Varying and unchanging whiteness on the wings of dusk-active and shade-inhabiting *Carystoides escalantei* butterflies

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Whiteness, although frequently apparent on the wings, legs, antennae, or bodies of many species of moths and butterflies, along with other colors and shades, has often escaped our attention. Here, we investigate the nanostructure and microstructure of white spots on the wings of *Carystoides escalantei*, a dusk-active and shade-inhabiting Costa Rican rain forest butterfly (Hesperiidae). On both males and females, two types of whiteness occur: angle dependent (dull or bright) and angle independent, which differ in the microstructure, orientation, and associated properties of their scales. Some spots on the male wings are absent from the female wings. Whether the angle-dependent whiteness is bright or dull depends on the observation directions. The angle-dependent scales also show enhanced retro-reflection. We speculate that the biological functions and evolution of *Carystoides* spot patterns, scale structures, and their varying whiteness are adaptations to butterfly's low light habitat and to airflow experienced on the wing base vs. wing tip.

butterfly wings | whiteness | angle dependent | retro-reflection | Area de Conservación Guanacaste

Butterflies and moths display on their wings a dazzling array of colors (1, 2)—from jet black, looking like a hole in the wing, to bright white like a bicycle reflector in automobile headlights—and with every imaginable color chart hue in-between. Not only are the colors there, but they occur in hundreds of thousands of patterns. Wings are flying billboards and exceedingly complex answers to the optimization that past and present natural selection imposes, whether to some aspect of courtship or escape from predators (3, 4), and even at times in regulating body temperature or as serendipitous outcomes of selection for texture, toughness, waterproofing, light weight, and air friction (5–7). To date, most attention has been on the mechanics and chemistry of a particular color of some easily accessible species that are particularly attractive to humans. For example, the bright blue morpho butterflies (8–12) have inspired many physicists, materials scientists, and engineers to investigate how the color is produced (9, 13–15), and then to mimic the color and its mechanisms, or the combination of color and water repellency (11, 16, 17).

Whiteness, although frequently apparent on the wings, legs, or bodies of many species of moths and butterflies, along with other colors and shades (18–21), has received relatively little attention and is easily ignored. Although “white” may often be simply one more color among many within a larger complex pattern, there are times when the whiteness itself appears to be a key signal. Note that there is no pigment for “whiteness.” Indeed, structural whiteness is technologically important in systems ranging from power-efficient computer displays, to sensors, to energy-efficient buildings, windows, and vehicles (22–24).

Natural whiteness is often attributed to the scattering or diffusion of light from (sub)micrometer-sized textures including ribs, ridges, pores, and others (25, 26) as seen as white spots and stripes on the wings of *Morpho cypris* and *Morpho rhetenor helena* (18, 27) in the Nymphalidae. Other species, such as *Pieris brassicae* and *Delias nigrina* in the Pieridae, have 100- to 500-nm beads randomly packed in-between ribs and cross-ribs, enhancing the whiteness (19). Metallic to silvery whiteness occurs on some butterfly wings as the result of mixing structure colors reflected from the membrane scale. For example, the thickness between ribs and scale membrane on *Argyrophorus argenteus* (Nymphalidae) wings changes across the wing; although multiple reflective colors can be seen at the microscopic scale, bright metallic white appears at the macroscopic scale (28). The ease with which shining metallic whiteness could be distinguished from dull whiteness and other colors could be important for communication between butterflies and for the butterfly's evolution–devolution.

Here, we investigate a dusk-active and shade-inhabiting Costa Rican hesperiid butterfly, also known as a “skipper butterfly,” *Carystoides escalantei* (janzen.sas.upenn.edu/) (29), which has different types of whiteness, including angle dependent (dull vs.

Significance

Whiteness, although frequently apparent on the wings, legs, antennae, or bodies of many species of moths and butterflies, has often escaped our attention. Here, we investigate the nanostructure and microstructure of white spots on the wings of *Carystoides escalantei*, a dusk-active and shade-inhabiting Costa Rican rain forest butterfly (Hesperiidae). We identify two types of whiteness: angle dependent and angle independent. We speculate that the biological functions and evolution of *Carystoides* spot patterns, scale structures, and their varying whiteness are adaptations to the butterfly's low light habitat and to airflow experienced on the wing base vs. wing tip during flight. Sex and species differences in the location of angle-dependent white spots on the wings may function in both intraspecific and interspecific communication.

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The whiteness of the spots *a1*–*a3* on the ventral side is shown in Fig. S1. All of these spots are bright white when viewed from the wing base. For comparison, another four species of *Carystoides*—*C. hondura*, *C. Janzen03*, *C. basoches*, and *C. Burns01*, which were reared from wild-caught caterpillars by the biodiversity inventory of the rain forests of ACG in northwestern Costa Rica (janzen.sas.upenn.edu/caterpillars/database.lasso) (29–32)—were also examined. As seen in Fig. S3, the spot *a3* (male, dorsal) always has the opposite reflection direction to *a1* and *a2*. However, there is inconsistency of spots *a4* (male, ventral, Fig. S4); whereas some showed bright white from the wing tip, others showed bright white from the wing base. We hypothesize that the difference in locations of the white spots and the resulting optical properties on male and female *Carystoides* are adaptations to some aspect of courtship and both interspecific and intraspecific recognition.

Closer examination of the scales on angle-independent white spots shows micrometer-sized periodic ridges with micropores in-between (Fig. 3C and Fig. S5B). The scales on the angle-dependent white spots, on the other hand, are undulated like ripples, consisting of periodic ridges and ribs that are perpendicular to the ridges on both sides of the scale (Fig. 3F; see definitions in Fig. 3G). From the 3D topography profile of the scale obtained by atomic force microscopy (AFM) (Fig. S6), the periodicity and height of the ridges are $\sim 2\text{ }\mu\text{m}$ and 400–500 nm, respectively, and the periodicity and the height of the ribs are ~ 165 and 50–100 nm, respectively. The thickness of the scale membrane is ultrathin, ~ 150 nm (Fig. 3F, *Inset*), and has low AR (AR of ~ 1 , estimated from the optical image in Fig. 3H). Such

Fig. 4. (A) Top-view SEM images of three typical types of scales from angle-dependent white spots. (B) Side-view SEM images of angle-dependent spots from (i) *a1* male, (ii) *a1* female, and (iii) *a3* male, respectively. (Insets) SEM images of the follicles (marked by the white dashed boxes) in the corresponding scales. The red arrows indicate the joints between the scale and the follicle. (C) Schematic illustrations of the change of scale angles θ along the wing body due to the airflow when flapping up and down.

structure seems to provide a stiff lightweight scale that resists gravity and alleviates aerodynamic stresses.

Based on the full-scale observation on different white spots, we categorize the standing scales into three types as summarized in Fig. 4A, including wavy scales (type A), lightly curled scales (type B), and highly curled scales, like cut-out cones (type C) with different tilting angles. As illustrated in Fig. S2, the tilting angle of the scales, marked as scale angle θ , indicates the intersection angle from the normal direction of wing membrane to the central axis of the scale. θ is set as positive if the scale tilts toward the wing tip. θ values from angle-dependent white spots were obtained and summarized in Table 1. Nearly all scales on the male *Carystoides escalantei* wings are wavy (type A), whereas those on the female *Carystoides escalantei* wings are curved (type B and type C). Furthermore, the scale angle decreases from the wing tip to the wing base on both male and female butterfly's forewings. On the dorsal side, the scales typically stand vertically on the wing membrane with smaller θ , whereas those on the ventral side tend to have a larger θ . Interestingly, the scale of *a3* (male, dorsal) is tilted to a negative angle (e.g., -15° as shown in Fig. 4Biii). In other words, the scale is tilted to the opposite direction of the scale holder at the root (see *Insets* and red arrows in Fig. 4B), and θ varies from specimen to specimen.

However, why do they present such different scale angles? The tilting angle of follicle and scale root are all $\sim 45^\circ$ and scales start to bend at the connection between the root and the main scale. Our hypothesis is that the variety of scale angles is the response of the standing scales to the airflow across the surface of the wing. As seen in Fig. 4C, the scales on the dorsal side could be pushed up by the airflow when the wing is flapping up because the airflow direction (from wing tip to wing base) is against the

scale tilting direction (from wing base to wing tip). When the wing is flapping down, scales are pushed down because the airflow is in the same direction as the tilted scales. As the push of airflow gradually increases in the direction of airflow (see bold arrows in Fig. 4C), the scale angle decreases when the airflow is against the scales' tilting direction but increases when the airflow follows the scales' tilting direction. This could explain why the scales of *a3* (male, dorsal) tilt to the opposite side of the scale holder because there should be stronger airflow near the wing base region. However, the scale angles of *a4* on the hind wings of male *Carystoides escalantei* vary from species to species (Table 1), which is consistent with their optical appearance on the wings (Fig. S4). We believe it may be attributed to the complex airflow on the hindwing created by the flapping of both forewing and hindwing.

Typically, whiteness is the result of scattering by random microstructures (Fig. 3C) accompanied by no pigment or dye beneath the scales. When the size of the features of the structures on the scales decreases to submicrometer sizes and becomes more regular (Fig. S4A and B), the scattering is decreased and whiteness becomes the result of mixing multiple grating colors as appears to be the case in the standing scales with angle-dependent white spots. As seen under the optical microscope at the microscale, *a1* (male, dorsal) appeared as purple head-on from the standing scales (Fig. 3D) but multicolored when viewed from the scale surface (Fig. 3H). The purple color could be attributed to the interference of the periodic nanoribs (see schematics in Fig. 3Gi). Supporting this is the prediction from multibeam interference (see detailed discussion in [Supporting Information](#)). We believe the multicolors to be the combination of thin film interference at air/scale membrane, air/(membrane + rib) and air/(membrane + ridge) interfaces of different thicknesses (see schematics in Fig. 3Gii). This is consistent with the prediction shown in [Supporting Information](#) (Table S1) and further supported when we put a thin poly(dimethylsiloxane) (PDMS) film in contact with the bottom side of the scale and the bright color was lost (see the dark region in Fig. 3I). This could be attributed to the decreased refractive index contrast, where air is replaced with PDMS, and thus decreasing the overall interference intensity. It is also consistent with the observed angle dependence of the white spots.

Enhanced Retro-Reflection on Angle-Dependent White Spots. To study the angle-dependent whiteness at a single scale level, we used backscattering, reflection probe (Fig. 2E, *Inset*), and fiber spectrometer to characterize the reflection of the white spots. Fig. 5A presents the retro-reflection intensity of two typical white spots with $\theta = -15^\circ$ (*a3*, male, dorsal) and 40° (*a1*, female,

Table 1. Scale angles of three types of scales on angle-dependent white spots

Location of the white spots	Male		Female	
	Scale type	Scale angle, θ	Scale type	Scale angle, θ
<i>a1</i> Dorsal	Type A	25°	Type B	20°
Ventral	Type A	52°	Type B	40°
<i>a2</i> Dorsal	Type A	20°	Type B	10°
Ventral	Type A	45°	Type C	35°
<i>a3</i> Dorsal	Type A	-15°	Type B	5°
Ventral	Type A	35°	Type C	35°
<i>a4</i> Dorsal	Type A	-12° to 5°	—	—
Ventral	Type A/type B	$\sim -10^\circ$ or $\sim 10^\circ$	—	—

The structure and optical characterization showed that spot *a3* (male, dorsal) had the opposite scale tilting angle and retro-reflection direction (Fig. 2 and Fig. 5D) in comparison with the spots *a1*, *a2* on male, dorsal wing, or *a1*, *a2*, *a3* on female, dorsal wing. As we speculate in Fig. 4, the main possibility to form opposite tilting scales on spot *a3* (male, dorsal) was the push force of airflow when *Carystoides* flies. In the evolution of these traits, *Carystoides* may recognize spot *a3* (male, dorsal) for courtship and male–male antagonism interactions.

Conclusions

White spots on the wings of the skipper butterfly *Carystoides escalantei* differ intersexually in number and location, and intrasexually in the constancy and variability of their whiteness. Two types of white spots, angle dependent (dull or bright) and angle independent, appear on both male and female wings, but differ from each other in the microstructure, orientation, and associated properties of their scales. The scales of angle-independent spots are long, flat against the wing membrane, and stacked atop one another; each has micrometer-sized periodic ridges with micropores between them. However, the scales of angle-dependent spots are vertical and variably tilted; and numerous closely set and slightly curved thin ribs are perpendicular to periodic ridges formed by overlapping segments. The whiteness appears to be a mixing of grating colors coming from the hierarchical structure and the tilting of the scales. The angle-dependent scales enhance retro-reflection. The bright vs. dull aspect of whiteness depends on whether the more or less vertically oriented scales are observed from the direction of the wing base or the wing tip. Scale structure also relates to differing airflow at the wing base vs. wing tip as the wing moves up and down during flight. This analysis may catalyze the development of materials mimicking structural whiteness for use in sensing and in displays with low energy consumption.

Evolution of the two kinds of white spots, one of which brightens when viewed at certain angles, is probably an adaptation to the dusky period of butterfly activity combined with the shady habitat of *Carystoides*. Altogether, the spots may function in communicative behavior such as camouflage, species and sex recognition, competition, and courtship.

Materials and Methods

Carystoides butterflies, including *Carystoides escalantei* [now known to be two extremely similar sympatric species, one undescribed (29)], *Carystoides hondura*, *Carystoides Janzen03*, *Carystoides basoches*, and *Carystoides Burns01*, were reared from wild-caught caterpillars by the biodiversity inventory of the rain forests of ACG in northwestern Costa Rica. Morphologies of the wing scales of *C. escalantei* were characterized by a field emission high-resolution scanning electron microscope (FEI Strata DB235) operated at 5.0 kV after sputter coating of a thin layer of gold/palladium (Au/Pd). The scale tilting angles were measured by tilting the sample stage in the scanning electron microscope. Optical microscopy images were taken by an Olympus BX 61 in the reflection mode. The surface topography of the scales, which were picked up by a 1-mm-thick PDMS film, were imaged by AFM (Bruker Icon) using a Si_3N_4 cantilever in the tapping mode. The angle-resolved reflection spectra were collected from a USB4000 fiber optical spectrometer (Ocean Optics) with home-built rotating stage, or connected to an optical microscope (BX 61; Olympus). Fig. S2 illustrates the definition of incident angle α , detecting angle β , and specular reflection angle γ . These angles are marked as positive if they move toward the wing tip side.

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