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RESEARCH ARTICLE

Coloration principles of nymphaline butterflies – thin films, melanin, ommochromes and wing scale stacking

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ABSTRACT

The coloration of the common butterflies *Aglais urticae* (small tortoiseshell), *Aglais io* (peacock) and *Vanessa atalanta* (red admiral), belonging to the butterfly subfamily Nymphalinae, is due to the species-specific patterning of differently coloured scales on their wings. We investigated the scales' structural and pigmentary properties by applying scanning electron microscopy, (micro)spectrophotometry and imaging scatterometry. The anatomy of the wing scales appears to be basically identical, with an approximately flat lower lamina connected by trabeculae to a highly structured upper lamina, which consists of an array of longitudinal, parallel ridges and transversal crossribs. Isolated scales observed at the abwing (upper) side are blue, yellow, orange, red, brown or black, depending on their pigmentation. The yellow, orange and red scales contain various amounts of 3-OH-kynurenine and ommochrome pigment, black scales contain a high density of melanin, and blue scales have a minor amount of melanin pigment. Observing the scales from their adwing (lower) side always revealed a structural colour, which is blue in the case of blue, red and black scales, but orange for orange scales. The structural colours are created by the lower lamina, which acts as an optical thin film. Its reflectance spectrum, crucially determined by the lamina thickness, appears to be well tuned to the scales' pigmentary spectrum. The colours observed locally on the wing are also due to the degree of scale stacking. Thin films, tuned pigments and combinations of stacked scales together determine the wing coloration of nymphaline butterflies.

KEY WORDS: Xanthommatin, 3-OH-kynurenine, Structural coloration, Pigmentary coloration, Scattering

INTRODUCTION

Butterflies are universally considered attractive because of their bright coloration patterns. The colour patterns are due to a tapestry of numerous small scales, each with a distinct colour, which together create the species-characteristic appearance as in pointillist paintings (Nijhout, 1991). The scale colours can have a structural and/or a pigmentary origin, depending on the scale anatomy and its pigmentation (Kinoshita and Yoshioka, 2005). Striking structural colours are widespread among butterflies, with *Morpho* butterflies being the most famous examples, but also many lycaenids and papilionids feature iridescent colours due to intricate photonic systems (Srinivasarao, 1999; Kinoshita et al., 2008; Michielsen et al., 2010).

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The scale elements creating structural colours can be almost perfect thin films, as in the papilionid butterfly *Graphium sarpedon* (Stavenga et al., 2012), (perforated) multilayers in the scale lumen of lycaenids (Wilts et al., 2009), multilayers in the scale ridges, as in *Morpho* butterflies, many pierids and nymphalids (Ghiradella et al., 1972; Ghiradella, 1989; Vukusic et al., 1999; Kinoshita et al., 2002), or complex three-dimensional photonic crystals, as in some lycaenids (Ghiradella, 1998; Michielsen and Stavenga, 2008; Michielsen et al., 2010) and papilionids (Saranathan et al., 2010; Wilts et al., 2012a).

The pigments contributing to coloration vary among the butterfly families. The black scales of all butterfly families contain melanin. White scales may be presumed to be unpigmented, but the white scales of pierids contain leucopterin, a purely UV-absorbing pigment, whereas the yellow, orange and red scales of pierids contain the violet- and blue-absorbing xanthopterin and/or erythropterin (Kayser, 1985; Wijnen et al., 2007). The pigments of the coloured scales of papilionids contain another pigment class, the papiliochromes, derived from the precursor kynurenine (Umebachi, 1985; Wilts et al., 2012b), which is also used by nymphalids to produce 3-hydroxy-kynurenine (3-OH-kynurenine) and various ommochromes, pigments that predominantly determine their wing coloration (Nijhout, 1997; Takeuchi et al., 2005; Nijhout, 2010). In a few cases, bile pigments and carotenoids colour butterfly wings (Barbier, 1981; Stavenga et al., 2010). The pterin pigments of pierids are concentrated in strongly scattering granules (Yagi, 1954; Ghiradella et al., 1972; Stavenga et al., 2004), but the other pigment classes are diffused throughout the scale components.

The wing scales of most nymphalids are essentially flattened sacs; in each, the lower lamina (the adwing side, i.e. facing the wing substrate) is connected by a series of pillars (the trabeculae) to the upper lamina, which consists of the ridges and crossribs (Ghiradella, 1998; Ghiradella, 2010). Light reflected by these components together with the wavelength-selective absorption by the scale's pigment determines the scale colour. Consequently, measured reflectance spectra are somewhat complementary to the pigment's absorbance spectra. Absorbance spectra of various ommochromes present in the wing scales of the buckeye, *Precis coenia*, which belongs to the nymphalid subfamily Nymphalinae, have been determined by extraction of the pigments with methanol (Nijhout, 1997). The synthesis of ommochromes and expression patterns in nymphalid wing scales has been studied in considerable detail in the peacock (*Aglais io*, previously called *Inachis io*), the small tortoiseshell (*Aglais urticae*), the map butterfly (*Araschnia levana*), the painted lady (*Vanessa cardui*), the red postman (*Heliconius erato*), the zebra longwing (*Heliconius charitonia*) and the gulf fritillary (*Agraulis vanillae*) (Nijhout and Koch, 1991; Reed and Nagy, 2005; Reed et al., 2008).

Structural and pigmentary coloration mechanisms are often encountered simultaneously. For instance, the wings of butterflies of the *Papilio nireus* group have conspicuous black margins

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surrounding blue–green bands. The lower lamina of the scales in the blue–green bands acts as a thin film, reflecting broad-band blue light. The thick upper lamina contains the violet-absorbing pigment papiliochrome II, and thus acts as an optical band-filter, limiting the reflected light to the blue–green wavelength range (Trzeciak et al., 2012; Wilts et al., 2012b). In the opposite way, in several pierid butterflies, in the short-wavelength range, where the wing pigments strongly absorb, ridge interference reflectors contribute to the reflectance (Rutowski et al., 2005; Stavenga et al., 2006). In many cases, melanin enhances the saturation of the colour signal. For example, in *Morpho* wing scales, the melanin deposited below the multilayered ridges effectively absorbs transmitted light, which potentially could be scattered back by the wing or other scale structures and thus result in a desaturating background signal (Kinoshita et al., 2008).

Here, we studied the wing coloration of a few common nymphaline butterfly species. We specifically investigated the optical properties and pigmentation that determine the colours of the various individual wing scales. We demonstrate that the thin film properties of the lower lamina are a dominant factor in determining the scale's colour. The reflectance spectrum of the lower lamina appears to be well tuned to the scale's pigmentation. We furthermore found that the wing coloration depends on the stacking of neighbouring scales.

RESULTS

Wing coloration and reflectance spectra

The wings of the small tortoiseshell [*A. urticae* (Linnaeus 1758)], peacock [*Aglais io* (Linnaeus 1758)] and red admiral [*Vanessa atalanta* (Linnaeus 1758)], all nymphaline butterflies, have strikingly colourful dorsal (upper) sides (Fig. 1A–C). In contrast, the ventral (lower) sides of the wings have a very inconspicuous dull brown–black colour, except for the red admiral where the ventral forewings feature some coloration (Fig. 1D). To unravel the various optical mechanisms underlying the different colours, we first measured the local spectral reflectance with a bifurcated probe. The reflectance spectra of the yellow to red–brown wing areas show characteristic long-pass features, i.e. a low reflectance at short wavelengths and a high reflectance in the long-wavelength range (Fig. 1E–H, nos 2, 3, 5, 6, 9 and 13); the number near the reflectance spectra corresponds with the number of the location where the spectra were measured (indicated in Fig. 1A–D). The reflectance spectra measured from blue areas reveal a distinct peak around 420 nm and a trough near 620 nm (Fig. 1E–H, nos 4, 7, 11 and 15). Interestingly, reflectance spectra obtained from white wing areas have a similar biphasic shape riding on a distinct plateau (e.g. Fig. 1G,H, nos 10 and 14). The black areas, not surprisingly, have a very low reflectance (Fig. 1F–H, nos 8, 12 and 16).

Scale anatomy

The wing colours reside in the tapestry of wing scales, and therefore we investigated single scales taken from the differently coloured wing areas. A red scale of a red admiral and a black scale of a peacock, immersed in a refractive index fluid (Fig. 2A,B), both show numerous longitudinal lines. These are due to the ridges, which are prominently revealed by scanning electron microscopy (SEM, Fig. 2C).

All nymphaline scale types appeared to have essentially the same basic bauplan, as shown in the diagram of Fig. 2D (Ghiradella, 1998; Ghiradella, 2010). The abwing side of the scales consists of parallel rows of ridges (Fig. 2D, labelled r) with slopes featuring microribs (Fig. 2D, labelled m). Some of the microribs are continuous with crossribs (Fig. 2D, labelled c), which connect adjacent ridges, thus marking open windows (Fig. 2C,D). The crossribs are connected by trabeculae (Fig. 2D, labelled t) with the approximately flat lower lamina.

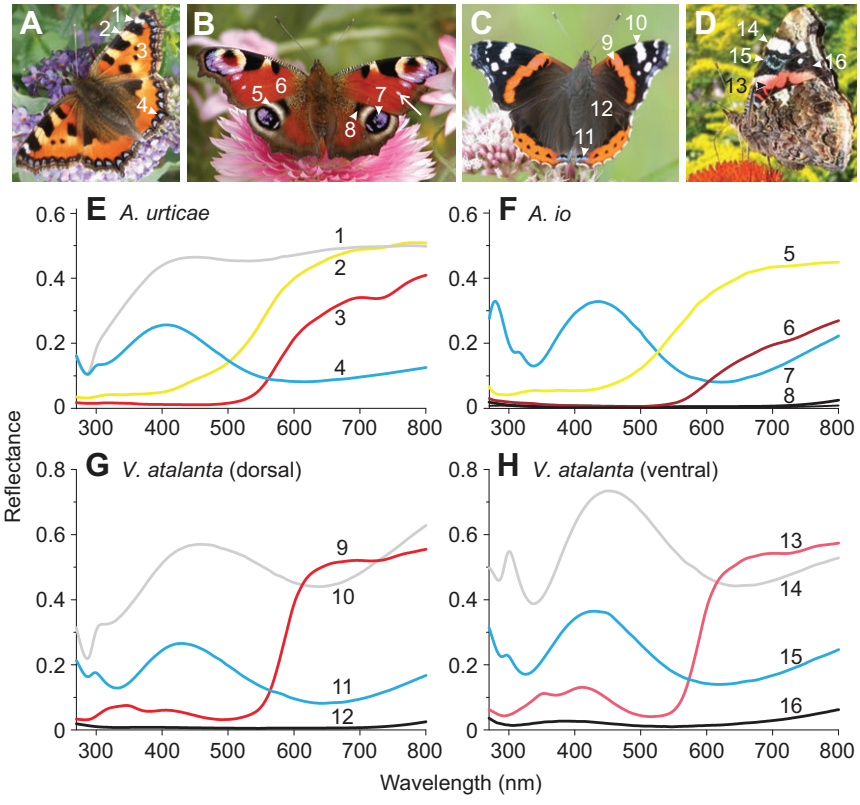

Fig. 1. Three common nymphaline butterflies and wing reflectance spectra measured with a bifurcated probe. (A,E) *Aglais urticae*, the small tortoiseshell; (B,F) *Aglais io*, the peacock; (C,G) *Vanessa atalanta*, the red admiral, wing dorsal surfaces; and (D,H) *V. atalanta*, wing ventral surfaces. The numbers in the photographs and spectra correspond. The arrow in B corresponds to Fig. 6C.

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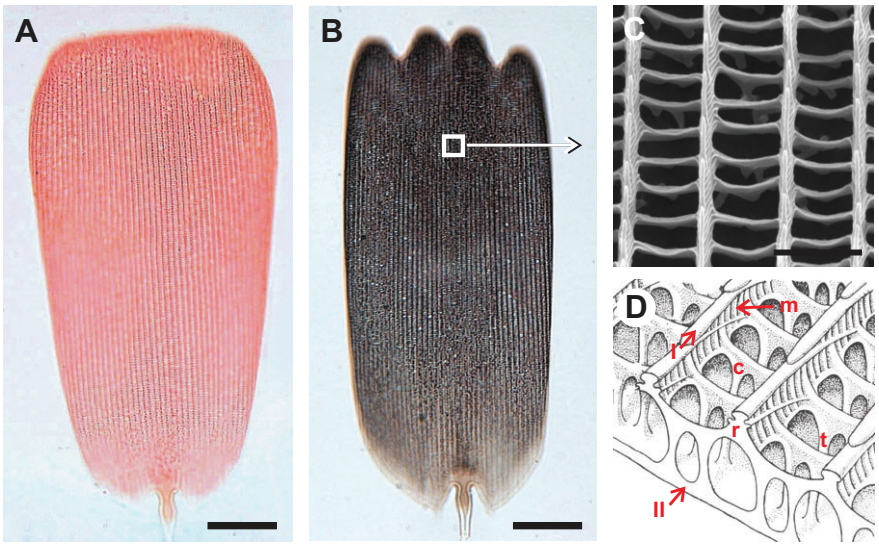

Fig. 2. Anatomy of nymphaline wing scales. (A,B) Transmitted light microscopy of a red scale of a red admiral and a black scale of a peacock immersed in a refractive index fluid (scale bars, 20 µm). (C) Scanning electron microscopy photograph of a black scale of a peacock corresponding to the square in B (scale bar, 2 µm). (D) Drawing of a nymphaline wing scale (modified from Ghiradella, 1998), showing the ridges (r) with lamellae (l) and microribs (m), some of which are continuous with the crossribs (c), which are connected by trabeculae (t) to the lower lamina (ll) of the scale.

Pigments of nymphaline wing scales

The scales of Fig. 2A,B evidently contain very different absorbing pigments. To identify the pigments in the wing scales of the investigated nymphalines, we isolated scales from the differently coloured wing areas of the three nymphalines of Fig. 1. We immersed the scales in immersion oil, so as to reduce light scattering and reflection that unavoidably occur at boundaries between media with different refractive index values, and we then measured the scale transmittance with a microspectrophotometer. The absorbance spectra thus obtained from the individual yellow, red and blue scales were rather varied (Fig. 3A–C). The absorbance of the orange and red scales peaked at 480–500 nm, and the absorbance of the yellow scales peaked in the ultraviolet, at around 380 nm, but often had a side band near 500 nm (Fig. 3B,C).

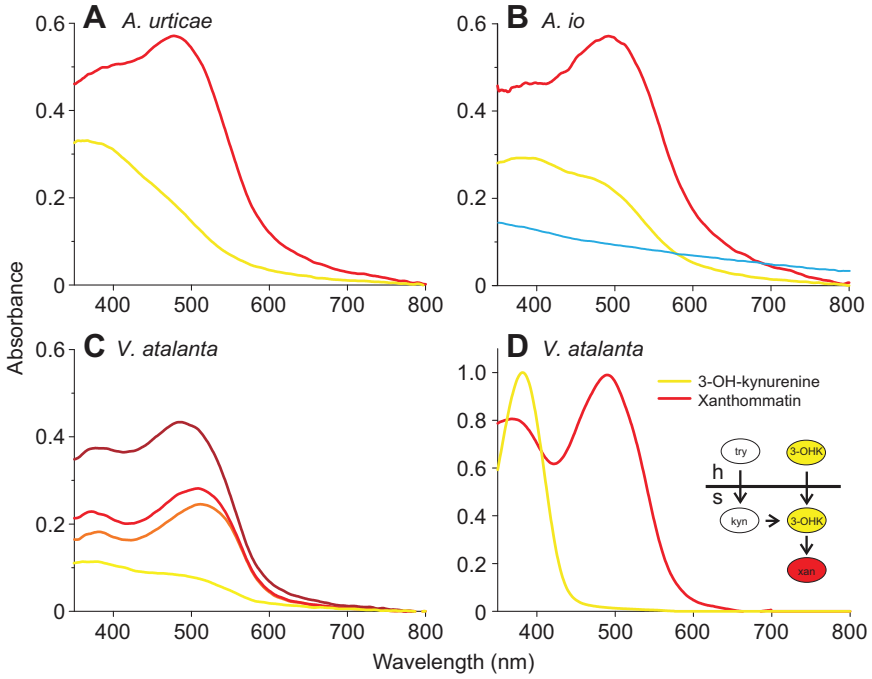

Fig. 3. Absorbance spectra of isolated yellow to red–brown wing scales in immersion oil (refractive index *n*=1.515) and pigments. (A) Small tortoiseshell. The red and yellow curves are from scales in areas 3 and 2, respectively, of Fig. 1A. (B) Peacock. The red curve corresponds to area 6 of Fig. 1B, and the yellow curve holds for scales from a small yellowish area on the ventral wing surface; the blue curve is the average absorbance spectrum of blue scales from the eyespot of the dorsal hindwing (Fig. 1B, area 7), showing some melanin pigmentation. (C) Red admiral. The brown and red curves are for scales from the orange–red band on the dorsal forewing (Fig. 1C, area 9), and the orange curve is for scales from the orange–red band at the ventral forewing (Fig. 1D, area 13); the yellow curve is from a small yellow area in the ventral forewing. (D) Normalised absorbance spectra of the pigments 3-hydroxy-kynurenine (3-OH-kynurenine), extracted from yellow scales of *Heliconius melpomene* with methanol (D.G.S., H.L.L. and B.D.W., unpublished) (see also Reed and Nagy, 2005) and xanthommatin (from Nijhout, 1997). Inset: hypothetical model of ommochrome synthesis in butterfly wing scales based on analyses of *Drosophila* colour mutants. The ommochrome precursor tryptophan (try) is thought to be transported from the haemolymph (h) into a scale (s) cell, where it is processed by several enzymes including vermillion to kynurenine (kyn) and cinnabar into 3-OH-kynurenine (3-OHK), which is processed further into xanthommatin (xan). 3-OH-kynurenine itself can be taken up directly into scales (after Reed and Nagy, 2005; Reed et al., 2008).

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The absorbance spectra of the orange and red scales are very reminiscent of the ommochromes, identified in related nymphalid butterflies (Nijhout, 1997; Reed and Nagy, 2005). Fig. 3D shows the absorbance spectrum of xanthommatin extracted from wing scales of the buckeye (Nijhout, 1997), together with the spectrum of its precursor, 3-OH-kynurenine (extracted from yellow scales of *Heliconius melpomene* with methanol; D.G.S., H.L.L. and B.D.W., unpublished) (see also Reed and Nagy, 2005). The spectra of the yellow scales (Fig. 3A–C) indicate that these scales contain mainly 3-OH-kynurenine with various traces of xanthommatin. The absorbance of the blue scales, taken from the eyespot of the peacock, was minor, decreasing monotonically with wavelength, indicating a rather small amount of melanin pigment (Fig. 3B). The absorbance spectra of the black scales (not shown), had a similar shape, i.e. monotonically decreasing with increasing wavelength, but the amplitude was quite variable, frequently exceeding an absorbance value of 1. Traces of ommochromes appeared to be often present, but the main component was clearly melanin.

Most of the absorbance spectra of Fig. 3, when compared with the reflectance spectra of Fig. 1, seem to confirm the common view that pigments determine the scale colour. For instance, the yellow, orange and red scales have a low reflectance in the wavelength range where the 3-OH-kynurenine and xanthommatin pigments strongly absorb. Melanin absorbs throughout the whole wavelength range and thus with sufficient density will cause low-reflecting, black scales. The blue scales, containing a small amount of melanin, have reflectance spectra that cannot be straightforwardly reconciled with the absorbance spectrum of its pigment, however. The conclusion therefore must be that the blue colour of these scales has a structural origin.

Thin films

The shape of the reflectance spectra of the blue wing areas (Fig. 1) suggests that the lower lamina of the local scales acts as an optical, dielectric thin film. We therefore calculated the reflectance spectrum of a thin film with various thicknesses using the classical Airy formula for normal light incidence (Yeh, 2005; Stavenga et al., 2012). For the refractive index of the thin film, we used the dispersion data determined for the glass wing scales of the papilionid butterfly *Graphium sarpedon* (Leertouwer et al., 2011). Those scales consist of two collapsed layers, each with a thickness of about 200 nm (Stavenga et al., 2010). The same thickness value was concluded for the lower lamina of the green scales of *P. nireus* butterflies (Trzeciak et al., 2012; Wilts et al., 2012b). However, rather different values exist in other cases; for instance, the thickness of the lower lamina of *Argyrophorus argenteus* scales is 120 nm (Vukusic et al., 2009), and the small white [*Pieris rapae*; see fig. 4a of Stavenga et al. (Stavenga et al., 2004)] and the angled sunbeam butterfly [*Curetis acuta*; see fig. 4c of Wilts et al. (Wilts et al., 2013)] have scales where the lower laminae have thicknesses well below 100 nm.

We therefore calculated reflectance spectra for five thin films with thicknesses of 125–225 nm (Fig. 4A). The reflectance spectrum for 200 nm (Fig. 4A, blue curve), with maximum at ~420 nm and minima at ~320 and 620 nm, closely resembles the spectra measured from the blue wing areas (Fig. 1) except for the latter's non-zero minima, which are most likely due to a slightly variable thickness of the lower lamina of the blue scales (Fig. 4B). The spectra of Fig. 4 hold for unpigmented scales. The refractive index is modified by high pigment concentrations (e.g. Stavenga et al., 2013), and therefore we also calculated the reflectance spectrum of a 200 nm thick scale containing the pigment measured in the blue scale of Fig. 3B; this yielded a virtually identical spectrum as that for the unpigmented scale (not shown).

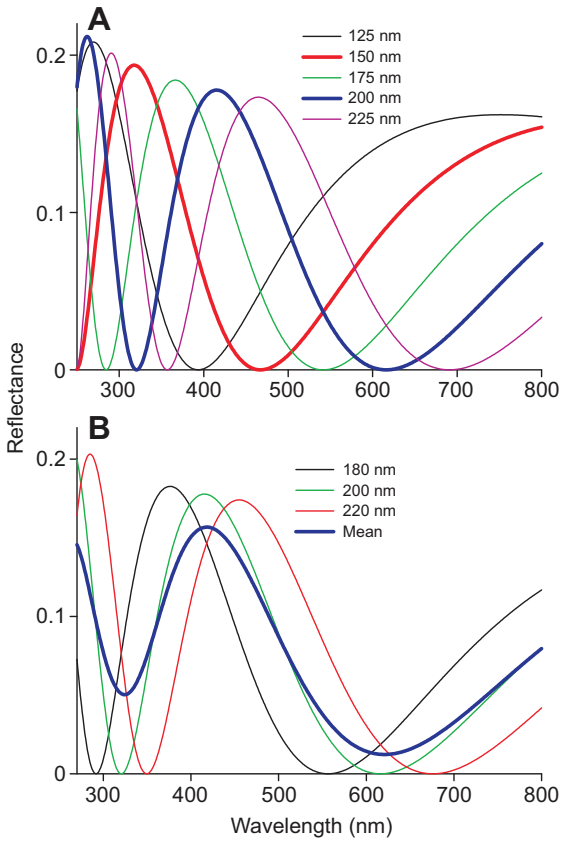

Fig. 4. Reflectance spectra of cuticle thin films in air. (A) Reflectance spectra of thin films with thicknesses of 125–225 nm and the refractive index of butterfly wing scales (Leertouwer et al., 2011). (B) Reflectance spectra of thin films with thicknesses of 180, 200 and 220 nm and their mean.

The conclusion that thin film reflection determines the colour of the blue scales suggests that thin film reflection can also contribute to the colour of the other scale types. We therefore performed a detailed study of the reflection properties of the variously coloured scales of the nymphalines by inspecting both sides of the scale.

Spatial and spectral reflection characteristics of single scales

To understand how the local wing colours are created, we investigated the spectral and spatial reflection characteristics of single scales. We glued single scales to the thin tip of a glass micropipette and observed the scales at both the abwing (upper) side and adwing (lower) side with an epi-illumination light microscope (Fig. 5, left). We measured scatterograms of both scale sides with a narrow aperture white light beam (Fig. 5, middle column) and we also measured the reflectance spectra with a microspectrophotometer (Fig. 5, right).

A blue scale (from a peacock's wing eyespot; Fig. 1B, no. 7) is blue on both sides (Fig. 5A, left). The adwing side, which is more or less smooth, creates a much glossier photograph than the abwing side, which is highly structured with ridges and crossribs. The scatterogram of the adwing side is a localised spot (Fig. 5A, middle). The aperture of the illumination beam was about 5 deg, but the reflection spot in the scatterogram is slightly larger, obviously due to the somewhat wrinkled adwing surface. In contrast, the abwing scatterogram shows

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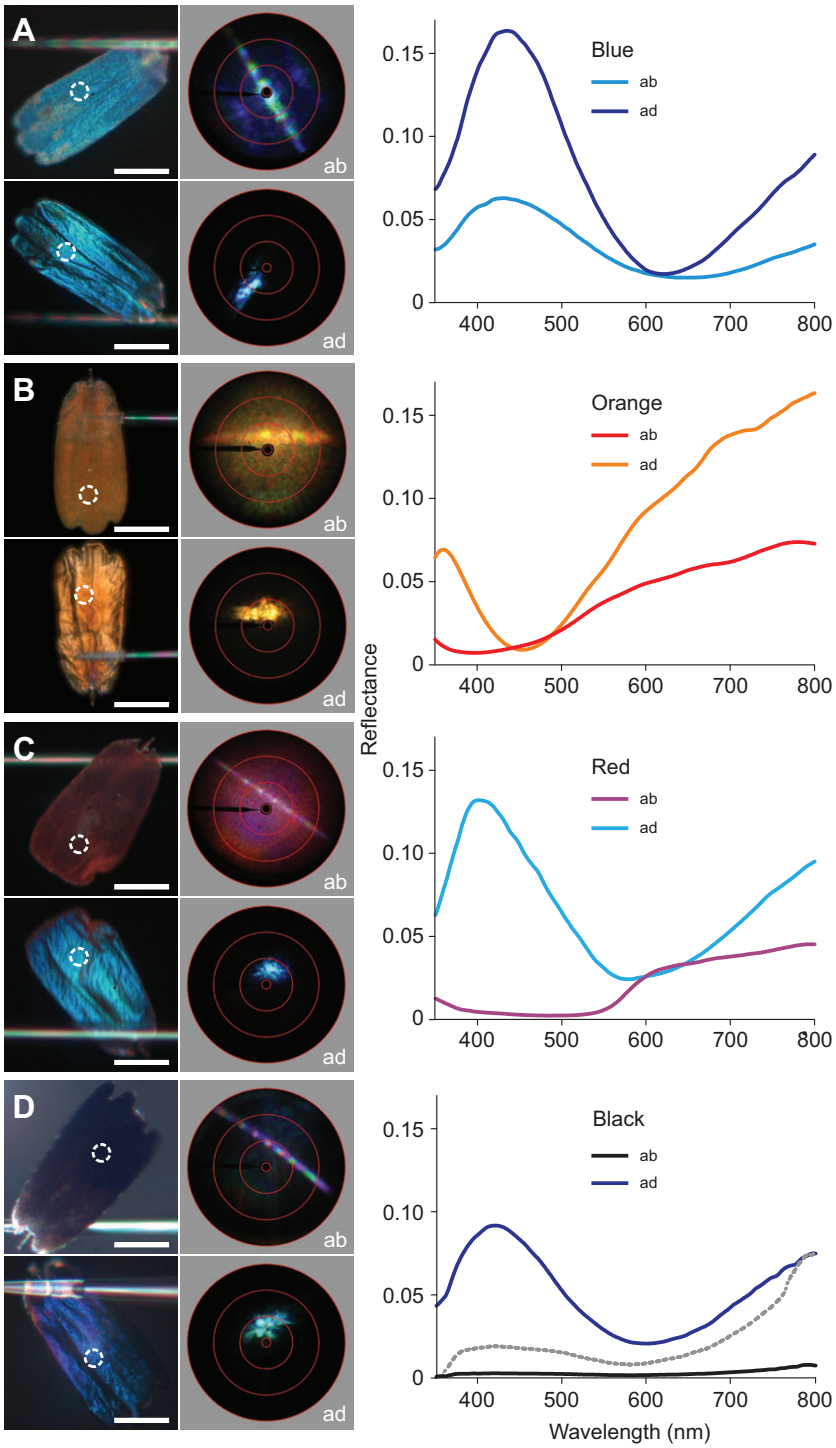

Fig. 5. Reflection properties of single nymphaline wing scales. Left column: photographs of abwing (top) and adwing (bottom) sides of a blue scale of a peacock (A), an orange scale of a small tortoiseshell (B), a red scale of a red admiral (C) and a black scale of a red admiral (D); scale bars, 50 µm. The wing scales were glued to a glass micropipette (shiny and/or coloured horizontal bars). Middle column: scattergrams of the four scales, obtained by illuminating a small area in the abwing (ab) and adwing (ad) sides (dashed circles in the photographs of the left column) with a narrow aperture light beam. The red circles indicate scattering angles of 5, 30, 60 and 90 deg (see supplementary material Fig. S1). Right column: reflectance spectra measured from a small area (similar to the dashed circle in the photographs of the left column). The dotted line in D (right column) is the black scale's abwing reflectance spectrum ×10.

a diffraction pattern, created by the array of parallel ridges, together with a diffuse pattern, presumably due to scattering by the crossribs (Fig. 5A, middle) (see also Stavenga et al., 2009).

The reflectance spectra of the two sides of the blue scale have a very similar shape (Fig. 5A, right) and closely resemble the spectrum calculated for a thin film with slightly varying thickness and a mean thickness of 200 nm (Fig. 4B, mean). This suggests that the thin film properties of the lower lamina of the blue scale dominantly determine the scale colour. The two reflectance spectra of the blue scale differ in amplitude, which is partly due to the minor amount of pigment (Fig. 3B), but the main effect must be attributed to the limited aperture of the objective of the microspectrophotometer, because it captures a much smaller part of the diffuse abwing reflection than of the directional adwing reflection.

An orange scale of the small tortoiseshell (Fig. 1A, no. 3,) is orange on both the abwing and adwing side (Fig. 5B, left). Similar to the blue scale, the abwing side has a matte colour, and the adwing side features a metallic reflection. Also similarly, the adwing scattergram reveals a spatially restricted reflection pattern (Fig. 5B, middle), while the abwing scattergram shows a bright orange line together with a diffuse background of the same colour. The adwing reflectance spectrum, which has a distinct trough at ~460 nm (Fig. 5B, right),

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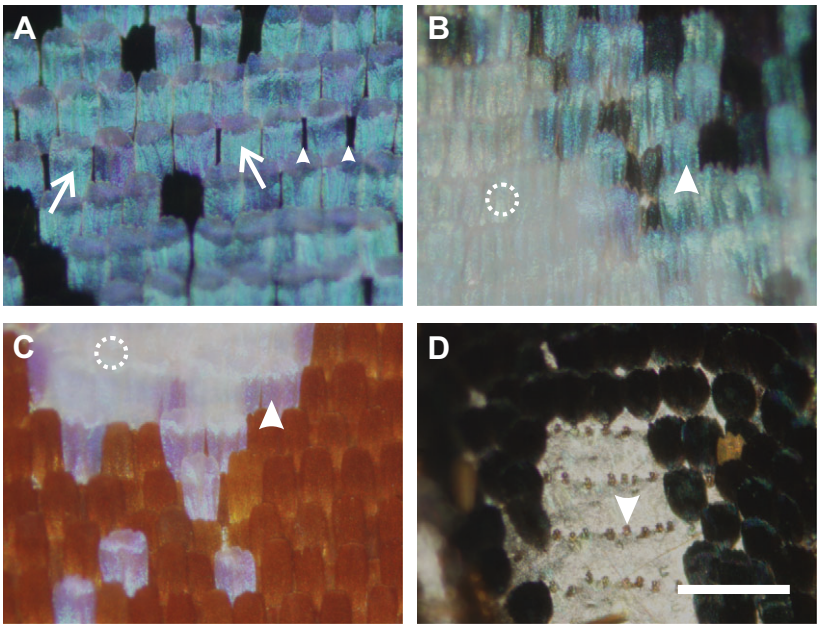

Fig. 6. Packing and stacking of scales on nymphaline butterfly wings. (A) Blue scales in the eyespot of a dorsal hindwing of a peacock butterfly (Fig. 1B, no. 7). Below the blue cover scales (arrows) and some black cover scales there exist black ground scales (small arrowheads). The tips of the blue scales are curved and thus show a violet colour (see Results, Scale stacking). (B) Border area of the white band on the ventral forewing of a red admiral butterfly (Fig. 1D, no. 14). Stacked unpigmented cover scales and ground scales, together with the wing substrate, yield a white colour (area with dotted white circle), but unpigmented cover scales above black ground scales appear blue (arrowhead). (C) Border of the white spot in the dorsal forewing of a peacock (indicated by the arrow in Fig. 1B). Stacked unpigmented cover scales, ground scales and wing membrane yield white (area with dotted white circle), but unpigmented cover scales above orange ground scales appear pink (arrowhead). (D) Damaged area with black scales on the dorsal forewing of a small tortoiseshell, showing the bare wing substrate with the sockets of the removed scales (arrowhead). Scale bar for A–D, 200 µm.

compared with the spectra of Fig. 4A, suggests that the lower lamina of the orange scale acts as a thin film with mean thickness ~150 nm. As with the blue scale, with abwing illumination, the light reflected by the lower lamina's thin film is diffracted by the ridges and more or less diffusely scattered by the crossribs. The abwing reflectance will be suppressed in the short-wavelength range, where the absorbance of the scale's main pigment, xanthommatin, is substantial (Fig. 3A,D), thus causing the almost monotonic increase of the reflectance with increasing wavelength (Fig. 5B, right).

A red scale of the red admiral (Fig. 1C, no. 9), is very different as the abwing side is red coloured, while the adwing side is metallic blue (Fig. 5C, left). The abwing scatterogram features a clear diffraction pattern, perpendicular to the ridge array, together with a diffuse red background, while the adwing scatterogram shows a local, blue spot, documenting a very directional, specular reflection pattern (Fig. 5C, middle). The adwing reflectance spectrum unequivocally indicates a thin film with thickness ~190 nm (Fig. 5C, right). With abwing illumination, the large amount of xanthommatin pigment in the scale will strongly suppress the blue light reflected by the lower lamina, while leaving the long-wavelength part unimpeded, thus yielding a substantial reflectance only in the red wavelength range (Fig. 5C, right).

An extreme case is a black scale of a red admiral, which has a metallic navy-blue coloured adwing side (Fig. 5D, left). The adwing scatterogram shows a localised spot, as expected from a directionally reflecting thin film (Fig. 5D, middle). The adwing reflectance spectrum indicates a thickness of ~200 nm (Fig. 5D, right). The distinct diffraction pattern in the abwing scatterogram was obtained with a relatively long exposure time, and accordingly the abwing reflectance was minimal, clearly because the scale's melanin pigment blocked the reflectance of the lower lamina. Interestingly, multiplying the abwing spectrum by a factor of 10 reveals a spectral shape reminiscent of the adwing reflectance spectrum (Fig. 5D, right, dotted curve). This confirms the interpretation that the lower lamina contributes to the abwing reflectance spectrum. The highly absorbing melanin suppresses the lower lamina's contribution, but the upswing in the far-red of the abwing reflectance spectrum can be immediately understood from the melanin absorbance spectrum, which decreases with increasing wavelength.

Scale stacking

Butterfly wing scales are arranged in regular, usually overlapping rows (Fig. 6). In the eyespots of the dorsal hindwings of the peacock, blue cover scales are backed by black ground scales (Fig. 6A), which thus ensure the blue coloration. When not backed by black scales, but stacked above one another, the blue scales have a whitish colour (Fig. 6B,C). The colour change is due to light that has passed one blue scale and is then reflected by the underlying one. In the case of stacked blue scales, the wing substrate can also contribute to the reflection (Stavenga et al., 2006), as removal of its scales shows that reflectance of the wing substrate is certainly not negligible (Fig. 6D). Blue scales above orange–red scales become purplish (Fig. 6C). The tips of the blue scales are curved and thus show a violet colour (Fig. 6A) because of the basic property of thin films that the reflectance spectrum shifts hypsochromically (towards shorter wavelengths) when the film plane is tilted (Fig. 6A).

Measurements on a bare, descaled area of a small tortoiseshell wing (Fig. 6D) show that the wing substrate has a rather constant reflectance over the whole wavelength range (Fig. 7). The reflectance spectrum features oscillations, indicating that the wing substrate has thin film properties. From the periodicity of the oscillations, a local wing thickness of ~1.1 µm can be derived [following a previously published method (Stavenga et al., 2011)]. The transmittance spectrum, $T(\lambda)$ (where λ is wavelength), measured at the same location yields identical oscillations to those seen in the reflectance spectrum, $R(\lambda)$ (Fig. 7). Combining the reflectance and transmittance spectra shows that the wing absorptance, $A(\lambda)=1-T(\lambda)-R(\lambda)$ (the area between $1-T(\lambda)$ and $R(\lambda)$ in Fig. 7) is not negligible. Presumably the wing contains traces of 3-OH-kynurenine and xanthommatin, similar to the yellowish scales (Fig. 3). This may not be surprising, as during development 3-OH-kynurenine is taken up from the wing haemolymph (Fig. 3D, inset) (see Koch, 1991; Reed and Nagy, 2005; Reed et al., 2008).

DISCUSSION

Pigments in the wing scales of nymphaline butterflies

The wing scales of nymphaline butterflies have structure- as well as pigment-based coloration. Following previous work on the closely related map butterfly (*A. levana*) (Koch, 1991) and painted lady (*V.*

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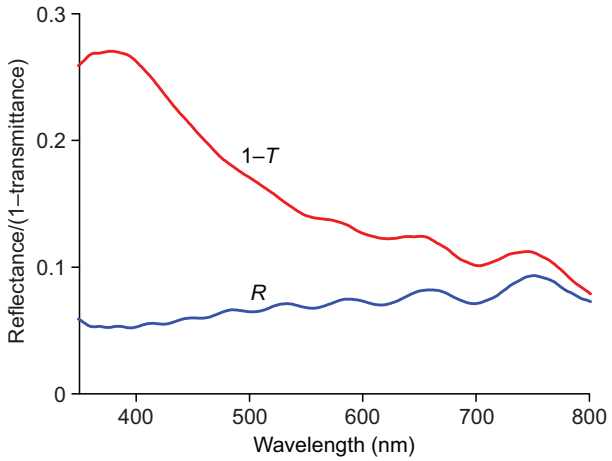

Fig. 7. Thin film properties of the clear wing of a small tortoiseshell. The absorptance $A=1–T–R$ (where T is transmittance and R is reflectance) indicates that the wing contains 3-OH-kynurenine as well as xanthommatin.

cardui) (Reed and Nagy, 2005), we identified as the important pigments xanthommatin and its precursor 3-OH-kynurenine. However, it is not unlikely that several ommochromes participate in the wing coloration of nymphaline butterflies. For instance, depending on the duration of the rearing conditions, the buckeye develops either a pale tan or a dark reddish-brown pigmentation, due to either xanthommatin or dihydro-xanthommatin and ommatin-D expression (Nijhout, 1997). The different ommochromes have absorbance spectra that all peak in the blue–green wavelength range (Riou and Christidès, 2010) and thus cannot be easily distinguished by *in situ* measurements. Presumably, therefore, the yellow to red scale colours can be finely tuned, not only by mixing 3-OH-kynurenine and xanthommatin but also adding other ommochromes. The situation may be even more complicated, as the absorbance measurements on transparent (blue) as well as ommochrome-pigmented (coloured) scales suggest that many of these scales contain traces of melanin.

A genome-wide survey of genes for enzymes involved in pigment synthesis in the ascidian *Ciona intestinales*, a marine invertebrate chordate, demonstrated that the genome contained a wide set of enzymes involved in the synthesis of melanin, ommochromes, papiliochromes, pterins as well as haemes (Takeuchi et al., 2005). Whereas pterins are the wing pigments of pierids, and papiliochromes colour the wings of papilionids, nymphalid butterflies have clearly favoured ommochromes and melanin for their wing coloration. The nymphalines have expressed these pigments in rather simply structured scales, in variable amounts and in different combinations, thus creating complex and striking patterns. Other nymphalids have diversified scale structures, thus creating much more intense structural colours. For instance, multilayered ridges cause the bright blue of the wings of *Morpho* species (Kinoshita et al., 2008), and closed windows result in silvery scales in some heliconiine butterflies (Simonsen, 2007).

Structural and pigmentary coloration of nymphaline scales

A central finding of the present study is that in all cases the lower lamina acts as a thin film, as is immediately demonstrated with illumination from the adwing side and diagrammatically shown in Fig. 8A for a red scale of the red admiral (Fig. 5C). In contrast, incident light at the upper, abwing side partly hits the ridges and crossribs, where it is scattered, but also enters through the large windows formed by the ridges and crossribs. The light flux reaching the lower lamina is partly reflected there and subsequently passes the ridges and crossribs. Pigment present in the scale's components will act as a spectral filter and thus modify the lower lamina's reflectance spectrum. In a simple interpretation, diagrammatically shown in Fig. 8B for the red admiral's red scale, the reflectance spectrum measured with abwing illumination is the reflectance spectrum of the lower lamina's thin film multiplied by the effective transmittance spectrum of the scale's pigment.

The spectral filtering of the thin film reflection is negligible when a scale has little or no pigment, and then the same reflectance spectrum will be obtained for both abwing and adwing illumination. This is the case of the blue scales (Fig. 5A). With a high concentration of melanin, which absorbs throughout the whole visible as well as ultraviolet wavelength range, abwing reflection is fully suppressed (Fig. 5D). With a considerable concentration of blue-absorbing xanthommatin, which is the case in the red scales, reflection in the short-wavelength range is largely suppressed, but the thin film reflection in the red wavelength range is left unaffected, resulting in a red colour with a slight purplish hue (Fig. 5C).

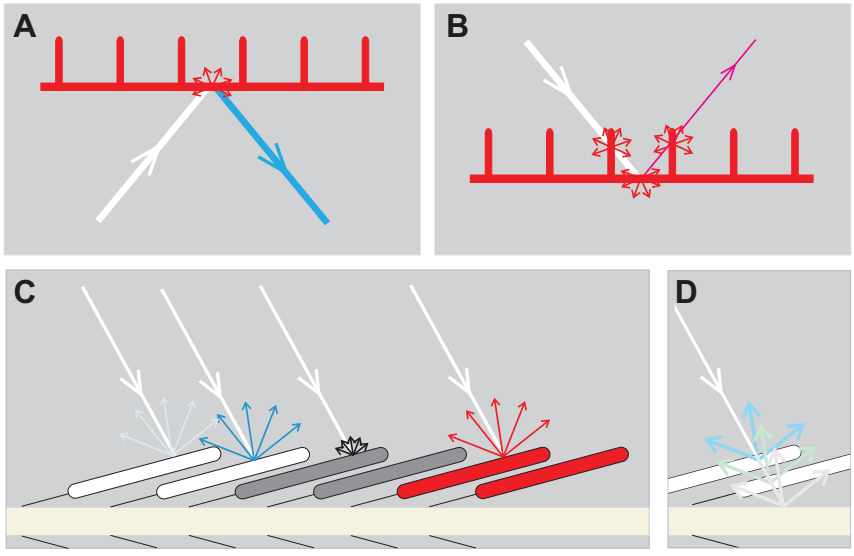

Fig. 8. Diagrams of light reflection and scattering by the wing scales of nymphaline butterflies. (A) Light reflection of a red pigmented scale illuminated at the adwing side is dominated by the thin film properties of the lower lamina, yielding a blue directional reflection. (B) Light reflection of a red pigmented scale illuminated at the abwing side is dominated by scattering at the ridges and crossribs, thus causing considerable absorption by the pigment in the scale. (C) Illumination of the scales on the wing results in coloration depending on the local stacking of the scales. An unpigmented scale on top of another unpigmented scale yields a white–bluish colour. An unpigmented scale on top of a melanin-pigmented, black scale yields a blue colour. A melanin-pigmented scale is black because of its low reflectance. A red-pigmented scale reflects and backscatters red light, which becomes more saturated with stacked red scales. (D) Detail of C to illustrate that the observed scale colour is the cumulative result of reflection and scattering by the stack of scales and the wing substrate.

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The thickness of the lower lamina of the various scales is not always the same. The lower laminae of the blue, black and red scales all have a blue-peaking reflectance, indicating a similar thin film thickness. The orange scales have a very different reflectance spectrum, however, indicating a much smaller thickness (Fig. 5B). The orange scales contain a modest amount of xanthommatin as well as some 3-OH-kynurenine, which together suppress the short-wavelength reflection of the lower lamina, and thus an almost monotonically increasing reflectance spectrum results (Fig. 5B). The orange colour hence is a combined structural and pigmentary coloration effect.

The reflectance spectrum of the orange scale's thin film suggests that it is tuned to the absorbance spectrum (or rather the transmittance spectrum) of the scale's pigment. A high concentration of xanthommatin in a scale with a reflectance spectrum of the lower lamina as that of the blue scale (Fig. 5A) will suppress the blue peak in the scale's (abwing) reflectance spectrum so that only the reflectance at the red wavelengths remains. This is in fact the case with the red scales (Fig. 5C). For a yellow or orange scale colour it is essential to shift the thin film reflectance minimum towards much shorter wavelengths. Pigments absorbing in the short-wavelength range, which act as long-pass spectral filters, can then effectively suppress the reflection at short wavelengths and leave the long-wavelength reflection relatively unhampered, resulting in an enhanced hue of the scale.

The survey of the optical properties of the various scales of the nymphalines leads to an important conclusion, namely that with negligible pigmentation the lower lamina determines the scale colour, while the pigment becomes a dominant factor at high pigment concentration, especially in the case of the red and black scales. The general conclusion is that thin film reflectance and pigment absorbance together determine the scale colour.

The importance of the lower lamina's thin film has been previously noticed for the blue cover scales of *Morpho didius* (Yoshioka and Kinoshita, 2004). However, rather than colouring the wings, these scales function as diffusers for the highly reflective, blue ground scales (Yoshioka and Kinoshita, 2004). Also, the green scales of butterflies of the *P. nireus* group have a lower lamina with a blue-reflecting thin film. The crossribs of these scales form a thick upper lamina, which contains the violet-absorbing pigment papiliochrome II. The upper lamina acts twice as an effective spectral filter for a broad-band white light beam, incident from the abwing side, which is reflected by the lower lamina, thus causing a narrow-band blue–green reflection (Trzeciak et al., 2012; Wilts et al., 2012b).

Wing coloration and scale stacking

The reflectance of the scales for about normal illumination is usually rather moderate, of the order of at most 20%, so that >80% is transmitted. Scales on the wing are stacked, so that a considerable fraction of the incident light passes the cover scales and reaches the overlapped ground scales. These scales thus can also contribute to the wing reflectance (Fig. 6). Because the reflectance of black scales is negligible, blue scales overlapping black ground scales create a blue wing colour, but blue scales on top of each other yield a desaturated bluish colour. When the wing substrate also contributes, the result is a whitish colour. The reflectance spectra of the whitish areas nevertheless have peaks in the blue wavelength range (Fig. 1G,H, nos 10 and 14), betraying the presence of blue-reflecting thin films. Similarly, a red scale on top of another red scale and/or the wing substrate will result in a more saturated red colour, due to the enhanced reflectance in the longer wavelength range. This is the case for the peacock wing areas with densely packed red scales (Fig. 1B, Fig. 6C). The consequences of wing scale stacking are diagrammatically shown in Fig. 8C,D (see also supplementary material Fig. S2).

Wing scale stacking appears to be an effective method to achieve bright coloration. At the level of a single scale, the ~200 nm thick lower lamina is an extremely lightweight, reasonably effective reflector. Its reflectivity is highly directional, and presumably therefore the upper lamina, consisting of ridges and crossribs, acts as a diffuser. When pigmented, the upper lamina additionally acts as a spectral filter. In other words, the asymmetrical anatomy of butterfly scales, with the well-reflecting lower lamina and the diffusive upper lamina, may in fact have a straightforward functional basis. Furthermore, the reflectance of a single scale may be moderate; by stacking a few scales, the additive effect yields quite appreciable reflectance values (Fig. 1).

Wing coloration and display

A special point to consider is the prominent red coloration of the studied nymphaline butterflies. Three different visual pigments have been characterised in nymphalines; ultraviolet-, blue- and green-absorbing rhodopsins (Briscoe and Bernard, 2005). The longest-wavelength receptor identified has a peak wavelength around 530 nm, meaning a very limited overlap of the receptor's spectral sensitivity and the red reflectance spectra of the wing (see also Kinoshita et al., 1997). The red wing parts hence may be bright signallers not for conspecific butterflies but, rather, for predatory birds, which have photoreceptors that are highly sensitive in the red (e.g. Hart, 2001). Whereas in the case of the peacock, the red coloration may add to the warning signal of the wings' eyespots (Blest, 1957), it is hard to conceive that a similar mimicry or threatening display is realised in the wings of the small tortoiseshell and the red admiral. Possibly, their wing patterns have a visually disruptive function.

Conclusions

A few levels of complexity determine the coloration of nymphaline wings. The lower lamina of the scales acts as a thin film reflector. Blue scales are virtually unpigmented and have a diffuse colour due to the light-scattering ridges and crossribs. When the scales are pigmented, however, spectrally selective absorption by the pigment of the light reflected by the lower lamina determines the scale colour. The local scale stacking as well as the reflecting wing substrate fine tune the wing colour.

MATERIALS AND METHODS

Animals

Specimens of the small tortoiseshell (*A. urticae*), the peacock (*A. io*; previously *Inachis io*) and the red admiral (*V. atalanta*), which are among the most common nymphalid butterflies in the northern part of The Netherlands, were captured locally in the summer of 2013.

Photography

Photographs of the tapestry of scales of different wing parts (Fig. 6) were taken with an Olympus SZX16 stereomicroscope equipped with an Olympus DP70 digital camera (Olympus, Tokyo, Japan). Photographs of both the adwing (lower) side and abwing (upper) side of single scales, obtained by gently pressing the wings on to a microscope slide and subsequently gluing the individual scales to a glass micropipette (Fig. 5), were made with a Zeiss Universal Microscope, using epi-illumination through a Zeiss Epiplan 16×/0.35 objective (Zeiss, Oberkochen, Germany). Photographs of single scales in immersion fluid (refractive index 1.60; Fig. 2) were made with a Nikon Fluor 40×/1.30 oil objective (Nikon, Tokyo, Japan). The digital camera was a Kappa DX-40 (Kappa Optronics GmbH, Gleichen, Germany).

Spectroscopy

Reflectance spectra of different wing areas (Fig. 1) were measured with a bifurcated probe (FCR-7UV200, Avantes, Eerbeek, The Netherlands), using an AvaSpec 2048-2 CCD detector array spectrometer (Avantes). The light source was a deuterium–halogen lamp [AvaLight-D(H)-S, Avantes], and the reference was a white diffuse reflectance tile (WS-2, Avantes). The bifurcated probe illuminated an area with a diameter of about 1 mm, and captured the light reflected in a small spatial angle, aperture ~20 deg. Reflectance spectra of both sides of single scales attached to a glass micropipette (Fig. 5) and of bare, descaled wing areas (Fig. 7) were measured with a microspectrophotometer (Ortholux microscope, Leitz, Wetzlar, Germany) connected to the detector array spectrometer, with a xenon arc light source. Absorbance spectra of single scales, immersed in immersion oil (refractive index 1.515), were also measured with the microspectrophotometer. We used immersion oil instead of fluids with higher refractive indices, because the refractive index fluids have a high absorption in the UV. The area measured with the microspectrophotometer was about square with sides typically ~15 µm. The microscope objective was an Olympus LUCPlanFL N 20×/0.45. Because of the glass optics, the microspectrophotometer spectra were limited to wavelengths >350 nm. For the reflectance measurements with the microspectrophotometer, the white diffuse reflectance tile was also used as a reference, but this causes severely overestimated reflectance values when the measured object is not diffuse but directionally reflecting. We estimated, by comparing the diffuse reflectance tile with a mirror, that the reflectance of specular reflecting objects (e.g. the adwing sides of the scales and the wing substrate) is about a factor 5, and therefore we divided the measured reflectance spectra in Figs 5 and 7 by that factor. We have to note, however, that this will inevitably cause artificially low values for the spectra of the abwing sides of the scales, which are more or less diffusely reflecting objects.

SEM

The scale anatomy was visualised by SEM (XL-30 ESEM, Philips, Eindhoven, The Netherlands). Prior to imaging, the samples were sputtered with palladium.

Imaging scatterometry

For investigating the spatial reflection characteristics of the wing scales, we performed imaging scatterometry (Stavenga et al., 2009; Wilts et al., 2009). An isolated, single scale (Fig. 5; or a wing patch: supplementary material Fig. S2) attached to a glass micropipette was positioned at the first focal point of the ellipsoidal mirror of the imaging scatterometer. The scatterograms were obtained by focusing a white light beam with a narrow aperture (<5 deg) on to a small circular area (diameter ~13 µm) of the isolated scale (or a scale of the wing patch), and the spatial distribution of the far-field scattered light was then monitored. A flake of magnesium oxide served as a white diffuse reference object (see Stavenga et al., 2009; Wilts et al., 2009).

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Competing interests

The authors declare no competing financial interests.

Author contributions

D.G.S., H.LL. and B.D.W. performed the experiments, and D.G.S. performed the analyses and wrote the paper.

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Supplementary material

Supplementary material available online at
http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.098673/-/DC1

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Supplementary Material

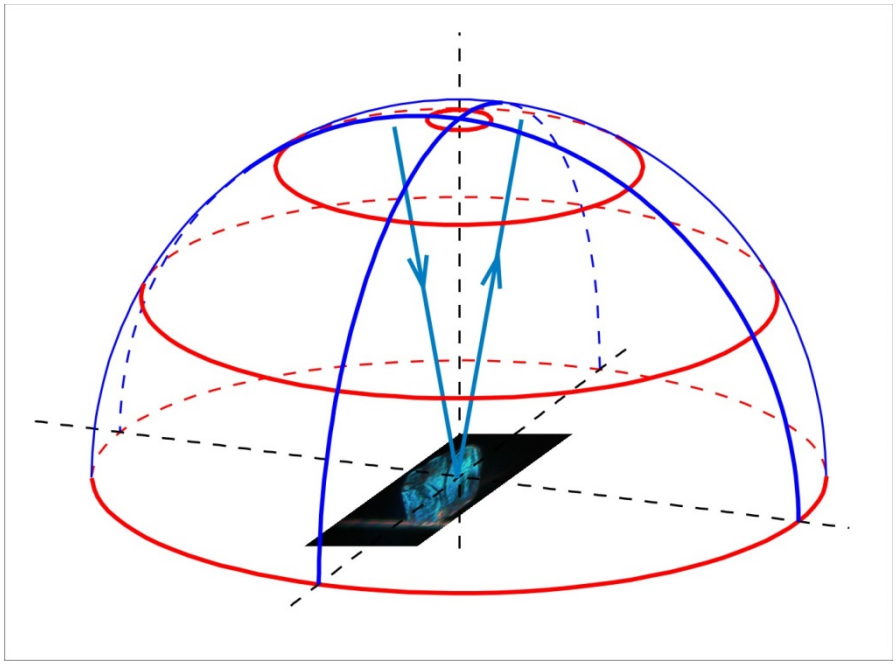

Fig. S1. Diagram of light reflection by the adwing side of a nymphaline butterfly wing scale. Locally the wing acts as a thin film reflector. The red circles correspond to the red circles in the scatterograms of Fig. 5 and Fig. S2, and indicate angular directions of 5, 30, 60 and 90 deg.

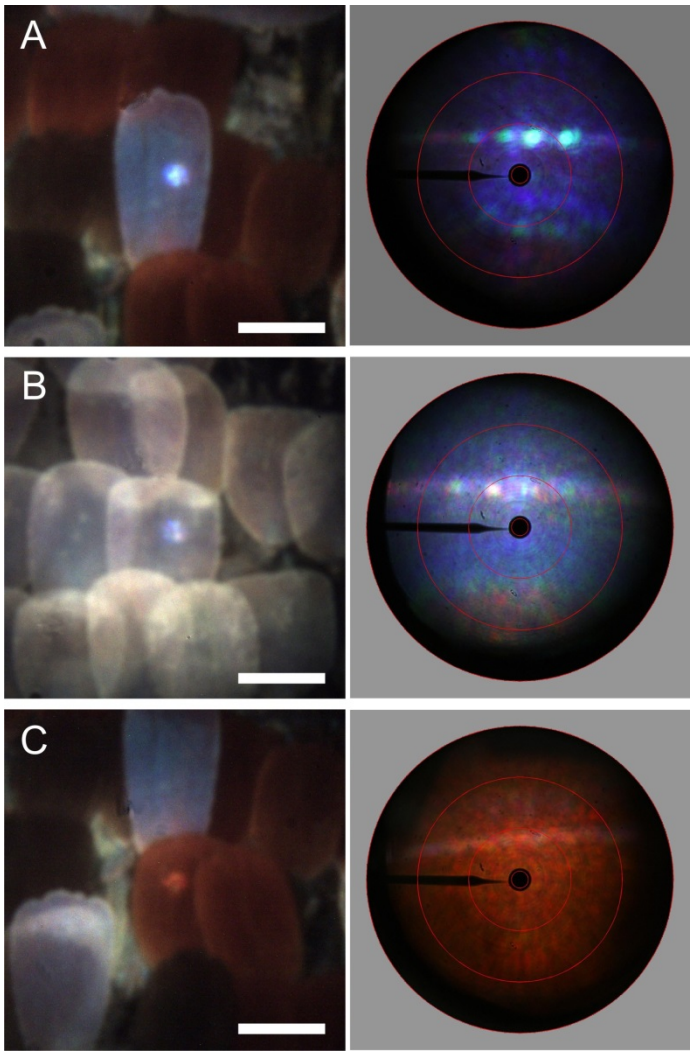

Fig. S2. Scales on the wing of a Peacock butterfly illuminated with a 5 deg aperture light beam on a ~13 µm diameter area (left) and resulting scatterograms (right). (A) A lowly pigmented (blue) scale on top of a black scale. (B) A blue scale on top of a similar scale yielding a white reflection. (C) A red-pigmented scale on top of a similar scale (bars: 50 µm).