Arthropod Structure & Development xxx (2016) 1–10

Contents lists available at ScienceDirect

Arthropod Structure & Development

journal homepage: www.elsevier.com/locate/asd

Structural and physical evidence for an endocuticular gold reflector in the tortoise beetle, *Charidotella ambita*

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ARTICLE INFO

Article history:
Received 4 July 2016
Received in revised form
3 October 2016
Accepted 5 October 2016
Available online xxx

Keywords:
Elytra
Ultrastructure
Structural color
Gold reflector
Tortoise beetle
Chrysomelidae

ABSTRACT

Charidotella ambita offers a unique opportunity for unambiguously locating its gold reflector by comparing the structure of reflecting and non-reflecting cuticle of the elytron and pronotum. Using light microscopy and TEM, the reflector was located underneath the macrofiber endocuticle just above the epidermis. The reflector is a multilayer comprising up to 50 bilayers alternating high and low density layers parallel to the surface of the cuticle. It is chirped, i.e., showing a progressive decrease in layer thickness from approximately 150 nm–100 nm across its depth. The high density layers in contact with the endocuticle fuse to the last macrofiber when the reflector is interrupted by a trabecula, demonstrating their cuticular nature. Simulated reflectance spectra from models of the multilayer matched the reflection spectra measured on the major gold patch of the elytron of living specimens.

Previous reports in adult insects exhibiting metallic colors located their reflector in the upper strata and structures of the cuticle, i.e., epicuticle, exocuticle, scales and hairs. Thus, the endocuticular location of the reflector in *C. ambita* (and other tortoise beetles) appears unique for adult insects. Gold reflection appears in *C. ambita* only when the synthesis of the macrolayer endocuticle is complete, which may take up to 2 weeks. The development of the gold reflector coincides with the start of mating behavior, possibly suggesting a signaling function in conspecific recognition once sexual maturity has been reached.

1. Introduction

The family name chosen by Linnaeus for leaf beetles, Chrysomelidae, was derived from the Greek for “gold beetles” (Greek χρυσομηλον, literally ‘golden apple’, influenced by χρυσομηλολόνθιον ‘little golden chafer’). No doubt Linnaeus who first coined the type genus name *Chrysomela* from which Chrysomelidae was later derived, was responding to the brilliant “metallic” colors of the beetles he included in this taxon (presently including both genera, *Chrysomela* and *Chrysolina*). Metallic colors are not just the privilege of leaf beetles, but occur widely throughout the Arthropoda, e.g., jumping spiders. The coloration in most of these derives from the multilayered structure of the hard, dry, peripheral layer of the exoskeleton (e.g., Neville and Caveney, 1969; Vigneron et al., 2006; Seago et al., 2009; Stavenga et al., 2011; Ingram et al., 2011), although an endocuticular reflector was reported in danaine pupae (Steinbrecht, 1985; Steinbrecht et al., 1985). Reflective metallic colors (or structural colors) produced by light interference are widespread in biology and are the subject of the active field of biophotonics, the search for bio-inspired materials (e.g., McPhedran and Parker, 2015). The layered exoskeleton of insects has presented natural selection many options for building structural colors, ranging from simple diffraction gratings to two- and three-dimensional photonic crystals in the scales of butterflies and beetles (Berthier, 2007). Metallic colors in Coleoptera are rarely pure gold, but instead are shiny, often iridescent, shades of yellow, green, blue and red. Pure gold reflectors are rarer, occurring in the

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http://dx.doi.org/10.1016/j.asd.2016.10.008
1467-8039/

elytra and pronota of scarab beetles in the genus *Chrysina* and in the discal elytra and pronota of Cassidini and Aspidimorphini tortoise beetles (where lateral and frontal margins are usually transparent). Metallic colors may be permanent as in scarab beetles and tortoise beetles in the tribe Omocerini or they may be transient, degrading rapidly from the live state and totally absent in dead specimens. Permanently metallic-colored species are often sought after by amateur "collectors" in commercial insect fairs. The transient metallic coloration of tortoise beetles in the tribes Cassidini and Aspidomorphini vanishes in dead and dried specimens, and are replaced by a dull yellow or reddish brown color. To a limited extent, the reflective gold color can be restored when dried specimens are rehydrated (Mason, 1929).

Yet other tortoise beetles have the ability to reversibly shut down their reflectivity upon disturbance, switching quickly from bright gold to reddish brown or vivid red if a sub-epidermal red pigment layer is present. Later and less quickly the same beetles, when alive, can restore their gold coloration (Mason, 1929; Hinton, 1973; Jolivet, 1994; Vigneron et al., 2007b). This unique capacity was investigated in two species, the South African *Aspidomorpha tecta* (Hinton, 1973; Neville, 1977; Parker et al., 1998) and the Panamanian *Charidotella egregia* (Vigneron et al., 2007b). In both species, a chirped multilayer reflector was described in the cuticle, and the switch of color attributed to the amount of liquid secreted (or resorbed) by the epidermal cells between the cuticular lamellae, although the precise mechanism remained to be disclosed. As in dead specimens, the reflector would be shut down, and dehydrated. In *Aspidomorpha tecta*, the chirped reflector was located in the endocuticle, just above the epidermis as illustrated by a single TEM micrograph, obviously from a freshly fixed elytron, published by Hinton (1973), and further used by Neville (1977) and Parker et al. (1998) in their later analyses. In *C. egregia*, however, the chirped reflector was located in the exocuticle as were the reflectors of other colored beetles exhibiting metallic colors. SEM and TEM micrographs obtained from dead, dry elytra were published in support of this location by Vigneron et al. (2007b).

Metallic coloration in some Cassidini species is limited to specific regions of the elytra and pronotum, alternating with reddish brown or black regions to form specific elytral designs. This configuration is present in *Charidotella ambita*, a species with a "fixed reflector", unable to change color when alive, but lacking the gold color entirely in dead, dry specimens, where it is replaced by dull yellow or brown colors.

C. ambita offers a unique opportunity for comparing the ultrastructure of reflecting and non-reflecting cuticle unambiguously identified within elytra from a single individual. Gold reflection is initially absent in immature adult individuals developing slowly over several days and in this way offering a way to observe ontogenetic changes in the ultrastructure of reflecting and non-reflecting cuticle. In this paper, we describe cuticle structure for elytra and pronotum of mature and immature adults of *C. ambita*. We deduce a putative reflector based on this morphological analysis. We simulate reflectance from the modeled reflector with this structure, which is then compared to that measured on living and freshly killed beetles.

2. Material and methods

Ten mature and two immature adults (age unknown) were collected on May 5, 2015, in El Copé (8.670°N, 80.593°W; Coclé Province, República of Panamá) while feeding on their host plant, *Ipomoea* sp. aff. *trifida* (Convolvulaceae). The immature adults were distinguished from mature adults by the lack of gold reflection and a softer cuticle. Five mature and two immature beetles from this collection were later fixed for TEM observations. Five mature beetles were kept alive for further spectroscopic measurements (in Namur, Belgium). Duplicate specimens were deposited as vouchers in the STRI reference insect collection and in the Museo de Invertebrados, Universidad de Panamá (MIUP).

Elytra were detached using microsurgery scissors and were cut transversely into anterior, median and posterior sections. The pronotal sclerite was cut longitudinally into two roughly equal pieces. The elytra and pronotal pieces were fixed overnight in cold 2% glutaraldehyde in a 50 mM Na-cacodylate buffer at pH 7.3 containing 150 mM saccharose. The pieces were rinsed in the buffer and kept in cold buffer until further processing.

After postfixation in 2% osmium tetroxide in the same buffer and dehydration in a graded acetone series, tissues were embedded in Araldite. Semi-thin sections with a thickness of 1 μm were made with a Leica EM UC6 ultramicrotome, stained with methylene blue and thionin before examination under an Olympus BX-51 microscope. Thin sections of 70 nm thickness were double stained with lead citrate and uranyl acetate, and examined under a Zeiss EM900 electron microscope. Material for scanning microscopy was mounted on a stub, coated with gold and examined in a JEOL JSM-6360 scanning microscope.

Reflection spectra ($R = I - B/W - B$) were measured using an Avantes AvaSpec-2048-2 fiber optic spectrophotometer and an Avantes AvaLight-DH-S-BAL deuterium/halogen light source. The measured spectra I were normalized with respect to the intensity W reflected by a white reference standard Avantes WS-2, with correction B for experimental optical and electronic noises and with respect to their respective maxima. The measurements were performed with a bifurcated optical fiber normal to the sample (guiding incident light to the sample and reflected light to the spectrophotometer). Live beetles were cooled during 20 min at 4 °C before measurement and positioned so that spectra were measured on the flatter section of the elytra.

The ultrastructure of the gold reflector was modeled as a planar multilayer stack. In different regions of the endocuticle (semi-ovoid central patch, explanate margin border), the multilayer stack could be recognized on the TEM micrographs from a typical pattern of alternating bright and dark layers. Along a line perpendicular to the stack, layers were counted and their thicknesses measured with the help of image processing software (ImageJ, Schneider et al., 2012). Thickness sampling error was estimated to 10%. Since the cuticle material consists of chitin microfibers in a protein matrix, density variations of chitin within the matrix modify the layer appearance (grey level) in TEM observations, i.e. dark layers are more dense than bright ones. Based on the reported refractive index value of pure chitin ($n_c = 1.56$ [Sollas, 1907; Leertouwer et al., 2011]), refractive indices of bright and dark layers were assumed to be around $n_b = 1.40$ and $n_d = 1.60$, respectively (combinations of slightly different values were tested in order to check the sensitivity of simulations to these parameters). From these refractive index values and layer thickness data, the reflectance spectrum of the multilayer stack was calculated at normal incidence using a computer code which implements the exact solutions of Maxwell's equations in arbitrarily stratified media (Vigneron and Lousse, 2006). The refractive indices of incidence and emergence semi-infinite media surrounding the multilayer stack were taken equal to $n_i = 1.0$ (air) and n_d (more dense cuticle), respectively.

From both experimentally normalized reflection and numerical reflectance spectra, chromaticity coordinates (x; y) were calculated (assuming a standard illuminant D65) following a method presented elsewhere (Judd and Wyszecki, 1975; Chamberlin and Chamberlin, 1980) and represented in the CIE 1931 color space chromaticity diagram.

Please cite this article in press as: Pasteels, J.M., et al., Structural and physical evidence for an endocuticular gold reflector in the tortoise beetle, *Charidotella ambita*, Arthropod Structure & Development (2016), http://dx.doi.org/10.1016/j.asd.2016.10.008