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Wing Scale Orientation Alters Reflection Directions in the Green Hairstreak Chrysozephyrus smaragdinus (Lepidoptera; Lycaenidae)

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There have been only a few reports on the directional reflection of light by butterfly wings. Here, we systematically investigated this phenomenon in a lycaenid butterfly, Chrysozephyrus smaragdinus, in which males have bright green wings based on structural coloration. We used a device that measures intensities of light in hemispherical space by vertical shifting of a sensor and horizontal rotation of the stage carrying the wing, which is illuminated from the top, to determine the direction of light reflected by the fore- and hindwings. The orientation and curvature of wing scales were examined microscopically. The forewing of this species reflected light shone from the top largely forward, whereas the hindwing reflected it slightly forward. This difference was attributed to the tilt angles of the wing scales. Light reflection by the forewing was relatively weak, and widely scattered, whereas that by the hindwing was rather concentrated, resulting in higher reflectance. This difference was attributed to difference in the curvature of the wing scales on the two wings.

Key words: directional reflection, orientation, wing scale, Lepidoptera, Lycaenidae, Chrysozephyrus

INTRODUCTION

Wing colors of butterflies are produced in two ways: pigmentarily and structurally. In the latter case, the systems responsible for color production have been categorized into three types (Ghiradella, 1984, 1989; Wilts et al., 2009): the Morpho type, in which multilayer systems composed of thin films are equipped on the ridge that runs along a scale’s upper lamina (Anderson and Richards, 1942; Giradella et al., 1972; Vukusic et al., 1999, 2002; Kinoshita et al., 2002; Yoshioka and Kinoshita, 2004); the Urania type, in which a multilayer system is involved in the scale body (Lippert and Gentil, 1959; Huxley, 1975; Vukusic et al., 2001; Wilts et al., 2009; Matějková-Plšková et al., 2011; Imafuku et al., 2012); and the three-dimensional photonic crystal, which is composed of regularly arranged spherical air spaces in a matrix of chitin (Morris, 1975; Allyn and Downey, 1976; Giradella, 1985, 1989; Vukusic, 2005; Kertész et al., 2006; Biró et al., 2007; Michielsen and Stavenga, 2008; Saranathan et al., 2010). Vukusic respectively refers to these three types as Types I, II, and III, respectively (2005).

Among the three types, the former two tend to reflect strong light in a color limited direction (Vukusic et al., 1999, 2001; Yoshioka and Kinoshita, 2004; Pirih et al., 2011), contrast to the last type in which angle-dependent color associated with layered structure is absent (Vukusic, 2005). Many studies have been performed on the mechanism that produces specific colors (Kinoshita and Yoshioka, 2005), whereas only a small number of studies have been made on the spatial distribution of reflected light (Pirih et al., 2011).

As early as 1880, Charles Darwin observed directional reflection by wings of butterflies, stating for Hypolimnas (Diadema in the original description) bolina that “the wings of the male, when viewed from behind, are black with six marks of pure white ...; but when viewed in front, ..., the white marks are surrounded by a halo of beautiful blue”. During the 1960s and 1970s, directional reflection was reported in association with ultraviolet (UV) light reflection by wings of pierid butterflies in Gonepteryx rhamni (Nekrutenko, 1965), Phoebis neocepris (Eisner et al., 1969), Eurema lisa (Ghiradella et al., 1972; Rutowski, 1977) and Eurema daira (Rutowski, 1977). These reports included photographs showing the wing of one side appearing with UV illumination from the opposite side.

Rigorous studies on mechanisms that produce directional reflection have been performed since 2000. Vukusic et al. (2002) found in Anchylura meliboeus that the reflection of light was limited to a narrow part of the entire hemispherical space over the wing surface, caused by the angle of multilayer ridging on the wing scales. Rutowski et al. (2007) revealed in Colias eurytheme that male-specific UV light was reflected nearly specularly. White et al. (2015) confirmed this phenomenon in Hypolimnas bolina. Rutowski et al. (2010) showed that the blue and iridescent patch of the hindwing of Battus philenor reflected light almost perpendicular to the wing surface. Pirih et al. (2011) measured the direction of light reflected by the wing of Gonepteryx rhamni along its proximal-distal axis, and revealed that the normally
applied incident light was reflected toward the wing base at a lower angle, due to the tilt of the reflectors on the wing scales with respect to the wing surface.

In preliminary observations, we found that the wing of the Chrysozephyrus smaragdinus male reflected light forward with respect to the body axis ("forward reflection") (Fig. 1). This species belongs to tribe Theclini, in which males of many species show brilliant green to blue and/or UV light on the dorsal surface (Imafuku et al., 2002; Imafuku, 2008) based on structural coloration categorized into Urania type; a multilayer structure in the scale body is observed in Chrysozephyrus ataxus (Matějková-Plšková et al., 2011; Imafuku et al., 2012), Favonius cognatus (Imafuku et al., 2012), F. jezoensis (Schmidt and Paulus, 1970), and Quercusia quercus (Tilley and Eliot, 2002). Another feature, "a minimal pepper-pot structure" (Tilley and Eliot, 2002) on the upper surface of the wing scale, is observed in the above species (no data for F. jezoensis), Chrysozephyrus brillantinus (Wilton et al., 2009), and Laeosopis evippus (Tilley and Eliot, 2002). This structure is also observed in the C. smaragdinus described here (Fig. 3).

To our knowledge, no study published to date has examined the forward reflection of light in butterflies. Wing scales are disposed roughly in straight lines perpendicular to the proximal-distal axis of the wing (Nijhout, 1980) or concentrically around the wing base (Yoshida et al., 1983). This is the case for C. smaragdinus (Fig. 2C, D). If wing scales were simply raised at the apex, light from the top would be reflected toward the wing base in both the fore- and hindwings. Forward reflection with respect to the body axis, therefore, should require tilting or twisting of the wing scales. In the present study, we examined how normally applied
light is reflected in the hemispherical space by the wings of this species, and whether there is tilting of wing scales, on the assumption that wing scales of this species show some degree of tilting, the angle of which differs between the fore- and hindwings. The results indicated that both the fore- and hindwings reflected light forward with respect to the body axis, and that this common directional reflection from the two wings was caused by difference in tilting angles of their wing scales.

MATERIALS AND METHODS

Butterflies
The species *Chrysozephyrus smaragdinus* (Bremer) belonging to tribe Theclini and family Lycaridae was studied. This species shows sexual dichromatism. Males of this species have metallic green wings, whereas females have dark brown wings with a large orange patch on the forewing (Imafuku and Kitamura, 2015). Specimens examined here were collected in Aomori, Nagano and Wakayama prefectures in Japan between 2002 and 2007, and maintained carefully in Paulownia boxes in a dark, cool room. Structural colors are shown to be stable against the effects of direct sunlight (Imafuku et al., 2002).

Measurement of directional reflection
The directional reflections of the fore- and hindwings were investigated separately. Measurements were performed in a dark room using the following setup (made by Verno-Giken, Japan, Fig. 4). A wing was set on the horizontal stage at the bottom with its centroid at the center of the stage, and then illuminated from the top with a xenon lamp (75 W, 6000 K) sealed in a box equipped with a hole for illumination and a cooling fan. Between the lamp and specimens were inserted a quartz lens and an interference filter, which together yield light of specific wavelengths focused on the area of the wing to be measured. Reflected light was measured with a sensor (S1226-5BQ, Hamamatsu Photonics, Japan) attached at the tip of an L-shaped arm rotating in a vertical plane. Rotation of the arm provided vertical component data (elevation, defined by Rutowski 2007) of reflected light at intervals of 15° from 15° to 75°, with 90° at the perpendicular. For each elevation, horizontal component data (azimuth) were obtained by rotating the horizontal stage carrying the wing at intervals of 15° from 0° to 345°. Regarding the azimuth, the direction from the centroid of the wing to the wing base was defined as the azimuth 0° (base line), with positive toward anterior and negative toward posterior.

The reflectance spectra of the wing of this species shows two peaks, at 318 nm (ultraviolet, UV) and 530 nm (green) (Table 1 in Imafuku et al., 2002), and thus reflection in these wavelength ranges was measured separately using an interference filter; wavelength at maximum transmittance and full width at half maximum (FWHM) were, respectively, 323 nm and 519 nm for the green filter. During measurements, a black cover with a hole 8 mm in diameter was placed on the wing, allowing use to measure light reflected from this area. All measurements were transformed into values relative to those of magnesium sulfate (a white reflectance standard) which were obtained under precisely the same conditions as the wing measurement with respect to measurement angles, cover and interference filter. When a diffuser is used as a reference, the reflectance spectra of the wing of this species shows two peaks, at 318 nm (ultraviolet, UV) and 530 nm (green) (Table 1 in Imafuku et al., 2002), and thus reflection in these wavelength ranges was measured separately using an interference filter; wavelength at maximum transmittance and full width at half maximum (FWHM) were, respectively, 323 nm and 15 nm for the UV filter, and 519 and 9 nm for the green filter.

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### Table 1. Azimuth, the maximum reflectance and the intensity of directionality of green light and UV light reflected by the fore- and hindwings in *Chrysozephyrus smaragdinus*. n = 10.

<table>
<thead>
<tr>
<th></th>
<th>Forewing</th>
<th>Hindwing</th>
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</thead>
<tbody>
<tr>
<td>Green</td>
<td>UV</td>
<td>Green</td>
</tr>
<tr>
<td>Azimuth (deg.)</td>
<td>60 ± 4</td>
<td>57 ± 4</td>
</tr>
<tr>
<td>Reflectance</td>
<td>0.79 ± 0.15</td>
<td>0.47 ± 0.12</td>
</tr>
<tr>
<td>Directionality</td>
<td>0.17 ± 0.03</td>
<td>0.10 ± 0.03</td>
</tr>
</tbody>
</table>

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### Determination of parameters of directional reflection
Four parameters of directional reflection were determined: the azimuth and the elevation of reflected light at maximum reflectance, the maximum reflectance, and the intensity of directionality (as a measure of concentration or scattering).
An example of the measurement results is shown in Fig. 5A. This wing reflects light more strongly at lower elevations relative to the wing base, azimuth ca. 0°. The result is shown three dimensionally in Fig. 5B, in which azimuth and elevation (as lengths of radius) are expressed in a horizontal plane (X-Y plane), and reflectance is shown in height (Z-axis). In order to determine the point of maximum reflectance, the measurements were approximated to a Gaussian plane (see Supplementary File S1 online). Fig. 5C shows the distribution of approximate values.

Within the measured range (0–360° azimuth, 15–75° elevation) of the approximate distribution, the maximum reflectance, the azimuth, and the elevation at the maximum reflectance were determined. The intensity of directionality was expressed with the standard deviation (SD) of all approximate values within the measured range.

Measurement of tilt angle of wing scales
To explore the cause of directional reflection by the wing of this species, the tilt angle of the central part of the wing scale relative to the wing surface was determined, as follows. In the upper surface of a wing scale, two points were defined near the edges of the scale in the line parallel to the base line (azimuth 0°) and passed through the center of the scale, and the heights, and of the distance between, the two points were measured under a microscope (Nikon 84803, Nikon, Tokyo) at a magnification of 600x with an oblique top illumination, to determine the X-component of the tilt of the scale. Similarly, the Y-component (azimuth 90°) was determined. Based on these components, an azimuth (facing the scale surface) and an elevation (the angle between the scale surface and the horizontal plane) were calculated (see Supplementary File S2 online).

Measurement was made on 20 scales for each wing, five scales from each of the four sections (a to d) shown in Fig. 2A–B. One forewing and one hindwing were used from each of 10 individuals.

Determination of curvature of wing scales
As seen in Fig. 2E and F, the shapes of the wing scales were different between the fore- and hindwings. The curvature of the wing scales on these wings was determined as follows.

Three points, one each near the base, near the apex, and near the center of the scale, were defined in the median line of a wing scale. The heights of the three points and the distances between them were measured under a microscope. From these measurements, the radius of the circle that included the three points was calculated, and then the curvature of the scale was determined as the reciprocal of the radius.

Measurement was made on 20 scales for each wing: five scales from each of the four sections (a to d) shown in Fig. 2A–B.

One forewing and one hindwing were used from each of 10 individuals.

Statistical analysis
All comparisons on reflected light and wing scale morphology were made by Wilcoxon signed-rank test, except for comparisons between direction of reflected light and orientation of wing scales which were made by Wilcoxon rank sum test, using JMP, version 5.1. For the data used for statistical analyses, see Supplementary File S3 online.

RESULTS

Directional reflection
The parameters of the light reflected by the fore- and hindwings of male Chrysozephyrus smaragdinus are shown in Table 1. The forewing reflects the normally applied incident light largely forward, with azimuth approximately 60°. The hindwing reflects light close to the wing base, with azimuth slightly less than 20°. The azimuths of the reflected light are significantly different between the fore- and hindwings (n = 10, Z = 27.5, P = 0.002 for both the green and UV ranges). They are also different between different wavelengths (Z = 27.5, P = 0.002 for forewing; Z = 22.5, P = 0.004 for hindwing).

The elevation of the reflected light was 15° for all wings examined, including under different wavelength conditions, suggesting that the light was most strongly reflected at a lower angle than this value, the lower limit of the measurement range.

The maximum reflectance was higher for the hindwing than the forewing (Z = 27.5, P = 0.002 both for green and UV ranges). In the same wing, the maximum reflectance was higher in the green range than in the UV range (Z = 27.5, P = 0.002 for both fore- and hindwings).

The intensity of directionality was higher in the hindwing than the forewing (Z = 27.5, P = 0.002 for both green and UV ranges). It was also different between different wavelengths (Z = 27.5, P = 0.002 for both fore- and hindwings).

Tilt angle and curvature of wing scales
The results of the measurements of tilt angle and curvature of wing scales are shown in Table 2. The azimuth of the wing-scale surface was significantly different between the...
fore- and hindwings \((n = 10, Z = 27.5, P = 0.002)\). It was also different from that of reflected light in the forewing \((45° \text{ vs. } 60°, Z = 3.74, P = 0.0002 \text{ for green light}; 45° \text{ vs. } 57°, Z = 3.52, P = 0.0004 \text{ for UV light})\), but not in the hindwing \((17° \text{ vs. } 17°, Z = 0.000, P = 1.00 \text{ for green light}; 17° \text{ vs. } 19°, Z = 0.42, P = 0.68 \text{ for UV light})\).

The elevation of the surface of the wing scales was 44° for the forewing and 37° for the hindwing. These angles indicate that the normally applied incident light is reflected at an elevation of 2° \((= 90 - 2 \times 44)\) in the forewing and 16° in the hindwing. The latter angle is not significantly different from 15°, the lower limit of the measurement of elevation \((Z = 5.5, P = 0.63, \text{ Wilcoxon signed-rank test})\).

The curvature of the wing scales was clearly larger in the forewings than in the hindwings \((n = 10, Z = 27.5, P = 0.002)\).

**DISCUSSION**

From measurements of the light reflected from the wings of *Chrysozephyrus smaragdinus* males, we found that the normally applied incident light was reflected largely forward by the forewing, and slightly forward by the hindwing, from the baseline connecting the centroid and the base of the wing. The azimuth of reflected light \((17° - 19°)\) agreed well with that of the surface of the wing scale in the hindwing \((17°)\), but such agreement was not found in the forewing \((azimuth 57° - 60° \text{ and } 45°, \text{ respectively})\). This lack of agreement in the forewing seems to be attributable to the morphology of the wing scales, and the area on the wing scale measured; the wing scales of the forewing were markedly curved \((\text{Fig. 2E})\), and a limited central area was used for assessing wing scale morphology, whereas the light reflected from the whole upper surface of the wing scale was used for reflectance. If wing scales on the forewing are twisted such that their apical areas are more forward-facing, then the observed angle of the reflected light would be understandable. Twisted wing scales are occasionally observed in an assembly of wing scale samples scattered over a microscope glass slide \((\text{Imafuku, personal observation})\).

The azimuths of reflected light were slightly \((2° - 3°)\), but significantly, different between different wavelengths both for the fore- and hindwings. This indicates that light of different wavelengths is reflected at different points on the convex wing scale, and that the wing scale is more or less twisted with respect to the median line. Regarding this problem, it will be necessary to examine the morphology and reflectance of wing scales at the single wing scale level \((\text{e.g., Vukusic et al., 1999; Yoshioka and Kinoshita, 2004; Giraldo et al., 2008})\).

In nature, males of this species engaged in territorial occupation \((\text{Takeuchi and Imafuku, 2005})\) usually assume a posture in which the wings are opened widely with the leading edge of the forewing lying roughly perpendicular to the body axis and the first anal vein \((1A, \text{ the Comstock-Needham system})\) of the hindwing lying parallel to the body axis \((\text{photos shown in Fukuda et al., 1984; Kurita, 1993, and Ohya, 2001})\). In this posture, light is expected to be reflected in almost a frontal direction: at 20° and 15° inward of the line parallel to the body axis in the hindwings, respectively \((\text{Fig. 6})\). The elevation of the reflected light is 15° or less for both of the wings. Thus, males of this species that settle in a territory facing an open space send their bright green light forward at a lower angle. This directional reflection observed in our territorial species is different from that observed in other species in which reflected light is nearly perpendicular to the wing surface \((\text{Rutowski et al., 2007, 2010; White et al., 2015, but for Pirih et al., 2011})\).

Color sense in butterflies is widely known \((\text{Arikawa et al., 1987; Bernard and Remington, 1991; Qiu and Arikawa, 2003})\), including Theclini species \((\text{Imafuku and Tsuji, 2008; Imafuku, 2013})\). Further, it has been observed that males of the present species discriminated a brown wing model of the female from a green wing model of the male \((\text{Imafuku and Kitamura, 2015})\), and that males of the closely related *Favonius taxila* \((\text{Bremen})\) alit on a site with a bright green male model less frequently than on a site with a dull green male model \((\text{Imafuku and Hirose, 2016})\), suggesting that the bright wing color of males of this species has a negative effect on establishment of territories by conspecific males.

Another feature found in our species is the differential light-scattering property of the two wings. As seen in Table 1, the hindwing reflected light more strongly \((\text{a higher maximum reflectance})\) in a narrower region \((\text{a higher intensity of directionality})\), whereas the forewing reflected light more weakly, and in a wider space. In *Morpho aega*, it has been shown that broad scattering is caused by a rotational variation of the scale plane \((\text{Giraldo et al., 2008})\). In our species, broadening was attributed to a curvature of the wing scales; the forewing possessed more strongly curved wing scales \((\text{Fig. 2E and F, Table 2})\). It has been shown that species

| Table 2. Tilt angles of the surface and curvature of wing scales in *Chrysozephyrus smaragdinus*, \(n = 10\). |
|--------------------------------|------------------|
|                                | Forewing         | Hindwing        |
| Azimuth (deg.)                 | 45 ± 5           | 17 ± 8          |
| Elevation (deg.)               | 44 ± 7           | 37 ± 3          |
| Curvature (1/mm)               | 17.8 ± 1.6       | 4.8 ± 1.5       |

**Fig. 6.** A schematic illustration of the light course at reflection on the fore- and hind wings of a butterfly in a natural posture. Light from the top is reflected forward at azimuth 60° or 20° by the fore- and hind wings, respectively, and at elevation less than 15°. These azimuths correspond to 20° inward to the body axis in the forewing, and 15° in the hindwing.
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with curved wing scales reflect light over a wider space than species with flat wing scales (Pirih et al., 2011). In our species, differential scattering properties were observed here within a single individual.

Why does *C. smaragdinus* have wings with differential scattering properties? The morphology of wing scales may be related to flight function. It has been shown in other butterfly and moth species that the presence of wing scales on the wing contributes to the production of dynamic lift (Nachtigall, 1965, 1967). The shapes of wing scales may also affect it such that more convex wing scales (Fig. 2E) enhance dynamic lift.

The possession of different shapes of wing scales by the present species may also be explained by differential functions in signaling. Light reflected strongly forward from the hindwings of territorial males that settle facing an open space may serve as a defensive signal against potential rivals. In contrast, cryptic females may appear from among the shadows of tree limbs behind (Fukuda et al., 1984), and the wider reflection of light by the forewing may contribute to attraction of potential mates.

In the present research, it was clarified that males of *C. smaragdinus* possessed wings that reflect light forward, and whose scattering property was different between the fore- and hindwings. These characteristics are inferred to have evolved in association with mate acquisition tactics by males. It will be necessary to investigate in the future whether forward reflection occurs widely in other territorial species, and the extent to which the differential scattering properties of the two wings observed here occur in various phylogenetic groups of butterflies.

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