Super black eyespots of the Eyed elater

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Abstract

Scattering of light by surface structures leading to near complete structural absorption creates an appearance of "super black." Well known in the natural world from bird feathers and butterfly scales, super black has evolved independently from various anatomical structures. Due to an exceptional ability to harness and scatter light, these biological materials have garnered interest from optical industries. Here we describe the false eyespots of the Eyed elater click beetle, which attains near complete absorption of light by an array of vertically-aligned microtubules. These cone-shaped microtubules are modified hairs (setae) that are localized to eyespots on the dorsum of the beetle, and absorb 96.1% of incident light (at a 24.8° collection angle) in the spectrum between 300 – 700 nm.

Introduction

Black in nature is often achieved by pigments (*e.g.* melanin) that absorb most visible light [**1**, **2**]. In some cases, only ultraviolet light (320 – 400 nm) is reflected, such as in Asian whistling-thrushes [**3**]. Often, black pigment is overlaid by a glossy surface thereby imparting specular reflection increasing at angles normal to the illumination source, for example in many beetles [**4**]. Among insects, black pigmentation is typically achieved during the process of molting and tanning including sclerotization and melanization. In contrast, super black in butterflies, moths, birds, and snakes is usually achieved by structural absorption of nearly all light [**5**]. Three-dimensional structures, such as forest-like arrays of microtubules on butterfly wings and the highly ramified barbules on Bird of paradise feathers, act as a baffle to light [**6**, **7**]. (But, there are instances of "pseudo"-black achieved through additive mixing of structural green and magenta iridescence [**4**]). In some of these instances, super black structures evolved to impart varying degrees of reflection (blackness) dependent upon angle [**7**]. In others, structural absorption is assisted by melanin, and in Jumping spiders, brush-like 3D scales absorb light and stray light is recaptured by an underlying melanin-containing microlens array [**8**]. Functional explanations of the evolutionary origins of super black include sexual selection [**7**], predator defense [**9**], hydrophobicity [**10**], and for scotopic vision [**11**]. In nearly all of these examples of structural black, there is an array of protuberances on the surface of the animal that are perpendicularly oriented. These protuberances, which vary in composition from setae, microtrichia, barbules, and scales, have evolved repeatedly across the Tree of Life. Super black surface structures from nature have been applied to human industry since they have application for solar technology, anti-reflective

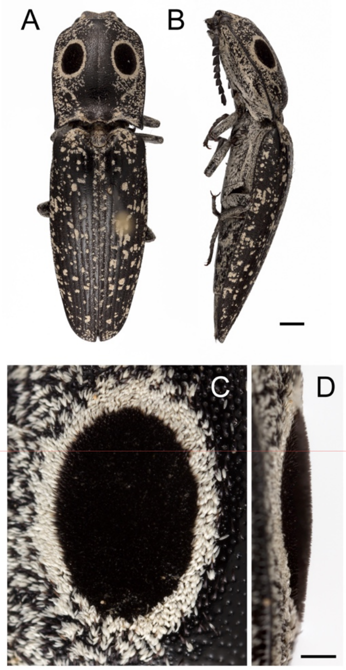

Figure 1 Eyed elater click beetle, *Alaus oculatus*, (A) dorsal habitus view, (B) right lateral view (scale bar = 2.0 mm); Eyed elater false eyespots, (C) left dorsal view, (D) right oblique view (scale bar = 0.5 mm).

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Comment [1]: This is capitalized throughout but probably shouldn't be, as it's neither a generic epithet or a proper noun

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Comment [2]: More arbitrary capitalization

coatings for the military, and a coating for the internal barrels of lenses in optical manufacturing [**8**, **12**].

Known colloquially as the Eyed elater or Eastern eyed click beetle, *Alaus oculatus* (Linneaus, 1758) (Fig. 1A, B) is a common beetle in the eastern U.S. with large and conspicuous eye-like spots on its back [**13**]. False eyespots have evolved independently in several lineages of insects including moths, cockroaches and mantises, butterflies, and beetles [**14**]. Two lineages of Click beetles (family Elateridae) in the subfamily Agrypninae possess ostensibly false eyespots, including some members of the genus *Alaus* and individuals of the tribe Pyrophorini [**15**]. The latter possess bioluminescent eyespots atop the pronotum and include the Headlight elater (*Pyrophorus noctilucus*), known colloquially as the "cucuyo", and other pyrophorine genera from the southern U.S. (*e.g. Deilelater*, *Ignelater*, *Vesperelater*). The bioluminescence of *P. noctilucus* is so bright it can be seen from afar and, according to ship logs, was confused by Spanish conquistadors with the smoldering matches of arquebusas held by indigenous inhabitants, thereby discouraging attack [**16**]. Eyespots are often used to deter predators and function to deflect attack to a non-vital body region or to startle predators [**17**]. These functions are the false-head ("lose-little-to-save-much" ref. **18**) and deimatic strategies [**19**]; however, in the case of the deimatic function it remains uncertain whether the eyespots deter attack because they appear as eyes (often of a larger, more intimidating animal) or due to their conspicuousness [**17**]. The false-head hypothesis for click beetles with eyespots atop their pronotum seems unlikely since the thorax houses vital organs such as the dorsal vessel and thoracic ganglia. Noting the similarity between the eyespots of the cucuyo and the Eyed elater, McDermott [**13**] examined the latter to determine if the eyespots were "luminous, or at least have beneath its chitin some structure indicating that the eyespots were a degradation of the photogenic organs of the cucuyo". Although he found thicker cuticle underlying the eyespots, potentially due to muscular attachments of the thoracic cavity, no structures consistent with the photogenic organs of the cucuyo were found. McDermott [**13**] remarked that the false eyespots may be "an extraordinary development of protective colouration."

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Comment [3]: It's okay to call them "eyespots" (or "false eyes"), I think the term "eyespot" implies that they're not true eyes. Perhaps "defensive eyespots?"

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As part of a study of structural coloration of insects in the Virginia Tech Insect Collection, we found striking examples of iridescence, but while observing the eyespots of *A. oculatus* in the in the collection and in the field in the Appalachian Mountains, we were struck by their profoundly black appearance at all angles. By depositing a thin metal film on the eyespots to exclude absorption by pigments, we tested if structural absorption provides the super black appearance. We compared the microstructures inside the periphery of the eyespots versus elsewhere on the exoskeleton of the beetle, and with the surface structural morphology of other instances of super black in nature.

Materials and methods

We used material preserved in the Virginia Tech Insect Collection for this study (VTEC, collection.ento.vt.edu). Three adult specimens of *A. oculatus* were selected for the analysis. The individuals were pinned dried specimens collected from Fredericksburg, Virginia and College Station, Texas (U.S.A.) with the following VTEC catalog numbers: VTEC000000784, 4961, and 4962. To visually examine gross morphology, the false eyespot of each specimen (right side) was examined at 45° and 90° angles with a Leica M125 stereomicroscope illuminated by a LED fiber optic light source. Setae composing the eyespot and the white ring encircling the eyespot (the "eyeliner") were removed with a straight-edge razor and mounted in glycerin on a microscope slide. Photographs of the setae were made with a Zeiss Axio Imager A2 microscope and AxioCam ERc5s camera. The beetle specimen was photographed with a Canon EOS 6D digital SLR camera illuminated with two Canon Speedlite 430EXII flashes diffused with a paper cylinder.

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Comment [4]: If you wanted to really go nuts (perhaps a future paper), you could split open some of the white setae and see if they have any specialized light-scattering structure inside. White setae in other beetle groups usually do.

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Comment [5]: This is an excellent word for it

To test the hypothesis that structural absorption contributes to the super black appearance, eyespots were plasma coated with a thin layer of platinum (Pt) and palladium (Pd) metals to control for absorption by pigments. From the middle of the right eyespot, including a piece of the eyeliner and surrounding cuticle, a 4.25 X 2.3 mm^2 tile was removed with a straight-edge razor and affixed on a 12.7 mm diameter aluminum scanning electron microscope (SEM) stub with double-sided carbon tape. The stub was plasma coated under stable argon pressure with 20 nm of Pt-Pd metals with a Leica EM ACE 600 high vacuum coater, and imaged on a FEI Quanta 600 FEG environmental SEM (5 kV, 3.5 spot size). A second round of 20-nm coating was carried out to ensure that pigmentation was entirely concealed. The width, spacing, and density of setae on the eyespots were calculated from the SEM images using the program ImageJ version 1.52k [**20**].

To measure reflectance, we used a spectrometer attached to a light source by a 400-µm diameter fiber core reflectance probe with a 24.8° acceptance angle (Ocean Optics USB 4000 spectrometer, QR400-7-UV fiber, and DH-BAL 2000 deuterium-halogen light source). A disc of PTFE was used as a reflectance standard to calibrate the measurements (Ocean Optics WS-1). Reflectance measurements were made in a dark room with the probe oriented at a 45° and normal incidence and at a detection distance of 3 mm. Units are in percent reflectance, and are reflection factors, or empirical measurements of intensity normalized by the intensity of the reflectance standard. Reflectance was measured between 300 – 700 nm, which encompasses the visible range of most animals. The eyespot, eyeliner, and the exoskeleton were measured from two individuals (VTEC000004961, 4962) three times. The R package pavo was used to analyze and visualize the reflectance measurements [**21**]. To calculate overall percent reflectance between 300 – 700 nm, area beneath the curve of the spectrum was summed with Riemann sums and divided by the total area of 100% reflectance between 300 – 700 nm. Reflectance spectra were averaged, standard error of the mean calculated, and plotted using the R package pavo.

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Comment [6]: Measuring reflectance from **both** the metal-coated and uncoated samples would be a nice empirical way to demonstrate how much light absorbance is purely structural. Would be great to add this if you have the time/access to spectrometer

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Comment [7]: Always from the same direction relative to the beetle's body axis? Or from front, back, and side?

Results

Based on visual examination with the light microscope, *A. oculatus* is generally black with white irregularly-shaped spots speckled across the body. The beetle possesses two large velvety black spots on the pronotum that are fringed in white eyeliner (Fig. 1C – D). The beetle is generally clothed with V-shaped seta of varying hue and texture, and the irregularly-shaped spots, white eyeliner, and false eyespots are made up of this seta. Outside of the false eyespots, and generally distributed across the cuticle of the beetle, the setae are brick red and have a smooth surface. The cuticle outside of the eyespots is smooth and glossy with lustrous specular reflection. The setae of the white eyeliner are translucent and lack pigment (Fig. 2A). Eyeliner setae and those outside of the eyespots are decumbent. In contrast the setae inside the periphery of the eyespots are erect, black with longitudinal grooves, and evenly spaced (Figs 2B – E, 3A). The V-shaped setae in the eyespots are more canoe-shaped than the others, with a flat slightly concave face opposite of the convex (hull) side (Fig. 2C, D). With the grooves, these setae appear as caraway seeds cut in half longitudinally, striped with lines running along its length (Fig. 2B – E). The cuticle underlying the eyespots is glossy and similar to the cuticle outside of the eyespots; however, its surface is recessed and dimpled around setal sockets (Fig. 3B). Some small pebbles and soil particles were trapped by the setae of the false eyespot.

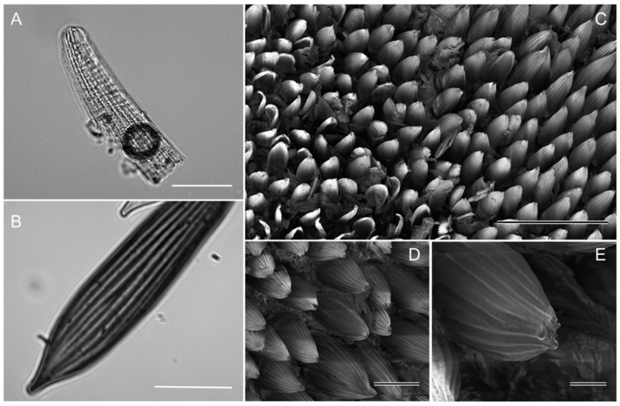

Figure 2 Eyed elater click beetle, *Alaus oculatus*, setal morphology, (A) transmitted light photograph of eyeliner setae (scale bar = 30.0 µm), (B) transmitted light photograph of eyespot setae (scale bar = 40.0 µm), (C) scanning electron micrograph of the eyespot setae, magnified 326X—scale bar = 0.2 mm, (D) magnified 1247X—scale bar = 0.05 mm, (E) magnified 5033X—scale bar = 0.01 mm.

When viewed at a 90° angle, even with the bright illumination of the microscope (illuminated at ca. 45°), little surface structure was apparent and the eyespots appeared profoundly black, appearing as voids in the body (Fig. 1C). When viewed at a 45° angle (and more acute angles), surface structure was discernable and the setae appeared regularly spaced with the convex side (hull) of the canoe-shaped hairs facing anteriorly (Fig. 3A). Along the periphery, the setae of the eyespots are bent at a ca. 60° angle and gradually change in angle to 90° at the center of the eyespot; in contrast, the setae at the posterior-facing margin remain upright and ca. 90° (Fig. 3A).

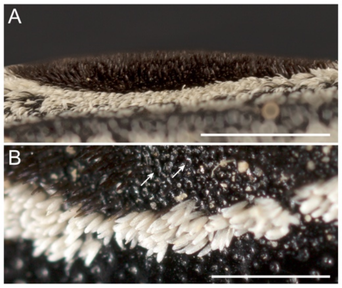

Figure 3 Eyed elater click beetle, *Alaus oculatus*, false eyespots, (A) lateral view (scale bar = 1.0 mm); (B) right dorsal view, dimples denoted by arrows (scale bar = 0.5 mm) [left side of A and B is anterior]

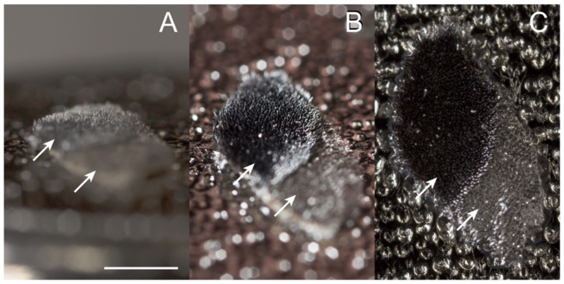

Figure 4 Eyed elater click beetle, *Alaus oculatus*, false eyespot (top arrows) and eyeliner (bottom arrows) coated with a 40-nm layer of platinum and palladium, (A) 30° view, (B) 45° view, (C) 90° view.

As a result of plasma coating, the SEM stub possessed a lustrous metallic surface, however the eyespot retained a deep black appearance at normal incidence (Fig. 4, Supplemental Movie). Based on examination of the eyespots with the SEM (between 326 – 5,033X magnification), the V-shaped seta are lined with about 14 longitudinal ridges (mean = 13.97, standard deviation = 1.92, n = 30) with a smooth somewhat concave opposing surface (Fig. 2B – E). The apices of the setae are dividing into two or occasionally three shallow furcations (Fig. 2E). Setae are about 38.33 µm in width (mean, standard deviation = 2.15, n = 30) and spaced about 10.30 µm edge-to-edge from one another (mean, standard deviation = 2.67, n = 30). Within in a 726.68 μm^2 area of the eyespot there are 250 setae, and 1,756 setae within the eyespot area in total.

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Comment [8]: And non-eyespot portions of the cuticle sample?

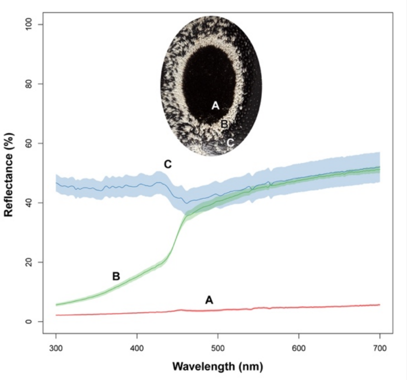

Figure 5 Reflectance spectra of the Eyed elater eyespot, (A) red line = eyespot, (B) green = white eyeliner, (C) blue = glossy cuticle

From the measurement of reflectance, the white eyeliner, the glossy exoskeleton and the black eyespot possessed spectra of different shapes (Fig. 5). The black eyespot spectrum was a flat profile, indicating a general lack of reflectance across a broad range of wavelengths. The white eyeliner spectrum had a plateau shape, lacked near-ultraviolet reflectance, and with uniformly high reflectance between 400 – 700 nm. The glossy exoskeleton spectrum was generally flat in profile but with consistently high overall reflectance (including ultraviolet) indicating a high glare from the lustrous cuticular surface. The overall reflectance of the black eyespot patch was 3.90%, the white eyeliner was 32.92%, and glossy exoskeleton was 46.14%. The overall reflectance of the black eyespot measured at a 45° specular orientation was three-fold less: 1.26%.

Discussion

We found that structural absorption gives a super black appearance to the eyespots of *A. oculatus*. By depositing a thin metal film on the eyespots to conceal light absorption by pigments, we demonstrated that pigmentation alone is not responsible for their deep black form. Based on our examination of the black eyespots, we found that their surface morphology was equivalent in shape, orientation, and general photonic properties of other super black structures in nature. In particular, the eyespot composed an array of perpendicularly aligned linear protuberances that absorb 96.1% of light and is analogous to the three-dimensional array of microtubules on

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Comment [9]: "Prohibit" might be a better word

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Comment [10]: "appearance" or "color?" Another way to describe this mechanism is that it "eliminates specular reflectance"

butterfly wings [**6**] and the ramified barbules on Bird of paradise feathers and Jumping spiders [**7**, **8**]. Other examples of structural super black in nature with similar perpendicularly aligned protuberances include the nipple array of moth eyes [**11**], cuticular papillae of stick insects [**10**], and leaf-like microstructures on viper and Peacock spider scales [**8**, **9**]. These surface structures scatter light, causing structural absorption and leaving little light available to reach the viewer's eye. While human-fabricated super black materials typically absorb more than 95.5% of light, in nature, Bird of paradise feathers come close to this with incident reflectance of about 0.5%. Butterflies and moths, snakes, stick insects, and click beetles with super black patches reflect more (incident) light with between about 3 – 11%.

Melanin is a ubiquitous black pigment of insect exoskeletons, and is an expected component of the setae of the Eyed elater's eyespots. Melanin, or another pigment, contributes to the super black form of the eyespots by absorbing light in concert with structural absorption. The pigment directly absorbs light and perhaps also recaptures light that strays from structural absorption. We showed that the eyespot setae sit in a concavity (Fig. 3B), and the cuticle underlying the eyespots has a dimpled topography. These concavities are smooth and have a black pigmentation. Other arthropods possess similarly shaped concavities that scatter light and impart additive mixing (of blue and yellow iridescence such as in the Emerald swallowtail [**22**]), or augment melanin absorption thereby decreasing reflectance and producing super black (as in Peacock spiders [**8**]). These lens-like concavities of the Eyed elater's eyespots and the 14 longitudinal ribs on the setae could be features that assist a super black. Additionally, scattering of light may be directional (*e.g.* backwards) given the shape and orientation of the setae on the eyespots. Optical modeling integrating these features would be fruitful to understand how this ensemble of features work in concert to affect light.

The function of super black eyespots in the Eyed elater may be for predator deterrence including aposematism, deimatism, or as a false head. A role as a false head is unlikely since the eyespots are in close proximity to the real head and not posteriorly located as in other insects (*e.g.* hairstreak butterflies). Since there are large dorsal intersegmental muscles directly beneath the eyespots [**23**], thermoregulation or muscle heating is another functional hypothesis. While the startle function is the most likely, click beetles have a powerful clicking mechanism that is noxious to birds [**23**, **24**] and disentangling aposematic versus startle function would ideally be tested using field experiments.

The study of super black structures in nature have uncovered a diversity of morphologies that cause near complete absorption of light. Some of these structures collimate light for subsequent excitation of photopigments of compound eyes (*e.g.* crepuscular moths, ref. **11**) and others act as a general baffle of light where light is scattered (*e.g.* crypsis in snakes, ref. **9**). Since super black materials have application for human industry (*e.g.* solar cells, anti-reflective coatings, etc.) the structural morphology of these various materials in nature are an ideal domain as creativeness for fabricating structures and as a means to understand the evolution of adaptive coloration and natural selection.

Acknowledgements

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Comment [11]: There are probably some reviews of lepidopteran eyespots in the literature, but I don't think we know anything about beetle eyespots! Review paper someday?

Thanks to Drs. Ellen Brown and Barry Lee Bressler for support of the Virginia Tech Insect Collection and a donation of beetle specimens, including the two Eyed elaters used in this study. Jackson Means assisted with counting the ridges on setae. Doro Tholl provided access to the Zeiss microscope. Steve McCartney and Chris Winkler at the Nanoscale Characterization and Fabrication Laboratory at the Virginia Tech Institute for Critical Technology and Applied Science assisted with SEM. We are grateful to Charity Hall for editing previous versions of the manuscript, and anonymous reviewers for suggestions and improvements.

Data accessibility

R script, uncompressed image files, and TXT text file spectral data are available to download from VTechData [**25**], https://data.lib.vt.edu/collections/rf55z781b

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