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

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Summary

The colouration 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 colouration of nymphaline butterflies.

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Keywords: xanthommatin □ 3-OH-kynurenine □ structural colouration □ pigmentary colouration □ scattering

Introduction

Butterflies are universally considered attractive because of their bright colouration 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 as 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).

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 colouration 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 and various ommochromes, pigments that predominantly determine their wing colouration (Nijhout, 1997; Takeuchi et al., 2005; Nijhout, 2010). In fewer 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 occur 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

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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 complimentary 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 other nymphaline butterflies, i.e., the Small Tortoiseshell (*Aglais urtica*), the Map butterfly (*Araschnia levana*; Koch, 1991), the Painted Lady (*Vanessa cardui*; Reed and Nagy, 2005), and also in a heliconine, the Red Postman (*Heliconius erato*; 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 surrounding blue-green bands. In the latter areas, the lower lamina of the scales 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 study the wing colouration of a few common nymphaline butterfly species. We specifically investigate 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 find that the wing colouration depends on the stacking of neighbouring scales.

Results

Wing colouration and reflectance spectra

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The wings of the Small Tortoiseshell, Peacock, and Red Admiral, all nymphaline butterflies, have strikingly colourful dorsal (upper) sides (Fig. 1A-C). The ventral (lower) sides of the wings have instead a very inconspicuous dull brown-black colour, except for the Red Admiral where the ventral forewings feature some colouration (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, # 2, 3, 5, 6, 9, 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 valley near 620 nm (Fig. 1E-H, # 4, 7, 11, 15). Interestingly, reflectance spectra obtained from white wing areas have a similar biphasic shape riding on a distinct plateau (e.g. Fig. 1G,H, #10, 14). The black areas, not surprisingly, have a very low reflectance (Fig. 1F-H, #8, 12, 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 (Fig. 2C).

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

Pigments of nymphaline wing scales

The scales of Fig. 2A,B evidently contain strongly 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 Figure 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).

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). Figure 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, 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 Figure 3, when compared with the reflectance spectra of Figure 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

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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 *Papilio nireus* butterflies (Trzeciak et al., 2012; Wilts et al., 2012b). However, rather different values exist in other cases, as 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*; Fig. 4a of Stavenga et al., 2004) and the Angled Sunbeam butterfly (*Curetis acuta*; Fig. 4c of 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 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 is most likely due to a slightly variable thickness of the lower lamina of the blue scales (Fig. 4B). The spectra of Figure 4 hold for unpigmented scales. The refractive index is modified by high pigment concentrations (see e.g. Stavenga et al., 2013), and therefore we calculated also the reflectance spectrum of a 200 nm thick scale containing the pigment measured in the blue scale of Figure 3B; this yielded a virtually identical spectrum as that for the unpigmented scale (not shown).

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 (under) side with an epi-illumination light microscope (Fig. 5, left column). 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 column).

A blue scale (from a Peacock's wing eyespot, area #7, Fig. 1B) 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 localized spot (Fig. 5A, middle column, ad). The aperture

of the illumination beam was about 5º, but the reflection spot in the scatterogram is slightly larger, obviously due to the somewhat wrinkled adwing surface. Quite differently, the abwing scatterogram shows 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 column, ab; see also Stavenga et al., 2009).

The reflectance spectra of both 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 mean thickness 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 (from area #3, Fig. 1A) is orange on both the ab- 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 scatterogram reveals a spatially restricted reflection pattern (Fig. 5B, middle), while the abwing scatterogram 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), 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 (from area #9, Fig. 1C) 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-

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wavelength part unimpeded, thus yielding a substantial reflectance only in the red wavelengths (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 localized 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 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 get 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), since removal of its scales shows that its reflectance 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 (for method, see Stavenga et al., 2011). The transmittance spectrum, $T(\lambda)$, measured at the same location yields identical oscillations as those seen in the reflectance

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spectrum, $R(\lambda)$ (Fig. 7; λ is the wavelength). Combining the reflectance and transmittance spectra shows that the wing absorptance, $A(\lambda) = 1-T(\lambda)-R(\lambda)$ (the area in between $1-T(\lambda)$ and $R(\lambda)$ in Figure 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 (inset Fig. 3D; 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 are structural as well as pigmentary coloured. Following previous work on the closely related Map butterfly (*Araschnia levana*; Koch, 1991) and Painted Lady (*Vanessa 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 colouration of nymphaline butterflies. For instance, dependent 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 peaking all 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 those 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 hemes (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 colouration. 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*

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species (Kinoshita et al., 2008), and closed windows result in silvery scales in some heliconiine butterflies (Simonsen, 2007).

Structural and pigmentary colouration 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 Figure 8A for a red scale of the Red Admiral (Fig. 5C). On the other hand, 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 Figure 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 times the effective transmittance spectrum of the scale's pigment.

The spectral filtering of the thin film reflection is negligible when a scale has no or little pigment, and then the same reflectance spectrum will be obtained for both ab- 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).

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 have all 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 colouration 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, with as result 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 for 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 *Papilio 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 colouration 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 contribute to the wing reflectance also (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, #10, 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

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the Peacock wing areas with densely packed red scales (Fig. 1B, 6C). The consequences of wing scale stacking are diagrammatically shown in Figure 8C,D (see also Supplementary Figure S2).

Wing scale stacking appears to be an effective method to achieve bright colouration. Already 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 colouration and display

A special point to consider is the prominent red colouration of the studied nymphaline butterflies. Three different visual pigments have been characterized 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 not be bright signalers 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 colouration 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 realized in the wings of the Small Tortoiseshell and the Red Admiral. Possibly, their wing patterns have a visually disruptive function.

Conclusion

A few levels of complexity determine the colouration 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 cross-ribs. 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.

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Materials and Methods

Animals

Specimens of the Small Tortoiseshell (*Aglais urticae*), the Peacock (*Aglais io*; previously *Inachis io*), and the Red Admiral (*Vanessa 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 made 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 16x/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 40x/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 (Avantes FCR-7UV200; Avantes, Eerbeek, Netherlands), using an AvaSpec 2048-2 CCD detector array spectrometer (Avantes, Eerbeek, Netherlands). The light source was a deuterium-halogen lamp (AvaLight-D(H)-S), and the reference was a white diffuse reflectance tile (Avantes WS-2). The bifurcated probe illuminated an area with a diameter of about 1 mm, and captured the light reflected in a small spatial angle, aperture about 20º. 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 (MSP), being a Leitz 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 MSP. We used immersion oil instead of fluids with higher refractive indices, because of the refractive index fluids' high absorption in the UV. The area measured with the MSP was about square with

sides typically ~15 µm. The microscope objective was an Olympus LUCPlanFL N 20x/0.45 (Olympus, Tokyo, Japan). Due to the glass optics, the MSP spectra were limited to wavelengths >350 nm. For the reflectance measurements with the MSP, 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 Figures 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.

Scanning Electron Microscopy (SEM)

The scale anatomy was visualized by scanning electron microscopy (Philips XL-30 ESEM; Philips, Eindhoven, 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 (< 5º) onto at 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 (for further details, see Stavenga et al., 2009; Wilts et al., 2009).

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Competing Interests

No competing interests declared.

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References

Barbier, M. (1981). The status of blue-green bile pigments of butterflies, and their phototransformations. *Experientia* **37,** 1060-1062.

Blest, A. D. (1957). The function of eyespot patterns in the Lepidoptera. *Behaviour* **11,** 209-256.

Briscoe, A. D. and Bernard, G. D. (2005). Eyeshine and spectral tuning of long wavelength-sensitive rhodopsins: no evidence for red-sensitive photoreceptors among five Nymphalini butterfly species. *J. Exp. Biol.* **208,** 687-696.

Ghiradella, H. (1989). Structure and development of iridescent butterfly scales: lattices and laminae. *J. Morphol.* **202,** 69-88.

Ghiradella, H. (1998). Hairs, bristles, and scales. In *Microscopic anatomy of invertebrates, Vol 11A: Insecta* (ed. M. Locke), pp. 257-287. New York: Wiley-Liss.

Ghiradella, H. (2010). Insect cuticular surface modifications: scales and other structural formations. *Adv. Insect Physiol.* **38,** 135-180.

Ghiradella, H., Aneshansley, D., Eisner, T., Silberglied, R. and Hinton, H. E. (1972). Ultraviolet reflection of a male butterfly: Interference color caused by thin-layer elaboration of wing scales. *Science* **178,** 1214-1217.

Hart, N. S. (2001). The visual ecology of avian photoreceptors. *Prog. Retin. Eye Res.* **20,** 675-703.

Kayser, H. (1985). Pigments. In *Comprehensive insect physiology, biochemistry and pharmacology* (eds. G.A. Kerkut and L.I. Gilbert), Vol. **10,** pp. 367-415. Oxford: Pergamon Press.

Kinoshita, S. and Yoshioka, S. (2005). Structural colors in nature: the role of regularity and irregularity in the structure. *ChemPhysChem* **6,** 1-19.

Kinoshita, M., Sato, M. and Arikawa, K. (1997). Spectral receptors of nymphalid butterflies. *Naturwissenschaften* **84,** 199-201.

Kinoshita, S., Yoshioka, S., Fujii, Y. and Osanai, M. (2002). Photophysics of structural color in the *Morpho* butterflies. *Forma* **17,** 103-121.

Kinoshita, S., Yoshioka, S. and Miyazaki, J. (2008). Physics of structural colors. *Rep. Prog. Phys.* **71,** 076401.

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

Koch, P. B. (1991) Precursors of pattern specific ommatin in red wing scales of the polyphenic butterfly *Araschnia levana* L.: Haemolymph tryptophan and 3-hydroxykynurenine. *Insect Biochem.* **21**, 785-794.

Leertouwer, H. L., Wilts, B. D. and Stavenga, D. G. (2011). Refractive index and dispersion of butterfly scale chitin and bird feather keratin measured by interference microscopy. *Opt. Express* **19,** 24061-24066.

Michielsen, K. and Stavenga, D. G. (2008). Gyroid cuticular structures in butterfly wing scales: biological photonic crystals. *J. R. Soc. Interface* **5,** 85-94.

Michielsen, K., DeRaedt, H. and Stavenga, D. G. (2010). Reflectivity of the gyroid biophotonic crystals in the ventral wing scales of the Green Hairstreak butterfly, *Callophrys rubi*. *J. R. Soc. Interface* **7,** 765-771.

Nijhout, H. F. (1991). *The development and evolution of butterfly wing patterns*. Washington: Smithsonian Institution Press.

Nijhout, H. F. (1997). Ommochrome pigmentation of the *linea* and *rosa* seasonal forms of *Precis coenia* (Lepidoptera: Nymphalidae). *Arch. Insect Biochem. Physiol.* **36,** 215-222.

Nijhout, H. F. (2010). Molecular and physiological basis of colour pattern formation. *Adv. Insect Physiol.* **38,** 219-265.

Reed, R. D. and Nagy, L. M. (2005). Evolutionary redeployment of a biosynthetic module: expression of eye pigment genes vermilion, cinnabar, and white in butterfly wing development. *Evol. Dev.* **7,** 301-311.

Reed, R. D., McMillan, W. O. and Nagy, L. M. (2008). Gene expression underlying adaptive variation in *Heliconius* wing patterns: non-modular regulation of overlapping cinnabar and vermilion prepatterns. *Proc. R. Soc. B* **275,** 37-45.

Riou, M. and Christidès, J. (2010). Cryptic color change in a crab spider (*Misumena vatia*): identification and quantification of precursors and ommochrome pigments by HPLC. *J. Chem. Ecol.* **36,** 412-423.

Rutowski, R.L., Macedonia, J.M., Morehouse, N. and Taylor-Taft, L. (2005) Pterin pigments amplify iridescent ultraviolet signal in males of the orange sulphur butterfly, *Colias eurytheme*. *Proc. R. Soc. B* **272**, 2329-2335.

Saranathan, V., Osuji, C. O., Mochrie, S. G., Noh, H., Narayanan, S., Sandy, A., Dufresne, E. R. and Prum, R. O. (2010). Structure, function, and self-assembly of single network gyroid (I4132) photonic crystals in butterfly wing scales. *Proc. Nat. Acad. Sci. USA* **107,** 11676-11681.

Simonsen, T. J. (2007). Comparative morphology and evolutionary aspects of the reflective under wing scale-pattern in Fritillary butterflies (Nymphalidae: Argynnini). *Zool. Anzeig. A* **246,** 1-10.

Srinivasarao, M. (1999). Nano-optics in the biological world: Beetles, butterflies, birds and moths. *Chem. Rev.* **99,** 1935-1961.

Stavenga, D. G., Giraldo, M. A. and Hoenders, B. J. (2006). Reflectance and transmittance of light scattering scales stacked on the wings of pierid butterflies. *Opt. Express* **14,** 4880-4890.

Stavenga, D. G., Giraldo, M. and Leertouwer, H. L. (2010). Butterfly wing colors: glass scales of *Graphium sarpedon* cause polarized iridescence and enhance blue/green pigment coloration of the wing membrane. *J. Exp. Biol.* **213,** 1731-1739.

Stavenga, D. G., Leertouwer, H. L., Pirih, P. and Wehling, M. F. (2009). Imaging scatterometry of butterfly wing scales. *Opt. Express* **17,** 193-202.

Stavenga, D. G., Leertouwer, H. L. and Wilts, B. D. (2013). Quantifying the refractive index dispersion of a pigmented biological tissue using Jamin–Lebedeff interference microscopy. *Light: Sci. Appl.* **2,** e100.

Stavenga, D. G., Mashushita, A., Arikawa, K., Leertouwer, H. L. and Wilts, B. D. (2012). Glass scales on the wing of the swordtail butterfly *Graphium sarpedon* act as thin film polarizing reflectors. *J. Exp. Biol.* **215,** 657-662.

Stavenga, D. G., Stowe, S., Siebke, K., Zeil, J. and Arikawa, K. (2004). Butterfly wing colours: scale beads make white pierid wings brighter. *Proc. R. Soc. Lond. B* **271,** 1577-1584.

Stavenga, D. G., Tinbergen, J., Leertouwer, H. L. and Wilts, B. D. (2011). Kingfisher feathers - colouration by pigments, spongy nanostructures and thin films. *J. Exp. Biol.* **214,** 3960-3967.

Takeuchi, K., Satou, Y., Yamamoto, H. and Satoh, N. (2005). A genome-wide survey of genes for enzymes involved in pigment synthesis in an ascidian, *Ciona intestinalis*. *Zool. Sci.* **22,** 723-734.

Trzeciak, T.M., Wilts, B.D., Stavenga, D.G. and Vukusic, P. (2012) Variable multilayer reflection together with long-pass filtering pigment determines the wing coloration of papilionid butterflies of the nireus group. *Opt. Express* **20**, 8877-8890.

Umebachi, Y. (1985). Papiliochrome, a new pigment group of butterfly. *Zool. Sci.* **2,** 163-174.

The Journal of Experimental Biology – ACCEPTED AUTHOR MANUSCRIPT

Vukusic, P., Sambles, J. R., Lawrence, C. R. and Wootton, R. J. (1999). Quantified interference and diffraction in single *Morpho* butterfly scales. *Proc. R. Soc. B* **266,** 1403-1411.

Vukusic, P., Kelly, R. and Hooper, I. (2009). A biological sub-micron thickness optical broadband reflector characterized using both light and microwaves. *J. R. Soc. Interface* **6,** S193-S201.

Wijnen, B., Leertouwer, H. L. and Stavenga, D. G. (2007). Colors and pterin pigmentation of pierid butterfly wings. *J. Insect Physiol.* **53,** 1206-1217.

Wilts, B. D., Leertouwer, H. L. and Stavenga, D. G. (2009). Imaging scatterometry and microspectrophotometry of lycaenid butterfly wing scales with perforated multilayers. *J. R. Soc. Interface* **6,** S185-S192.

Wilts, B. D., Michielsen, K., De Raedt, H. and Stavenga, D. G. (2012a). Iridescence and spectral filtering of the gyroid-type photonic crystals in *Parides sesostris* wing scales. *Interface Focus* **2,** 681-687.

Wilts, B. D., Trzeciak, T. M., Vukusic, P. and Stavenga, D. G. (2012b). Papiliochrome II pigment reduces the angle dependency of structural wing colouration in *nireus* group papilionids. *J. Exp. Biol.* **215,** 796-805.

Wilts, B. D., Pirih, P., Arikawa, K. and Stavenga, D. G. (2013). Shiny wing scales cause spec(tac)ular camouflage of the angled sunbeam butterfly, *Curetis acuta*. *Biol. J. Linn. Soc*. **109,** 279-289.

Yagi, N. (1954). Note of electron microscope research on pterin pigment in the scales of pierid butterflies. *Annot. Zool. Jap.* **27,** 113-114.

Yeh, P. (2005). *Optical waves in layered media*. Hoboken NJ: Wiley-Interscience.

Yoshioka, S. and Kinoshita, S. (2004). Wavelength-selective and anisotropic light-diffusing scale on the wing of the Morpho butterfly. *Proc. R. Soc. B* **271,** 581-587.

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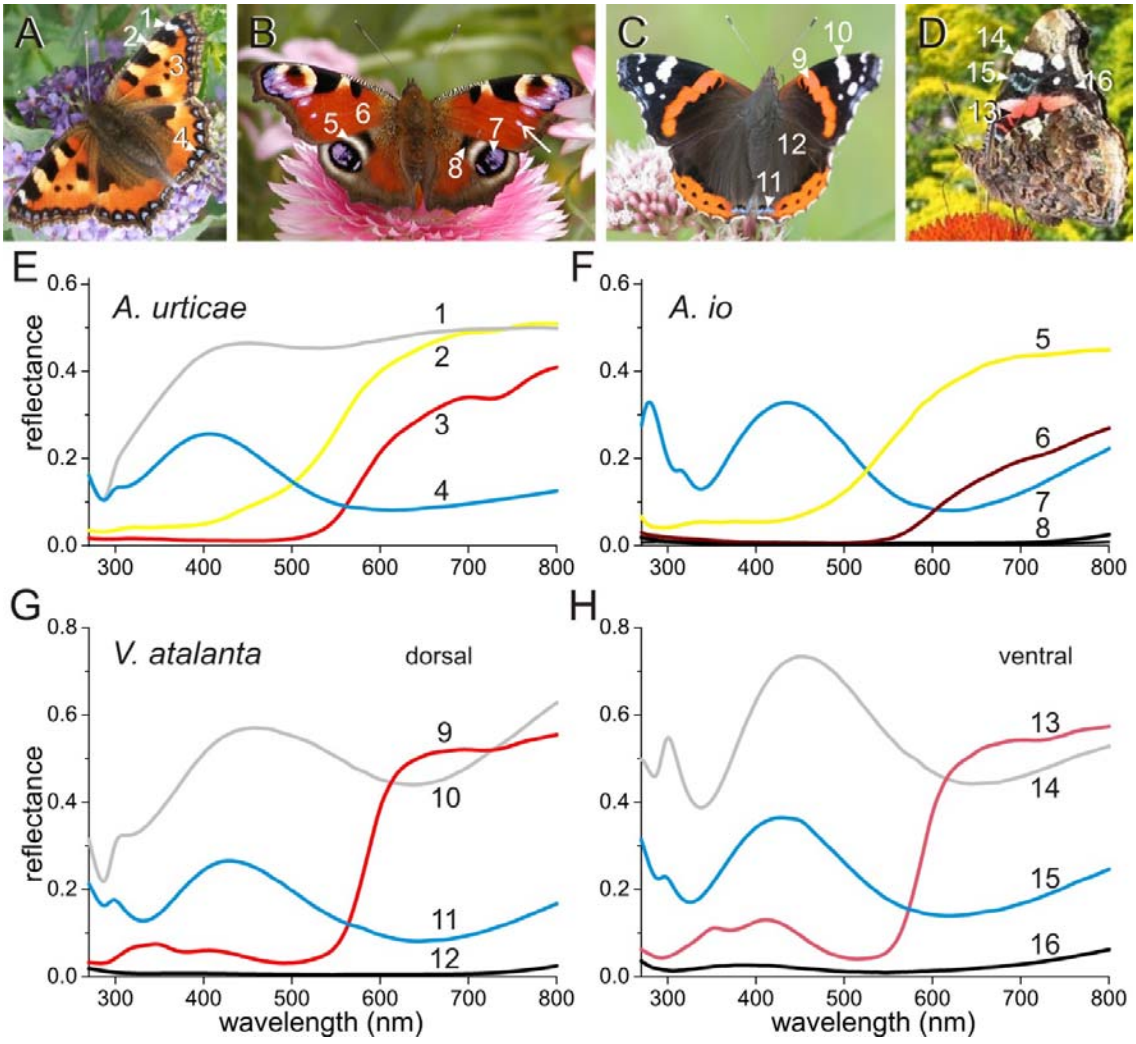

Fig. 1. Three common nymphaline butterflies and wing reflectance spectra measured with a bifurcated probe. **A**, **E** *Aglais urticae*, the Small Tortoiseshell. **B**, **F** *Aglais io*, the Peacock. **C**, **G** *Vanessa atalanta*, the Red Admiral, wing dorsal surfaces. **D**, **H** *V. atalanta*, wing ventral surfaces. The numbers in the photographs and spectra correspond. The arrow in **B** corresponds to Fig. 6C.

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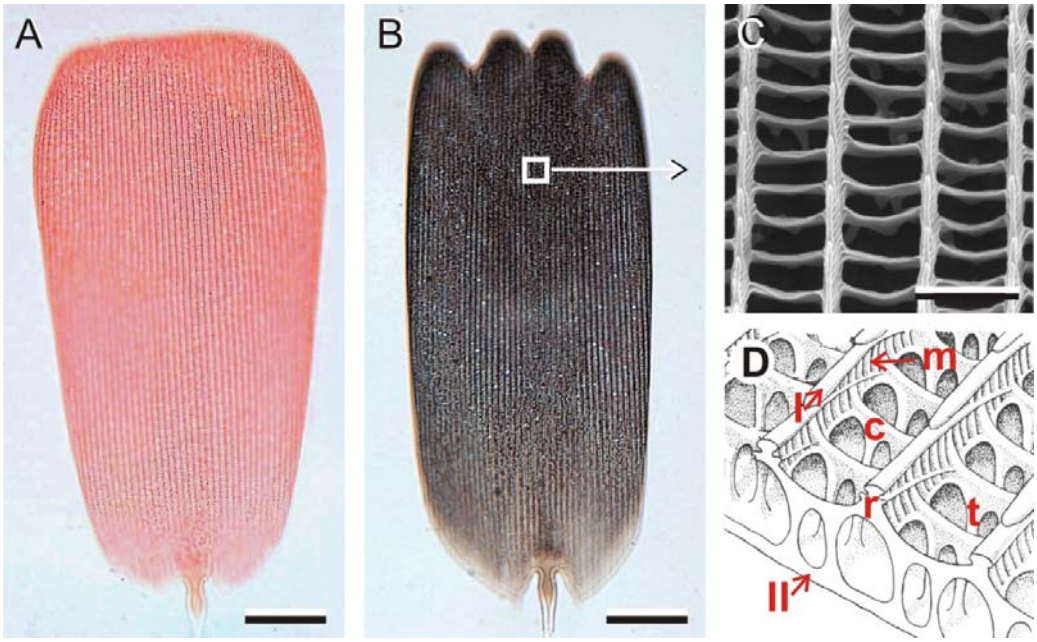

Fig. 2. Anatomy of nymphaline wing scales. **A**, **B** Transmitted light microscopy of a red scale of a Red Admiral and a black scale of a Peacock immersed in a refractive index fluid (bars: 20 µm). **C** Scanning electron microscopy photograph of a black scale of a Peacock (bar: 2 µm); the size of **C** corresponds to the square in **B**. **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.

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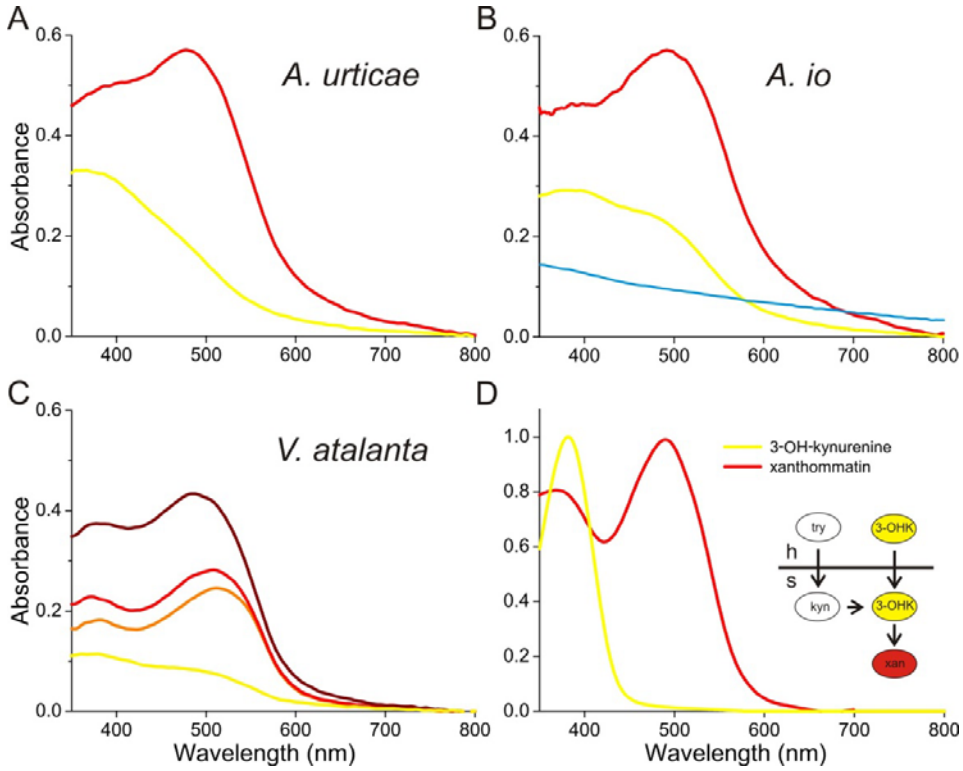

Fig. 3. Absorbance spectra of isolated yellow to red-brown wing scales in immersion oil (refractive index n = 1.515) and pigments. **A** Small Tortoiseshell. The red and yellow curves are from scales in areas #3 and 2, respectively, of Fig. 1A. **B** Peacock. The red curve corresponds to area #6 of Fig. 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, extracted from yellow scales of *Heliconius melpomene* with methanol (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-hydroxy-kynurenine (3-OHK), which is processed further into xanthommatin (xan). 3-OHK itself can be taken up directly into scales (after Reed and Nagy, 2005; Reed et al., 2008).

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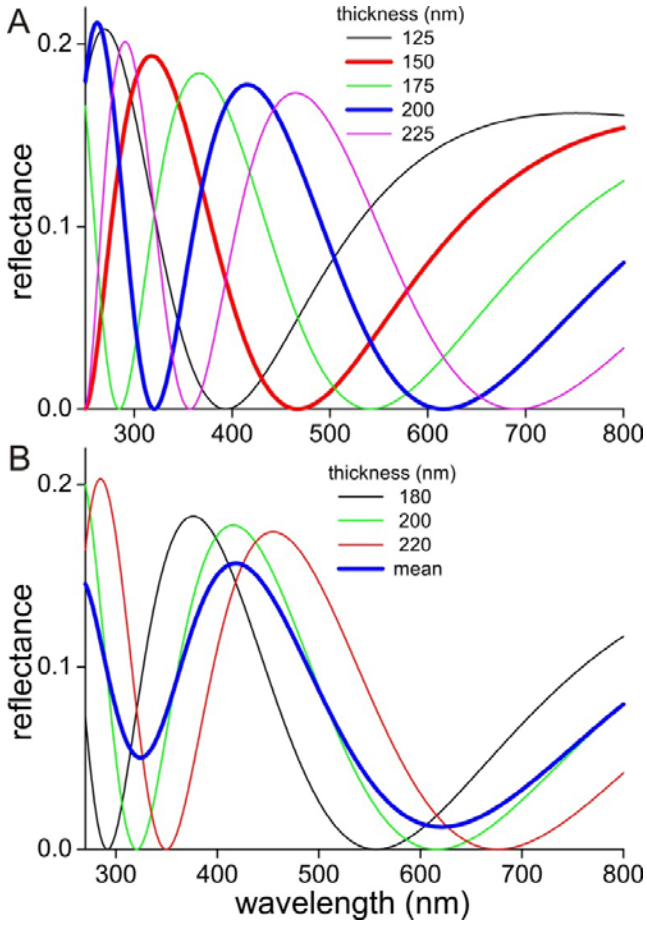

Fig. 4. Reflectance spectra of cuticle thin films in air. **A**. Reflectance spectra of thin films with thicknesses 125 to 225 nm and the refractive index of butterfly wing scales (Leertouwer et al. 2011). **B** Reflectance spectra of thin films with thicknesses 180, 200 and 220 nm and their mean.

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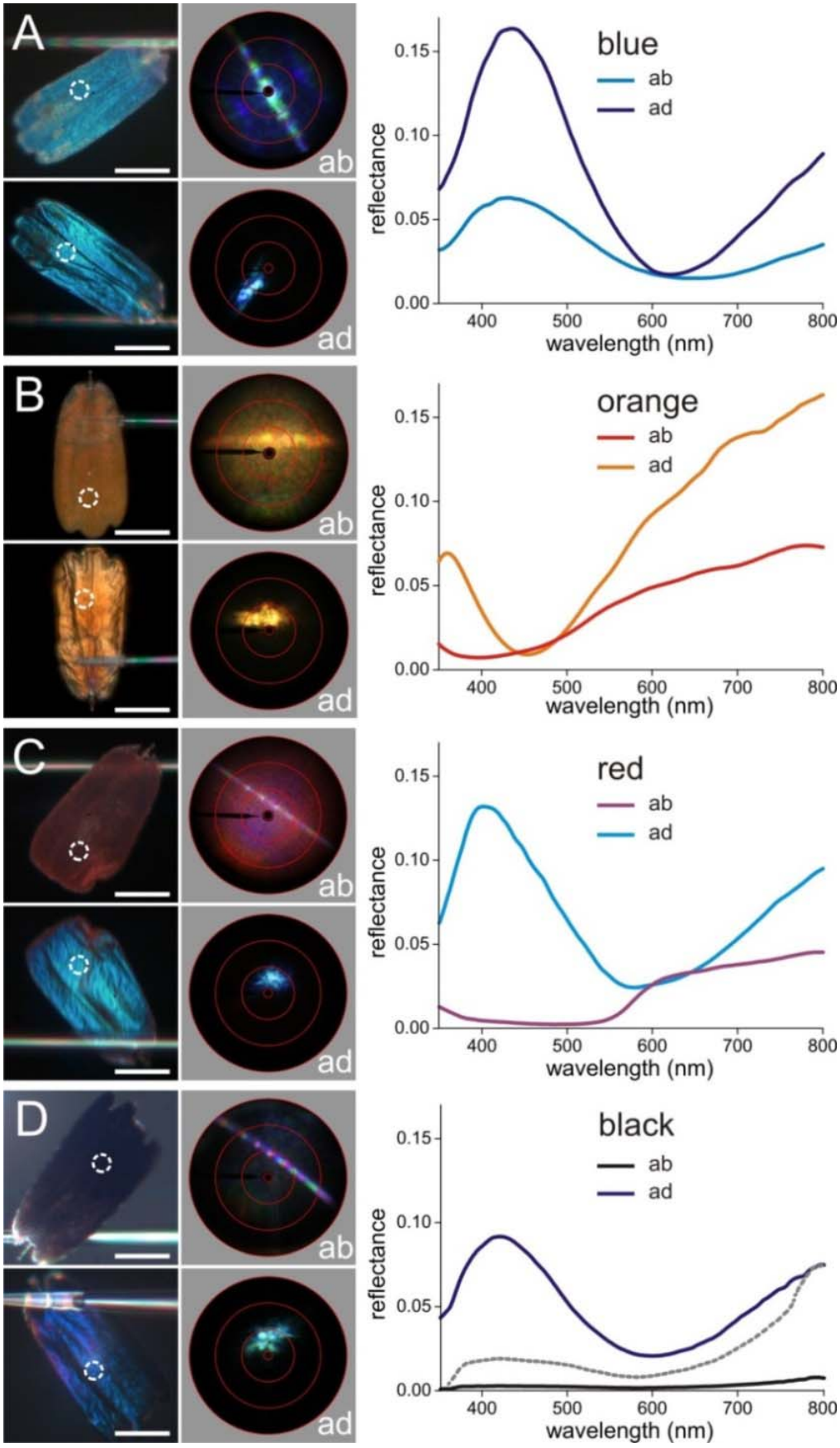

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Fig. 5. Reflection properties of single nymphaline wing scales. Left-hand column: photographs of abwing (top) and adwing (bottom) sides of a blue scale of a Peacock (**A**), an orange scale of a Small Tortoiseshell (**B**), a red scale of a Red Admiral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

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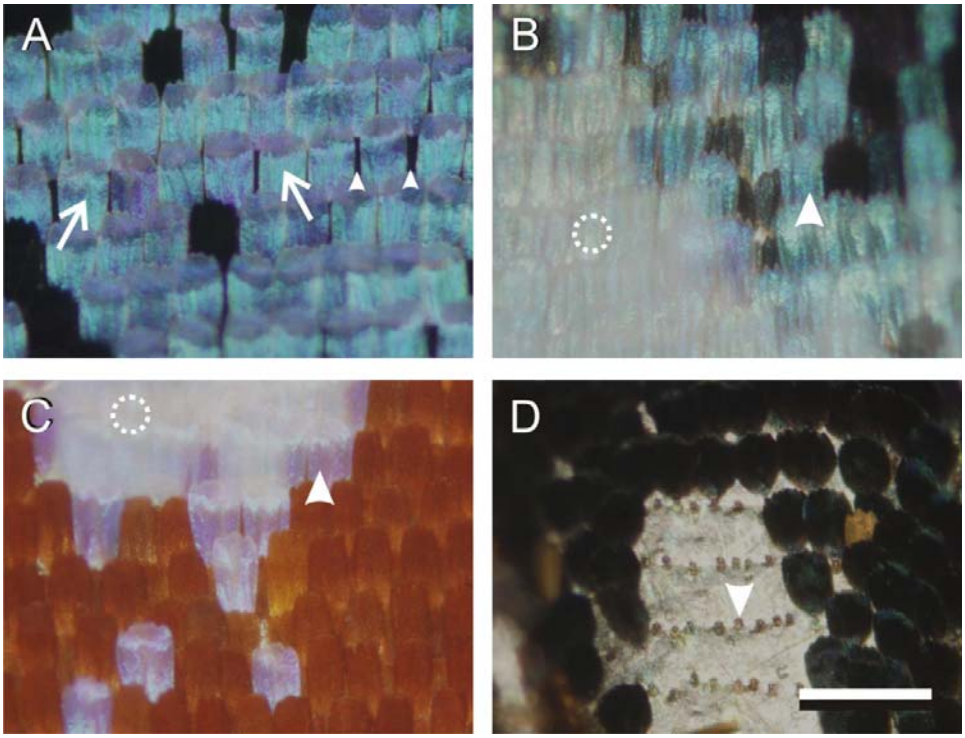
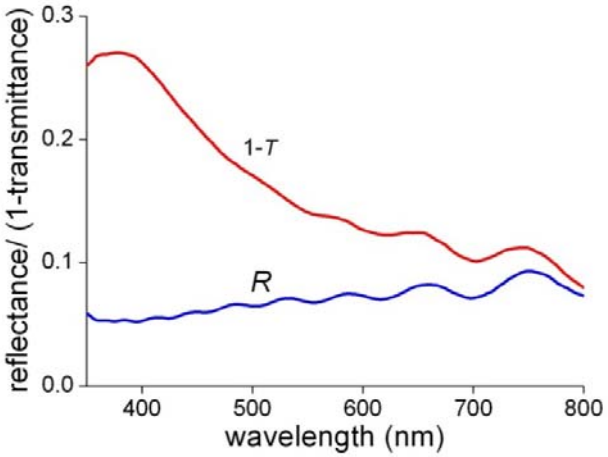


Fig. 6. Packing and stacking of scales on nymphaline butterfly wings. **A** Blue scales in the eyespot of a dorsal hindwing of a Peacock butterfly (Fig. 1B, area #7). Below the blue cover scales (arrows) and some black cover scales there exist black ground scales (small arrowheads). The tips of the blue scales are curved and thus show a violet colour (see text). **B** Border area of the white band on the ventral forewing of a Red Admiral butterfly (Fig. 1D, area #14). Stacked unpigmented cover scales and ground scales yield together with the wing substrate 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). Bar for **A**-**D**: 200 µm.



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670 Fig. 7. Thin film properties of the clear wing of a Small Tortoiseshell. The absorptance

671 $A = 1\text{-}T\text{-}R$ indicates that the wing contains 3-OH-kynurenine as well as xanthommatin.

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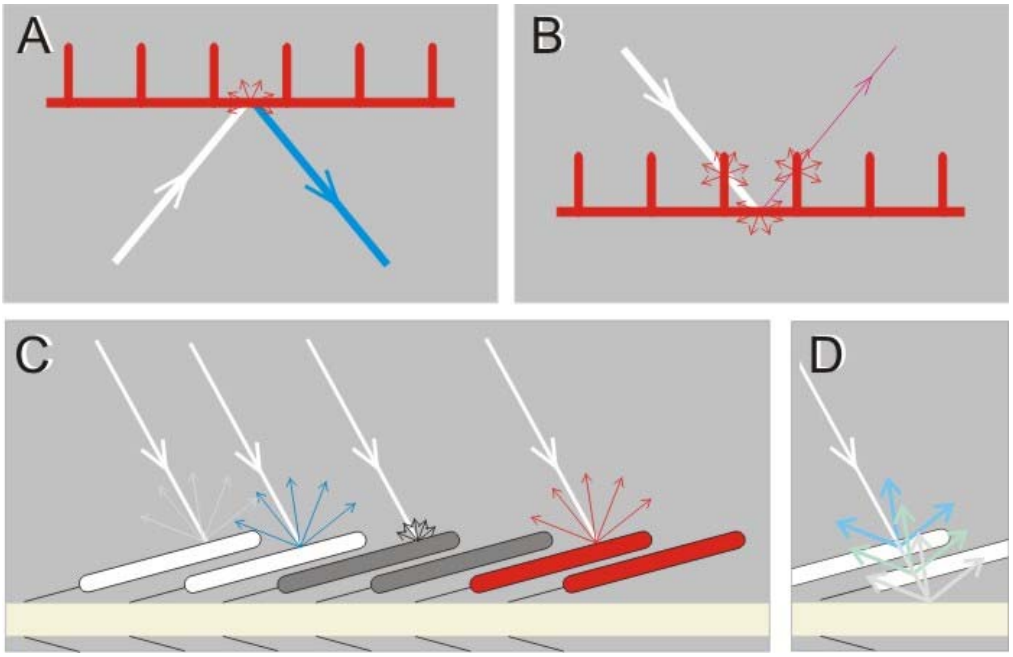

Fig. 8. Diagrams of light reflection and scattering by the wing scales of nymphaline butterflies. **A** Light reflection of a red pigmented scale illuminated at the adwing side is dominated by the thin film properties of the lower lamina, yielding a blue directional reflection. **B** Light reflection of a red pigmented scale illuminated at the abwing side is dominated by scattering at the ridges and cross-ribs, thus causing considerable absorption by the pigment in the scale. **C** Illumination of the scales on the wing results in colouration 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 due to its low reflectance. A red-pigmented scale reflects and back-scatters 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.