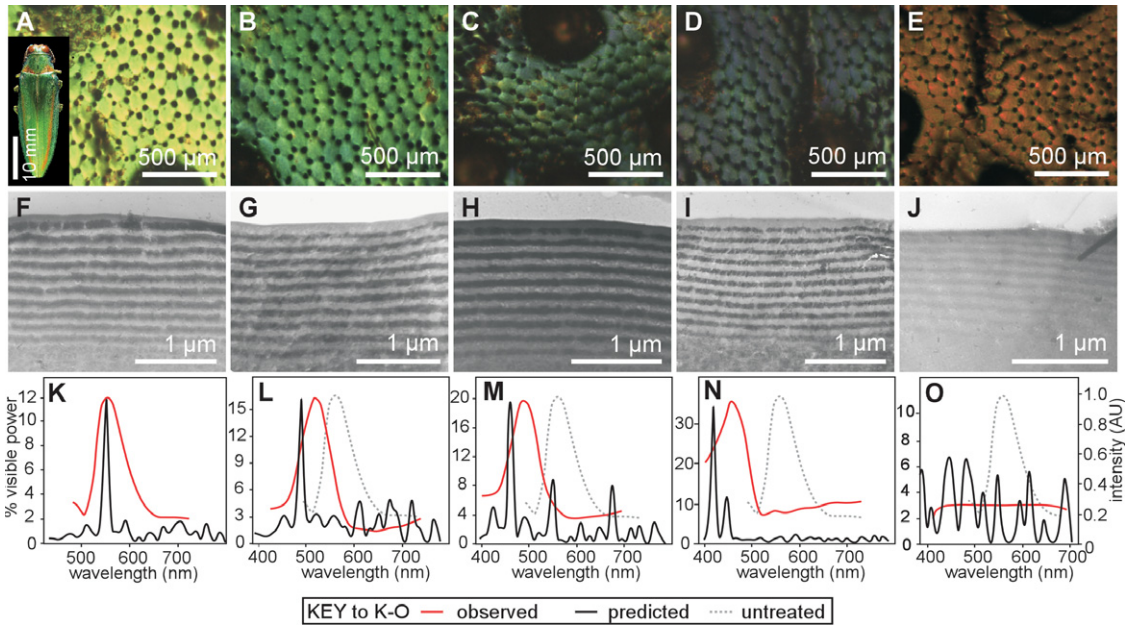

Figure 1. Experimentally induced color change in *Chrysochroa raja* cuticles. A–E: Light micrographs of the cuticle surface of untreated specimens (A) and specimens treated to 200 °C, 117 bar (B); 200 °C, 250 bar (C); 200 °C, 500 bar (D); and 270 °C, 500 bar (E). F–J: Transmission electron micrographs of the epicuticular multilayer reflector for samples in A–E. High- and low-refractive index layers exhibit high and low electron contrast, respectively. K–O: Experimentally measured (observed) and predicted (using Fourier analysis) reflectance spectra for cuticles in A–E.

Cuticles for gas chromatography–mass spectrometry (GC-MS) were stored at −80 °C for 24 h, crushed, and extracted in 2:1 CH_2CL_2:CH_3OH. The total lipid extract was analyzed by an Agilent 7890A gas chromatograph interfaced to a 5975C mass spectrometer. Gas chromatography was performed at 70eV using an HP-1MS 60 m capillary column with 320 mm diameter and 0.25 mm film thickness, and He as carrier gas; concentrations of lipid compounds were evaluated using a 7683B Series autosampler. The GC oven was programmed from 40 °C (held 1 min) to 150 °C (10 °C min^{-1}) and then to 315 °C (6 °C min^{-1}; held 20 min).

RESULTS

The hue generated by a biophotonic nanostructure is determined by both the periodicity and refractive index of its constituent materials (Schultz and Rankin, 1985); any color change observed in our experiments thus reflects alteration of one, or both, of these. The hue of *C. raja* cuticles was unaltered after 18 mo. decay (Figs. DR1A and DR1B). Fresh (i.e., undecayed) cuticles heated to 200 °C and treated to pressures of 117 bar, 250 bar, and 500 bar for 24 h altered from green (λ_{max} = 554 nm) to cyan (λ_{max} = 519 nm), blue (λ_{max} = 485 nm), and indigo (λ_{max} = 453 nm), respectively (Figs. 1A–1D). Coloration disappeared during the 270 °C, 500 bar treatment (Fig. 1E). Pressure and peak wavelength are positively correlated (Fig. DR2). Similar results in experiments using degraded cuticles (Figs. DR1C–DR1G) confirm that decay processes have no discernable effect on the physical properties of the multilayer reflector or the resultant hue. During pressure-temperature treatment, the decrease in wavelength is accompanied by a decrease in the periodicity of the multilayer reflector from 161 nm to 128 nm (Figs. 1F–1J; Table DR1); its layers decrease in thickness from ~100 nm to 76 nm (high index) and from ~61 nm to 52 nm (low index). Other cuticular ultrastructures (Fig. DR3) also decreased in thickness (Table DR2), but the cuticles did not show fissures, wrinkling, or delamination (Fig. DR3). Two-dimensional Fourier analysis of the multilayer reflector from each experimental treatment revealed a consistent offset (5.3% ± 2.1%) between the observed and predicted spectra of specimens with visible color: the observed peak hue was shifted to longer wavelengths than predicted (Figs. 1L–1N; Table DR1).

The multilayer reflector in *C. raja* comprises the bulk of the epicuticle, which in beetles is composed of volatile lipids cross-linked to protein; lipids are rare in the remainder of the cuticle (Neville, 1975). The lipid component of the cuticle therefore indicates the extent to which the chemistry of the epicuticle, and thus the multilayer reflector, is altered. Total lipid extraction of fresh *C. raja* cuticles generates

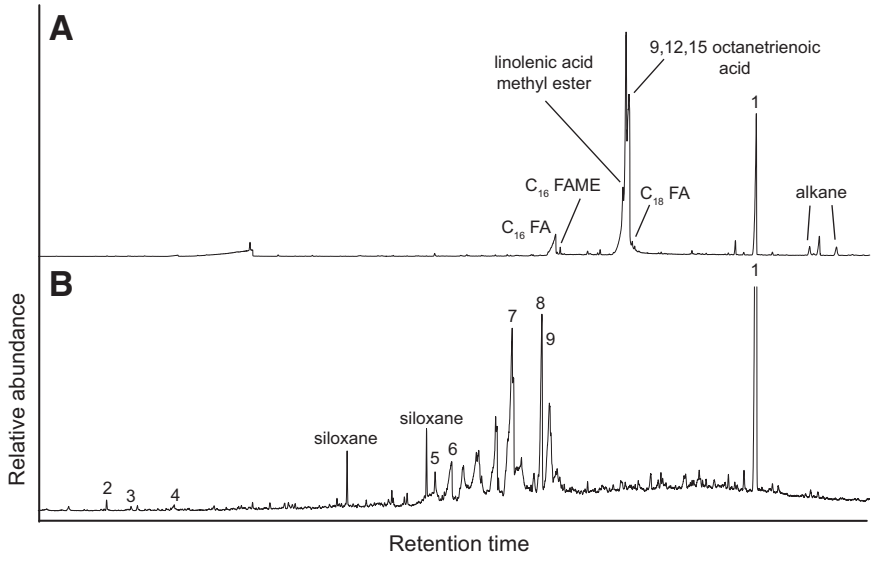

Figure 2. Total lipid extraction of fresh (A) and matured (B) *Chrysochroa raja* cuticles. Gas chromatography–mass spectrometry identification of compounds in matured cuticle: 1—1,2 benzenedicarboxylic acid mono (2-ethyl hexyl) ester; 2—acetophenone; 3—methyl-3-butenoate; 4—3-piperdidinone; 5—dimethyl amino (dimethyl) fluorophosphorane; 6—1H-pyrazole-1-carboxaldehyde,4-ethyl-4,5 dihydro-5-propyl; 7—piperazine derivative; 8—2,5 cyclohexadien-1-one 3,5 dihydroxy-4-4-20 dimethyl; 9—pyrolo (1,2-a) pyrazine 1-4 dione hexahydro-3-2 methyl propyl. C—carbon chain length; FA—fatty acid; FAME—fatty acid methyl ester.

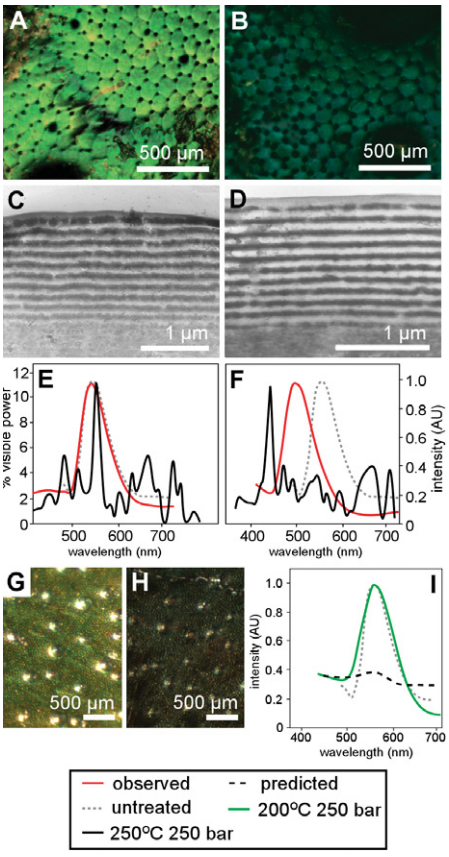

Figure 3. Relative impact of pressure and temperature on color in *Chrysochroa raja* cuticles. A,B,G,H: Light micrographs of the cuticle surface of specimens treated to 20 °C, 500 bar (A), 200 °C, 1 bar (B), 200 °C, 250 bar (G), and 250 °C, 250 bar (H); experiments lasted 24 h in A and B and 1 h in G and H. C,D: Transmission electron micrographs of the epicuticular multilayer reflector for specimens in A and B. E,F: Measured and predicted reflectance spectra for cuticles in A and B. I: Measured reflectance spectra for cuticles in G and H.

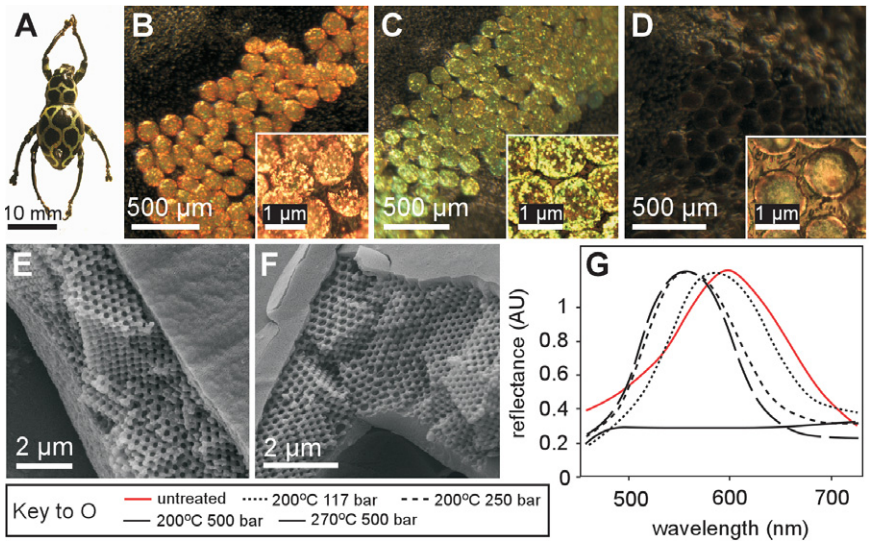

Figure 4. Experimentally induced color change in *Pachyrrhynchus reticulatus* cuticles. A: Dorsal aspect of untreated specimen. B–D: Light micrographs of structurally colored scales on elytra showing bands of densely packed, structurally colored scales in untreated specimens (B) and specimens treated to 200 °C, 250 bar (C) and 270 °C, 500 bar (D) with inset details of scales. E,F: Scanning electron micrographs of three-dimensional photonic crystals within the scale lumen in specimens in B and D. G: Measured reflectance spectra for untreated and experimentally treated cuticles.

a profile dominated by C_{16} and C_{18} straight-chain fatty acids and their methyl ester counterparts (Fig. 2A). The major lipid components of cuticles from the 270 °C, 500 bar treatment are markedly different. They are dominated by aromatic ketones (e.g., acetophenone) and derivatives of piperazine, pyrazine, and pyrazole (Fig. 2B), i.e., reaction products of functionalized lipids with thermally decomposed proteinaceous moieties.

Additional autoclave experiments on *C. raja* investigated the impact of pressure and temperature, each in isolation, on structural color (Fig. 3). The hue of cuticles treated to 500 bar at room temperature in Ar remained green (λ_{max} = 550 nm; this value is within the standard deviation of the entire sample set of untreated cuticles [548.24 ± 3.07 nm]) (Fig. 3A). Cuticles heated to 200 °C in Ar at atmospheric pressure altered from green (λ_{max} = 551 nm) to blue (λ_{max} = 496 nm) (Fig. 3B). The blueshift was accompanied by a decrease in the periodicity of the multilayer reflector from 161 nm to 145 nm (Table DR1).

Supplementary experiments were run at 200 °C or 250 °C at 250 bar for just 1 h; under the former conditions, cuticles remained green (λ_{max} = 552 nm) (Fig. 3G), but after the latter, cuticles were essentially black (Fig. 3H), with a near-flat profile characteristic of black materials (Fig. 3I) and micron-scale textures indicative of thermal degradation (Fig. DR4).

Structural colors generated by photonic crystals in *P. reticulatus* did not change during experimental degradation (Figs. DR5A and DR5B) but were altered during pressure-temperature treatment (Fig. 4). Scales on cuticles heated to 200 °C and treated to pressures of 117 bar, 250 bar, and 500 bar for 24 h altered from orange (λ_{max} = 591 nm) (Fig. 4B) to yellow-orange (λ_{max} = 582 nm), yellow-green (λ_{max} = 550 nm) (Fig. 4C), and green (λ_{max} = 541 nm), respectively; coloration disappeared during the 270 °C, 500 bar treatment (Fig. 4D). Electron microscopy revealed no obvious alteration in the structure of the photonic crystals (Figs. 4E and 4F).

DISCUSSION

Our experiments demonstrate that structural colors generated by multilayer reflectors are unaffected by decay but are blueshifted by the combined effects of pressure and, especially, temperature. The spectra observed in treated cuticles are, however, consistently offset (redshifted) from those predicted from the structure of the multilayer reflector. Thus the color changes observed in our experiments are not derived solely from alteration of the periodicity of the multilayer reflector. Small differences in the chemistry of biophotonic nanostructures can result in significant changes in refractive index and peak reflectance (Schultz and Rankin, 1985). Our experiments resulted in significant modification of the chemistry of the lipid component of the cuticle. Indeed, most of the primary components of the lipid extract of the experimentally treated cuticles have higher refractive indices than those of the fresh cuticles (Table DR3). The discrepancy between the predicted and observed spectra in our experiments therefore results from alteration of the chemistry, and thus refractive index, of the epicuticle. Our GC-MS data show that a key element of this transformation is reactions between lipids and proteinaceous moieties within the epicuticle. There is no evidence that dehydration of the epicuticle contributes significantly to the increase in refractive index: the thickness of the low-index, water-rich layers of the multilayer reflector does not alter preferentially during treatment. The observed color change in treated samples therefore reflects a decrease in the periodicity and refractive index of the multilayer reflector in combination; the former produced a significant color blueshift that is partially offset by a redshift induced by changes in cuticle chemistry.

Our results demonstrate that, as with other organic substrates (Price and Wenger, 1992), elevated temperature is the primary agent of alteration of biophotonic nanostructures during burial; elevated pressure alone has no discernible effect. The experiments undertaken at 250 bar for 1 h indicate that structural colors transform to black above a threshold temperature. Thus the absence of structural colors in certain insect-rich fossil biotas may indicate that burial temperatures

exceeded such a threshold. This hypothesis can be tested by relating data on the burial history of host sediments to the preservation of the cuticle. Insects are abundant and diverse in the Eocene Green River Formation (Utah and Wyoming, USA) but do not preserve structural colors or cuticular ultrastructures (McNamara et al., 2012a); geochemical studies of sedimentary organic matter indicate that the sediments experienced higher burial temperatures (~65–180 °C) (and pressures [~400–2200 bar]) than coeval biotas that preserve structural colors (≤40 °C, <100 bar) (McNamara et al., 2012a). Although other factors (e.g., Holocene weathering and microbial degradation of cuticles) can also induce color loss (McNamara et al., 2012b), burial temperature is identified as the fundamental taphonomic filter for fossilization of structural colors.

Three-dimensional photonic nanostructures and multilayer reflectors respond similarly under identical experimental conditions; the preservation potential of each is therefore similar. Multilayer reflectors are preserved in Pleistocene (Tanaka et al., 2010) and many Cenozoic biotas (Parker and McKenzie, 2003; McNamara et al., 2012b), and fossil dragonflies with metallic colors occur in the Cretaceous Santana Formation (Martill et al., 2007). Three-dimensional photonic crystals, however, have not been reported in fossils. Among extant beetles, biophotonic lattices occur in longhorn beetles (e.g., Colomer et al., 2012) (Cerambycidae) and many weevils (e.g., Wilts et al., 2012). We examined more than 200 fossil weevil specimens, including representatives of extant families that contain taxa with 3-D photonic crystals (e.g., Entiminae), from various Cenozoic fossil biotas (see McNamara et al., 2012a, 2012b), but found no evidence for 3-D photonic crystals. Assuming at least some of these taxonomic determinations are reliable (the preservation of many specimens is inadequate for detailed identification), the absence of 3-D photonic crystals in the fossil record cannot be an artifact of the fossilization process but instead reflects a late Cenozoic evolutionary origin.

CONCLUSIONS

Our experimental data resolve the principal controls on the fossilization of biophotonic nanostructures. Burial temperature is a fundamental taphonomic filter; fossil insect biotas dominated by biological signals can thus be identified and used in evolutionary studies of structural coloration. Previous studies have reconstructed the color of Cenozoic insect fossils using preserved cuticular nanostructures that show no evidence for physical modification such as fracturing or wrinkling (McNamara et al., 2012a). Our results, however, show that increased pressure and temperature can alter the dimensions of cuticular nanostructures without other modifications. Morphological analyses can reveal the organization of the nanostructure and identify optical effects (e.g., McNamara et al., 2011), but the original color is retained only where the effects of pressure and temperature are not significant (e.g., Tanaka et al., 2010). The lipid profile of the cuticle is a proxy for the extent to which the chemistry and, by extension, the observed color is modified. Future investigations will determine the extent to which this proxy varies between insect taxa and with the age and diagenetic history of the biota.

Our results show that biophotonic nanostructures survive in cuticles even when the original hue is no longer apparent following pressure-temperature treatment. Hence, it is likely that structural color in fossil insects is not restricted to visibly colored specimens but has an extensive cryptic history. This conclusion is supported by the discovery of a multilayer reflector preserved in a black fossil beetle from the Oligocene of Florissant, Colorado (USA) (Fig. DR6). Ultrastructural and geochemical investigation of such specimens has the potential to yield critical data on the evolution of structural color.

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