Species-dependent microarchitectural traits of iridescent scales in the triad taxa of Ornithoptera birdwing butterflies

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Abstract

Ornithoptera birdwing butterflies have blue, green, or orange iridescent scales in different species or subspecies. To understand the species- or subspecies-dependent scale color differences, we performed comparative morphometric analyses of iridescent scales from three closely related taxa: O. priamus priamus (green), O. priamus urvillianus (blue), and O. croesus (orange). The three types of Ornithoptera wings exhibited reversible color changes to longer wavelengths with different kinetics upon immersion in methanol, suggesting that their color differences are at least partly based on differences in the size of air cavities made by nanostructures. Cover scales of all three color types were visually semi-transparent glass scales that exhibited color when placed on a dark background. The dorsoventral differences in coloration were observed in single scales, suggesting the optical importance of scale surfaces. Scanning electron microscopy of cover scales in cross section revealed that all color types exhibited finely sculpted tapered ridges and thick, irregular basal multilayers containing tandemly clustered granular objects and air cavities. Scale thickness, ridge height, and multilayer thickness were significantly different among the three color types, and granular object size was significantly different between orange scales and blue and green scales. We conclude that each of the three taxa of Ornithoptera butterflies possesses unique quantitative size values on tapered ridges and irregular multilayers with granular objects and air cavities to express unique structural color. These species- or subspecies-dependent structural colors might have evolved via quantitative shifts in these microarchitectural traits rather than via changes in the basic developmental or architectural plan for color expression.

Key words: butterfly wing, comparative morphometry, Lepidoptera, Papilionidae, scale microarchitecture, structural color.

INTRODUCTION

Butterflies are conspicuous insects because of their highly visible and diverse wing color patterns. These color patterns are achieved by changing two factors: pattern itself (or “design”) and coloration. Because butterflies generally use their wings as visual signals in mate recognition, both design and coloration are important to identity species. The diversity of the former is mainly attained through evolutionary modifications of an “ideal” scheme referred to as the nymphalid groundplan (Nijhout 1991, 2001; Otaki 2009, 2012; Taira et al. 2015). The nymphalid groundplan is the positional arrangement of pattern elements on a plain background; changes in the size, shape, and position of pattern elements produce a variety of elemental patterns. The nymphalid groundplan itself does not impose any restrictions on colors of pattern elements. However, the basic pattern elements are likely black (or dark color) on the white (or light) background, which is known as the binary rule (Otaki 2011). In contrast, diversity of coloration is achieved by the evolutionary invention of chemical pigments, structural colors, and other photonic manipulations such as scale...
stacking (Nijhout 1991; Wickham et al. 2006; Stavenga et al. 2014; Wilts et al. 2014). Pattern elements with various designs (i.e. size, shape, and position) can be expressed in various colors, and non-elemental regions (i.e. background) can also be colored variously. Evolutionary and developmental studies on how butterfly color patterns are formed are now an active field in biological sciences. Understandably, however, because of complex physicochemical nature of butterfly coloration, studies of butterfly wings involve not only classical fields of the natural sciences (e.g. biology, chemistry, and physics) but also engineering fields, such as materials science, that explore photonic materials.

Colors are generally classified into two categories: pigmentary, or chemical, colors and photonic, or physical, colors referred to as “structural” colors (Nijhout 1991). Structural colors are produced when scales have fine structures that are comparable to a certain wavelength range of light. Butterflies frequently employ glittering structural colors, most often blue or green, that may function as visual signals, often together with pigmentary colors. Striking butterfly structural colors can be observed in a group of *Morpho* butterflies distributed across South America. Photonics and materials science studies have revealed that lamellar structures on scale ridges (ridge lamellae) are the major nanostructural basis of blue color in these *Morpho* butterflies (Anderson & Richards 1942; Ghiradella 1984, 1989, 1994, 1998, 2005; Kinoshita et al. 2002a,b; Yoshioka & Kinoshita 2004; Kinoshira & Yoshioka 2005). Structural colors of other butterfly species have different photonic mechanisms (Ghiradella 1984, 1985, 1994, 1998, 2005; Biró et al. 2007; Biró 2010; Dechkrong et al. 2011). In another typical case, thin-film basal multilayers (body lamellae) contribute to structural coloration (Ghiradella et al. 1972; Vukusic 2005). Yet another case is photonic crystals made of regular arrangement of small objects, usually two transparent materials of different refractive indices (Welch 2005).

Among many iridescent scales from different species that have been examined, *Ornithoptera* scales have been shown to have unique nanostructures. Scanning and transmission electron microscopy analyses of sectioned and fractured *Ornithoptera* scales have revealed: (i) tapered ridges with fine microribs on their sides; and (ii) basal multilayers composed of stacks of “chips” but not smooth sheets (Ghiradella 1985; Prum et al. 2006). Associated with the chips are “spacer-rods” (Ghiradella 1985), which may comprise bead-like granular objects that are clustered together. The tapered ridges appear to function to generate polarized light (Zhang et al. 2014). Recently, *Ornithoptera* scales were examined with transmission electron microscopy (TEM), scanning electron microscopy (SEM), scatterograms, and absorbance and reflectance spectra, stressing the importance of the basal multilayers (Wilts et al. 2015). Interestingly, Wilts et al. (2015) mentioned, “the multilayers are rather disordered and chirped”. Moreover, it was optically demonstrated that *Ornithoptera* iridescent scales contain fluorescent pigments (Wilts et al. 2015). We believe that the “disordered and chirped” multilayers need further morphometric studies based on SEM from the viewpoints of evolutionary color differences among *Ornithoptera* species.

These previous studies on scale microarchitectures are to be incorporated into butterfly biology with evolutionary, developmental, and physiological perspectives to obtain a synthetic view on *Ornithoptera* speciation. We have been studying speciation processes using butterflies, such as *Vanessa* (Otaki & Yamamoto 2004; Otaki et al. 2006; Otaki 2007, 2008) and *Zizeeria* (Otaki et al. 2010; Buckley et al. 2010; Hiyama et al. 2012), with a primary focus on changes in wing elemental patterns rather than coloration. The structural colors of *Ornithoptera* birdwing butterflies have provided us with a unique opportunity to focus on changes in wing coloration. Of note is the *O. priamus* species complex (subgenus *Ornithoptera*), which is distributed in Papua New Guinea and its surrounding islands (Parsons 1999; Matsuoka 2001; Deslisle & Sclavo 2015). This species complex exhibits three different iridescent colors (blue, green, and orange) depending on the species or subspecies (Fig. 1A), among which the green type is by far the most widely distributed.

Individuals belonging to this triad exhibit one of the three colors on the dorsal side of their wings. The color likely serves as the main species or subspecies identifier for mating, as only minor differences are noted in their patterns (Fig. 1A). As the orange type is recognized as an independent species, the evolutionary invention of the color orange would have been associated with its speciation. Thus, understanding the mechanisms responsible for orange coloration should help to clarify one process involved in the speciation of *Ornithoptera croesus* Wallace, 1859. Similar efforts have recently related butterfly scale microstructures to species taxonomic status in Papilionidae, Nymphalidae, and Lycaenidae (Wickham et al. 2006; Bálint et al. 2012; Wilts et al. 2014).

Here, we study the photonic characteristics and microarchitectural traits of iridescent scales in three taxa of *Ornithoptera* butterflies in the subgenus *Ornithoptera*. We first perform a wing-in-methanol experiment to understand a role of scale nanostructures and air cavities in color expression. This is a simple way to appreciate nanostructural differences among the triad. We also examine photonic characteristics of cover and ground
scales at the individual scale level. These simple experiments have not been reported before in the literature, making the present study unique. We then obtain SEM images for qualitative and quantitative analyses of microarchitectures to understand structural differences among the triad. Comparisons with the scales of *Morpho didius* were also made to highlight structural differences. These detailed microarchitectural comparisons have not been performed before.

Complementary to optical characterization by Wilts et al. (2015), our study focuses on mesoscopic aspects based on methanol treatment and bright-field observations, nanostructural morphometry of the basal multilayers based on SEM, and evolutionary aspects of the triad.

**MATERIALS AND METHODS**

**Butterflies**

Wing scales from three taxa of *Ornithoptera* birdwing butterflies (Lepidoptera, Papilionidae) (Fig. 1A) and one taxon of *Morpho* butterfly (Lepidoptera, Nymphalidae) were used. The butterfly specimens were purchased from Nautilus, Nagoya, Japan (http://nautilus-world.com). These specimens were sold in full compliance with all laws and regulations associated with these species.

Information associated with these specimens is as follows: (i) Orange scales were sampled from the Wallace’s golden birdwing, *Ornithoptera croesus*: *O. c. lydius* (Felder, 1865) from Helmahera Island in the Moluccas (Maluku), Indonesia, November 2003 (*n* = 1); (ii) green scales were sampled from the common green birdwing, *Ornithoptera priamus* (Linnaeus, 1758): *O. p. priamus* (Linnaeus, 1758) from Seram Island, Indonesia, June 2003 (*n* = 1); (iii) blue scales were sampled from *Ornithoptera priamus urvillianus* (Guérin, 1830) from Buin, Baugainville Island, Solomon Islands, Papua New Guinea, December 2003 and August 2008 (*n* = 2); and (iv) glossy blue scales were sampled from the giant blue morpho, *Morpho didius* Hopffer, 1874, from Las Palmas, Peru, in October, 2004 (*n* = 1). All specimens were male.

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Figure 1 *Ornithoptera* butterflies and their reflectance spectra. (A) The three taxa of *Ornithoptera* butterflies used in this study. Three color types were investigated: blue, green, and orange. (B) Reflectance of *Ornithoptera* and *Morpho* wings.
For all analyses, wing fragments were isolated with scissors from the iridescent areas of the *Ornithoptera* forewing. For structural comparisons, wing fragments were also isolated from the central black band of the forewing of *O. priamus priamus*.

**Reflectance measurements**

A square area (14 mm × 14 mm) of structural coloration was excised from the anterior part or the cell (central compartment) of the forewing. The excised wing portion was glued on the surface of a piece of glass slide with double-faced adhesive tape so that the dorsal portion to be measured was faced upward. The glass slide was stacked on a Spectralon Diffuse Reflectance Standard (Labsphere, North Sutton, NH, USA). The specimens were measured using a JASCO V-660 UV–VIS Spectrophotometer (JASCO, Tokyo, Japan). This is a double-beam spectrophotometer equipped with a double monochromator and a photomultiplier tube detector that highly reduce stray light. This spectrophotometer was also equipped with a JASCO ISV-722 UV–Visible 60 mm Integrating Sphere. For measurements, bandwidth was set at 2.0 nm. Before the measurements of wing specimens, the baseline reflectance of the standard was established with a Spectralon Diffuse Reflectance Standard. The reflectance spectra were obtained using a software Spectra Manager II (JASCO). A given specimen was measured three times, and the output data (mean values of three measurements) were graphically presented using Microsoft Excel.

**Color change experiment in methanol**

Color change experiments in methanol were performed at room temperature and were recorded by a digital video camera with a timer. The refractive index is 1.33 for methanol and 1.00 for air, justifying the use of methanol for this experiment. Digital images were cut as still images at 60-s intervals and other relevant time points for measurements of brightness using Adobe Photoshop Elements 11. Brightness values in the HSB (hue, saturation, brightness) color mode were recorded five times from a given wing fragment at a given time. These five values were averaged and graphically presented using Microsoft Excel.

**Examination at low magnification**

The desktop digital microscope SKM-2000-PC (Saitou Kougaku, Yokohama, Japan) and other conventional stereomicroscopes were used to examine low magnification images of the wings. To characterize scales from both the dorsal and ventral sides, isolated scales were placed between two pieces of cover glass.

**Scanning electron microscopy**

To obtain SEM images, wing pieces were cut out with scissors and mounted with double-faced adhesive tape so that the cross sections were exposed to an observational plane. After platinum coating with dotite (JEOL DATUM, Tokyo, Japan) using the JEOL DATUM ion-sputtering device JFC-1100E, specimens were observed with a JEOL JSM-840A scanning electron microscope equipped with a SemAfore digitizer system (JEOL DATUM).

**Quantification of microstructures**

Scale thickness, ridge height, and basal multilayer thickness were measured in SEM images using ImageJ (Abramoff et al. 2004). Measurements were obtained at 5 to 30 locations (n = 5–30) within a single scale. A mean value was assigned to each scale because scale thickness, ridge height, and basal multilayer thickness may vary even within a scale and may potentially be influenced by cut angles and other artifacts. We used 20 to 30 scales to determine scale thickness (n = 25 for each color type), ridge height (n = 20 for each color type), and basal layer thickness (n = 30 for each color type). The means and standard deviations (SD) were obtained from these values (shown as the mean ± SD).

Similarly, sizes of the granular objects observed in the basal multilayers were measured from SEM images using ImageJ. Three SEM images (three scales) of cross sections (n = 3) were used for each color type, and 30 easily measurable granular objects (n = 30) per image were selected and measured. Thus, 90 granular objects in total were measured from each color type (n = 90), and these values were used directly for statistical analysis. To obtain diameters of granular objects, area values were measured using ImageJ and, assuming that the objects were circular, diameters were calculated. The means and SD values were then obtained (presented as the mean ± SD).

**Statistical analyses**

Two-sided unpaired Student’s *t*-tests were performed after confirmation of the equality of two variances by *F*-tests, using Microsoft Excel and JSTAT (Yokohama, Japan). When Student’s *t*-test was not appropriate, Welch’s *t*-test was performed. Because multiple comparisons were performed, Bonferroni-corrected *P*-values were generated. The results were graphically displayed using Microsoft Excel.
RESULTS

Reflectance of wings
To highlight optical differences among the triad as well as between Ornithoptera and Morpho, we first measured the reflectance of green, blue, and orange portions of Ornithoptera and Morpho wings (Fig. 1B). Each of the three Ornithoptera wings had a single peak at the following wavelengths: blue at 482 nm, green at 533 nm, and orange at 640 nm.

The Morpho wing had a peak at 462 nm, although there was an increase at ultraviolet wavelengths. Thus, the peak wavelength differed between Morpho blue (462 nm) and Ornithoptera blue (482 nm), and their reflectance levels differed markedly, partly explaining their different quality of blue coloration.

Reversible color changes in methanol
Butterfly scale structural coloration often depends on repetitive nanostructures between which air may be present. When a non-air substance with a higher refractive index occupies the air cavities between nanostructures, the structural color shifts to a longer wavelength in a reversible manner (Nijhout 1991; Kinoshita & Yoshioka 2005). Reversible color shifts can thus be considered proof of structural coloration in butterflies. We first examined whether Ornithoptera scale colors meet these criteria (Fig. 2; Movie S1) because they are less metallic and glittering than Morpho scales, which display a well-characterized structural color. Besides, kinetics of color changes can be compared among the triad to elucidate the relative size of air cavities.

In methanol, Morpho wing segments turned dark green within 1 s. In contrast, the three Ornithoptera wing segments required more time to change color. A blue wing segment turned dark blue in 5 s and then gradually turned greenish within 2 min. A green wing segment began turning dark green in 1 min and then gradually became brownish or yellowish. An orange wing segment turned brown in 1 min and then gradually darkened. In all three

Figure 2 Methanol-induced color changes of Ornithoptera and Morpho wing fragments. (A) Wing fragments in air (right) and in methanol (left). This image was obtained after color change had ceased. The species or subspecies used are as follows: O. priamus priamus (OPP; green), O. priamus urvillianus (OPU; blue), O. croesus lydius (OCL; orange), and Morpho didius (MOD). Scale bar, 10 mm. (B) Changes in the brightness of wing fragments in methanol over time. Methanol was added at 0 s and removed at 210 s. The inset depicts enlargements at early time points. (C) Images of wing fragments in methanol over time. The upper images show changes after methanol addition, whereas the lower images present changes after methanol removal. The time points of methanol addition and removal were adjusted to 0 s in this figure for convenience. For example, 2:30 means 2 min 30 s.
cases, the color change required time in the order of minutes rather than seconds.

When methanol was removed, the *Morpho* wing segment was the fastest among the four to revert to its original color; its brightness value increased within 2 min of the removal of methanol and was back to its original level in 3 min. In contrast, all *Ornithoptera* wing segments displayed slower kinetics, suggesting that *Ornithoptera* has finer air cavities than *Morpho*. The segments began to change color within 3 min of methanol removal. Among the three, orange was the fastest, followed by green and then blue, suggesting that the size of air cavities differs among the triad.

**Functions of cover and ground scales**

We next observed scale arrangement by stereomicroscopy and SEM at low magnification (Fig. 3). Cover scales were regularly aligned, with black ground scales observed in between (Fig. 3A). Again, it was clear that the blue color in *Ornithoptera* (Fig. 3A) was qualitatively different from that of *Morpho* (Fig. 3B). The scale arrangement pattern was similar in all three types of *Ornithoptera* (Fig. 3C).

The isolation of individual scales revealed that cover scales were visually semi-transparent; they appeared to contain no pigment in a visually recognizable way. They had high levels of transparency so that they could be called glass scales (Ghiradella 1994; Vukusic 2005). In contrast, ground scales were black or dark brown (Fig. 3D). In addition, ground scales had deep teeth that were similar in shape and color to black scales in other regions of the same wing (Fig. 4D–F). When placed on a black background or on a ground scale, cover scales exhibited unique colors (Fig. 3D,E). The expression of colors was dependent on cover scales but not on ground scales. For example, placing a cover scale from a blue-type wing on a ground scale from a green-type wing yielded a blue color (Fig. 3F).

We further demonstrated that *Ornithoptera* scale colors could be observed from both the dorsal and ventral sides of the scale under a vertical white light (Fig. 3G). We observed that one side (likely the dorsal side) was slightly brighter than the other side (likely the ventral side). Moreover, scale coloration was slightly different between the dorsal and the ventral sides; one side exhibited color of a longer wavelength. These results suggest that there may be a weak dorsoventral polarity in *Ornithoptera* scales.

For comparison, we isolated scales from *Morpho didius* (Fig. 3H). As previously shown in detail (Vukusic et al. 1999), *Morpho* wings contain semi-transparent cover scales and dark ground scales. However, when placed against a white background, ground scales exhibited a blue color on one side (likely the dorsal side). When placed against a black background, both cover and ground scales exhibited a blue color on both the dorsal and the ventral sides, but ground scales clearly exhibited a dorsoventral polarity. That is, blue color was observed more strongly on one side than on the other side in ground scales. These results can be explained by the existence of the functional thin-film lower lamina on both cover and ground scales, the presence of denser ridges on ground scales than on cover scales, and the existence of ridges lamellae on only one side of the ground scales in *Morpho* wings (Ghiradella 1984, 1989, 1994, 1998, 2005; Vukusic et al. 1999; Kinoshita et al. 2002a,b). The fact that dark pigments exist only in the ground scales may also contribute to the clear dorsoventral polarity of the ground scales. These results demonstrated that the functions of cover and ground scales differ between *Ornithoptera* and *Morpho* butterflies.

**Scanning electron microscopy**

As indicated in Figure 3, cover and ground scales within a colored area had different shapes in *Ornithoptera*. Low magnification SEM images revealed that these two scale types differed in surface structure. Cover scales had fine and dense ridges in the upper lamina, whereas ground scales had sparse ridges (Fig. 4A–C). Scales in non-structural black areas were similar in shape and in ridge structure to ground scales in colored areas (Fig. 4D–F).

Cross-sectional views confirmed that ground scales had relatively large ridges and wide ridge intervals, whereas cover scales had finer ridges and narrow ridge intervals in green *Ornithoptera* (Fig. 5A–E). However, dense ridge lamellar structures, which are known to reflect blue light in *Morpho*, did not exist in *Ornithoptera* at least in relatively low resolution images: the ridges of cover scales simply appeared smooth and tapered (Fig. 5B–F). However, at higher resolutions, fine sculptures, likely microribs, were observed at regular intervals on ridge sides (Fig. 5G–K). Interestingly, the fine sculptures were not in parallel with the basal parts; they were tilted at approximately 45° to the basal plane at least in one scale (Fig. 5G), although we were not able to confirm this angle in other scales. Furthermore, the ridges varied like waves (Fig. 5F,G). Importantly, cover scales had thick basal multilayers (Fig. 5B–L). These basal multilayers were not composed of stacks of even sheets but rather of irregular layers; each layer appeared to be composed of “irregular granular objects” (Fig. 5H–L). Several of them were likely tandemly clustered together to form a short stretch corresponding to a single

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Figure 3 Characterization of *Ornithoptera* and *Morpho* wing scales. (A) Bright-field (left) and SEM (right) images of blue *Ornithoptera* scales. Cover scales appear blue and have a smooth edge, whereas ground scales appear black with a highly jagged edge. (B) Bright-field image of *M. didius* under the same optical conditions. Note the difference in the quality of blue color between *Morpho* and *Ornithoptera*. The surface of the *Morpho* wing glitters. (C) Bright-field images of the three types of scales under a stereomicroscope. (D) Isolated cover and ground scales from blue (left), green (middle), and orange (right) wings. Cover scales are placed against a white background in the upper images and against a black background in the lower images. Cover scales are essentially semi-transparent and show color when placed against a black background. (E) The function of ground scales in *O. priamus priamus* (green). Cover scales display green color when placed against a black background (left). Cover and ground scales are placed against a white background (middle). A cover scale is placed on top of a ground scale, resulting in the appearance of green coloring in the area in which the two scales overlapped (right). (F) The function of ground scales. A blue-type cover scale is placed on a green-type ground scale, resulting in blue coloration in the area in which the two scales overlapped. (G) The bi-sided nature of scales. A single *Ornithoptera* scale is observed from the dorsal and ventral sides by bright-field epi-illumination. The scales exhibit slightly different coloration on their dorsal and ventral sides. (H) Isolation of *Morpho* scales for comparison. Images of both sides are presented as mirror images with the axis of symmetry in the middle. Numbers are assigned to each scale for the purpose of identification. The scale specimens bound by two pieces of cover glass were placed against a white background (upper images) or a black background (lower images). Ground scales: 1, 3, 6, and 8. The ground scales express dorsoventral polarity. Cover scales: 2, 4, 5, 7, and 9.
layer. This single layer is probably a small chip-like sheet. The granular objects may be a component of the irregular chips identified in the previous study (Ghiradella 1985). Parts of the chips were likely occasionally pulled out of a cross-sectional plane (Fig. 5K). The ridges also appeared to comprise similar granular objects (Fig. 5I–K). Stacking layers were often tilted (Fig. 5L). Air cavities were also observed at irregular positions. However, some portions showed relatively regular stacks of sheets (Fig. 5J). The very bottom of cover scales below the stacking multilayers was a smooth sheet, which may be called the ventral sheet as a part of the lower lamina (Fig. 5G,H).

For comparison, we examined *Morpho* scales, which showed dense ridge lamellae (Fig. 5M–O). Overall, *Ornithoptera* exhibited very different scale microarchitectures from *Morpho*. We also examined blue and orange *Ornithoptera* scales and found that the three color types possessed essentially similar structures (Fig. 6): cover scales had smooth ridges (at least at low resolution) and thick basal multilayers.

**Scale thickness, ridge height, and basal multilayer thickness**

To obtain insights into structural differences among the green, the blue, and the orange scales, we measured scale thickness (\(n = 25\); the number of scales for each color type in this case and hereafter), ridge height (\(n = 20\)), and basal multilayer thickness (\(n = 30\)) (Fig. 7A). Scale thickness (3.15 ± 0.41 \(\mu\)m for blue, 4.10 ± 0.31 \(\mu\)m for green, and 4.98 ± 0.44 \(\mu\)m for orange) significantly differed among the three color types in multiple comparisons (\(t = 9.20, df = 48, P < 0.0001\) between blue and green; \(t = 8.21, df = 48, P < 0.0001\) between green and orange; \(t = 15.24, df = 48, P < 0.0001\) between orange and blue) (Fig. 7A, left). Ridge height (1.41 ± 0.18 \(\mu\)m for blue, 1.74 ± 0.31 \(\mu\)m for green, and 1.99 ± 0.20 \(\mu\)m for orange) also differed significantly (\(t = 4.24, df = 31, P < 0.001\) between blue and green; \(t = 3.01, df = 38, P = 0.014\) between green and orange; \(t = 9.72, df = 38, P < 0.0001\) between orange and blue) (Fig. 7A, middle). Likewise, basal multilayer thickness (1.75 ± 0.19 \(\mu\)m for blue, 2.53 ± 0.37 \(\mu\)m for green, and 2.98 ± 0.41 \(\mu\)m for orange) differed significantly (\(t = 10.27, df = 43, P < 0.0001\) between blue and green; \(t = 4.48, df = 58, P = 0.00011\) between green and orange; \(t = 15.09, df = 41, P < 0.0001\) between orange and blue) (Fig. 7A, right).

**Size of granular objects**

Because the irregular multilayers likely provide the most important mechanism underlying color expression, and because the thickness of each layer was difficult to measure consistently due to their irregularity, we measured the size of the granular objects that constituted the multilayers of each color type (Fig. 7B,C). Blue,
Figure 5 Cross-sectional scanning electron microscope images of (A–L) *Ornithoptera priamus priamus* (green) and (M–O) *Morpho didius* scales. (A) Low magnification image; some scales are cut, whereas some are intact. (B) High magnification image of A. Cover and ground scales are indicated. (C) Cover and ground scales. Cover scales are composed of three parts: R (ridges), BM (basal multilayers), and VS (ventral sheet). (D) A cover scale. (E) Cover and ground scales. (F,G) Ridge structures in cover scales. Waving heights are indicated by arrowheads in G. Fine sculptures on ridge sides are indicated by an arrow in G. (H–K) Ridges and basal multilayers of cover scales. The ventral sheet at the very bottom of the cover scale is physically separated from the basal multilayers in H (an arrow). Irregular stacks of multilayers are observed. However, relatively regular stacks are also seen in J (upper part of the image). Fine sculptures on ridge sides are indicated by arrows in I–K. Air cavities are indicated by arrowheads in J. Granular objects in basal multilayers and ridges are indicated by arrowheads in K. Pulled-out chips are indicated by asterisks in K. (L) High magnification of basal multilayers. Tilted layers are indicated by consecutive arrows. Tandemly clustered granular objects are indicated by arrowheads. (M) Cover and ground scales. (N,O) Cover scale ridges with extensive laminar structures.
green, and orange types showed the following sizes: 0.13 ± 0.03 μm (n = 90; the number of particles from three scales in this set of measurements) for blue, 0.13 ± 0.03 μm (n = 90) for green, and 0.16 ± 0.04 μm (n = 90) for orange. We found that the size of the granular objects in *O. croesus* (orange) was significantly larger than those of the *O. priamus* blue (t = 7.13, df = 164, P < 0.0001) and green (t = 7.04, df = 164, P < 0.0001) types (Fig. 7B). No difference was noted between the green and blue types (t = 0.094, df = 178, P = 1.0).

**DISCUSSION**

**Basic characteristics of *Ornithoptera* scales in comparison with *Morpho* scales**

In this study, we examined the structural colors of three color types of *Ornithoptera* scales to reveal species- or subspecies-dependent traits. *Morpho* scales were examined for comparison to highlight the unique physical characteristics of *Ornithoptera* scales. We demonstrated that *Ornithoptera* scales differ from *Morpho* scales in
the following five characteristics: (i) the kinetics of solvent penetration and evaporation; (ii) the functions of cover and ground scales; (iii) the dorsoventral polarity of scales; (iv) the presence or absence of clear ridge lamellae in the upper lamina; and (v) the presence or absence of basal multilayers (basal lamellae). In contrast to Morpho, specific color expression is executed almost exclusively by cover scales in Ornithoptera, and ground scales simply provide a dark background color beneath semi-transparent cover scales. The relatively slow methanol penetration and evaporation confirmed that the physical mechanism for Ornithoptera color expression differs markedly from that present in Morpho. The difference in dorsoventral polarity between Ornithoptera and Morpho further confirmed this idea.

We did not observe well-developed ridge lamellae in Ornithoptera. Rather, thick basal multilayers, which do not exist in Morpho scales, likely play a major role in reflecting a specific wavelength of light in Ornithoptera scales. Considering that the thin-film basal lamellar structure is the defining feature of Type II (Vukusic 2005), Ornithoptera scales may be categorized as Type II. Indeed, Ornithoptera structural coloration has been understood among researchers to be mainly based on a multilayer mechanism (Vukusic 2005; Prum et al. 2006; Zhang et al. 2014; Wilts et al. 2015). Interestingly, however, the basal multilayers of Ornithoptera scales are not simple. An excellent electron microscopic study has reported that the multilayers are composed of irregular stacks of chips of irregular shapes (Ghiradella 1985), to

Figure 7 Scale structure measurements in the three Ornithoptera color types. (A) Scale thickness (left), ridge height (middle), and basal multilayer thickness (right). All combinations exhibited significant differences. Insets indicate the length measured. (B) Diameters of granular objects. Granular objects in orange scales are significantly larger than those in the other scales, as indicated by triple asterisks. (C) Examples of cross-sectional SEM images for size measurements of granular objects. Numbers (1–30) were assigned to measurable granular objects.
which our cross-sectional SEM images are similar. Furthermore, irregular multilayers have also been indicated in a recent study (Wilts et al. 2015). Thus, we believe that the irregularity of the basal multilayers in our images was not an artifact.

The basal multilayer structures of *Ornithoptera* are illustrated schematically (Fig. 8). In contrast to stacks of regular smooth sheets like lasagna (wide flat pasta) (Fig. 8A), “chips” of irregular shapes are stacked irregularly like lasagnette (fragmented lasagna) (Fig. 8B). Between these chips, the granular objects exist as spacers. However, the granular objects may also be a component of the stacking chips themselves.

Slower solvent penetration in *Ornithoptera* than in *Morpho* likely indicates that a smaller physical space is responsible for color production in *Ornithoptera* compared with *Morpho*. In *Morpho*, ridge lamellar structures are quickly filled with methanol, leading to an instant color change, as the physical spaces of the lamellae that are responsible for blue coloration are relatively large. In contrast, basal multilayers of the *Ornithoptera* scales are composed of tightly packed irregular objects and air cavities. Methanol must penetrate these tight air spaces to change structural color, which would be a relatively slow process. These qualitative observations support the idea that basal multilayers play a major role in determining the structural colors of *Ornithoptera*.

However, the existence of granular objects in the basal multilayers may suggest a possible mechanism in which photonic crystals express coloration. Indeed, several cases of photonic crystals in butterfly wings have been described and are referred to as Type III (Ghiradella 1984, 1989, 1994, 1998, 2005; Vukusic & Sambles 2003; Vukusic 2005; Welch 2005; Wickham et al. 2006; Mika et al. 2012; Yoshioka et al. 2014). An important example of Type III wings is *Paridessesostris* (Lepidoptera, Papilionidae). Both *Paridessesostris* and *Ornithoptera* belong to the tribe Troidini. However, the multilayer structures of *Ornithoptera* scales are likely too irregular to produce photonic crystal effects, although the possibility of a Type III mechanism (in addition to a Type II mechanism) cannot be ruled out.

**Figure 8** Schematic illustrations of the basal multilayer structures. (A) A cross-sectional view of the simplest multilayers with alternating chitin and air layers. Chitin layers are supported physically by spacers. (B) A cross-sectional view of fragmented multilayers. This model represents *Ornithoptera* microarchitectures. The thin chitin layers are supported by spacers (i.e. granular objects). The thin layers are fragmented, tilted, and often weakly curved. The layers themselves may be composed of granular objects (not shown in this illustration).

**Differences among the triad: possible functions of basal multilayers and granular objects**

In the present study, we quantitatively measured microstructures to examine structural differences within the triad of *Ornithoptera* species and subspecies. To our knowledge, such comparative morphometric analyses of scale structures among closely related taxa have been scarcely undertaken from a biological viewpoint. An important attempt is found in Kemp et al. (2006), which showed that intraspecific variation of scale microstructures was induced under nutritional or temperature stress in *Colias eurytheme*.

The present study has revealed that the granular objects that constitute the basal multilayers differ in size between orange and green (or blue) scales, suggesting that the size of the objects, which approximates the thickness of a unit layer, is likely responsible for the production of different structural colors. Large granular objects in the orange type probably help to reflect longer wavelengths of light when other photonic conditions for coloration, such as the refractive index of the layer materials, are not significantly different among the three types of *Ornithoptera*. Large granular objects also result in larger air spaces between them. Consistently, we observed that orange scales exhibited the fastest recovery rate upon the removal of methanol among the three color types of *Ornithoptera*.

The blue-green color difference cannot be explained by the sizes of granular objects, because they were similar between the blue and the green types. However, there was a difference in the thickness of the basal multilayers and also in the ridge height. Fluorescent pigments may also explain the blue-green difference (Wilts et al. 2015). An example that uses both pigmentedary and structural coloration is the swallowtail butterfly *Papilio xuthus* (Stavenga et al. 2015).

It should be noted that scale structures and coloration may also differ across a wing, even in a single individual or a single taxon (Kusaba & Otaki 2009; Dhungel & Otaki 2014; Iwata & Otaki 2016); this possibility was not examined herein, but may be an interesting topic in developmental physiology of butterfly wings.
Possible functions of ridges

We detected dorsoventral differences in color and brightness as well as ridge height, suggesting that ridges also likely contribute to different color expression. It has been shown that ridges play a role in the polarization of light (Zhang et al. 2014). Additionally, ridge volume may contribute to coloration directly, as ridges likely contain more fluorescent pigments than basal multilayers (Wilts et al. 2015).

Another possibility is that ridges function as multilayers, like the ridge lamellar structures found in Morpho. At first glance, the ridges of Ornithoptera are smooth, but at higher magnifications, they exhibit regularly spaced fine sculptures, which are most likely microribs. Similar ridge structures have been reported previously in Troides (a genus closely related to Ornithoptera) (Vukusic 2005) as well as in Ornithoptera (Ghiradella 1985). Interestingly, they were tilted to the plane of the basal multilayers. Furthermore, the ridge was likely wavy rather than uniform. This is reminiscent of Morpho scales where ridge height variation functions to destroy the interference of light between adjacent lamellae (Kinoshita et al. 2002a,b; Kinoshita & Yoshioka 2005). In any case, we believe that the photonic mechanisms of Ornithoptera are more complex than simple multilayers. Similar combinatorial mechanisms have recently been proposed in Morpho butterflies (Giraldo & Stavenga 2016) as well as in the Rajah Brooke’s birdwing butterfly Trogonoptera brookiana (Wilts et al. 2016).

Scale structures and speciation

According to molecular phylogenetic analyses (Morinaka et al. 1999, 2000), the three Ornithoptera taxa that are used in the present study are phylogenetically closely related to one another and diverged relatively recently. A species exhibiting blue color, O. aesacus, is the sister species of O. priamus. We hypothesize that the ancestral species in this genus was likely green. If this hypothesis is true, then blue color may have independently evolved twice in the lineage leading to O. aesacus and O. priamus and in another lineage of O. alexandrae. Orange color evolved only once in O. croesus.

We measured the scale thickness by separating it into two components, ridge height and basal layer thickness, and discovered that both measures varied in parallel among the triad: the smallest in blue scales, intermediate in green ones, and the largest in orange ones. In other words, evolutionary pressure acted on both ridge height and basal layer thickness simultaneously (probably on the scale thickness as a whole) but not independently.

The reduction of cover scale thickness may be a sole mechanism for the evolution of blue wing from the ancestral green one through subspeciation in O. priamus. Oppositely, the increase of scale thickness would have caused the wing color change from green to orange in the course of speciation of O. croesus, but in this case, an increase in size of granular objects should have been accompanied as well. Therefore, the green-to-orange evolution is likely more costly, in terms of material compensation, than the green-to-blue evolution. This is also consistent with the finding that the green–orange difference in wavelength (107 nm) is twice as much as the green–blue difference (51 nm). The evolutionary cost may also explain partly the fact that only a single orange-wing species, in contrast to three blue wing species, has evolved in the genus Ornithoptera.

Ohno and Otaki (2015a) observed, in Junonia orithya, that inhibition of calcium waves resulted in a failure to produce the brilliant blue color, and suggested that developmental production of blue structural coloration depends on long-range slow calcium waves. Identification of the genes responsible for production of calcium waves is thus of great interest. Furthermore, real-time in vivo observations (Iwata et al. 2014; Ohno & Otaki 2015b) may be helpful to understand scale-forming processes in the future.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Movie S1 Sequential color changes of Ornithoptera and Morpho wing segments in response to methanol addition and removal.