RESEARCH ARTICLE

Papiliochrome II pigment reduces the angle dependency of structural wing colouration in *nireus* group papilionids

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SUMMARY

The wings of four papilionid butterfly species of the *nireus* group, *Papilio bromius*, *P. epiphorbas*, *P. nireus* and *P. oribazus*, are marked by blue-green coloured bands surrounded by black margins. The cover scales in the coloured bands contain a violet-absorbing, blue-fluorescing pigment. The fluorescence and absorbance spectra of the *nireus* group wings are very similar to those of the wings of the Japanese yellow swallowtail, *Papilio xuthus*, and thus the pigment is presumably papiliochrome II. The scale structures of *P. xuthus* are arranged irregularly, and both the fluorescence and light reflection are diffuse. In the *nireus* papilionids, the spatial fluorescence distribution of the scales is also diffuse, but the reflection is specular. The scales have a multilayered structure, consisting of two main laminae. We show that the papiliochrome II pigment in the upper lamina of the scales functions as a violet-blocking long-pass filter in front of the lower lamina, thus limiting the reflectance spectrum to the blue-green wavelength range. Optical modelling showed that the papiliochrome II filter effectively removes the angle dependency of the reflectance spectra – that is, it reduces the wing iridescence. The contribution of the fluorescence signal to the visual appearance is minor.

Key words: fluorescence, papiliochrome II, thin film, multilayer, scattering

INTRODUCTION

The beautiful patterns displayed by butterflies are determined by the assembly of colourful scales that imbricate the wings (Nijhout, 1991). Butterfly wing scales commonly consist of two layers, a flat, solid basal lamina and a structured upper lamina, which are joined by pillar-like trabeculae (Ghiradella, 1998; Vukusic et al., 2000). The components of the upper lamina – that is, the parallel ridges and the connecting crossribs – are often rather irregularly organized, so that, in the absence of absorbing pigments, the scattering of incident light is wavelength independent, resulting in a white scale colour (Mason, 1926; Gilbert et al., 1988; Stavenga et al., 2004; Luke et al., 2009; Stavenga et al., 2010).

Not all white scales are unpigmented, however; for instance, the white wings of cabbage butterflies contain a pterin pigment, leucopterin, which absorbs exclusively in the ultraviolet (UV) (Yagi, 1954). The spectral sensitivity of butterflies extends into the UV, and therefore the Whites (Pieridae) see each other as brightly coloured. The absorption of other pterins common in pierid butterflies, xanthopterin and erythropterin, extends into the blue and green wavelength ranges, respectively, and thus the wings have a yellow, orange or red colour (Morehouse et al., 2007; Wijnen et al., 2007). Similarly, several *Heliconius* species have yellow scales owing to the violet-absorbing pigment 3-hydroxykynurenine (3-OHK) (Gilbert et al., 1988; Reed et al., 2008; Briscoe et al., 2010), which is the precursor of the ommochromes xanthommatin and dihydroxanthommatin that, in turn, cause orange- and red-coloured scales (Reed et al., 2008). The pale-yellow scales of the Japanese yellow swallowtail, *Papilio xuthus*, and several other papilionids contain papiliochrome II, which is a combination of N-β-alanyldopamine and L-kynurenine (Umebachi, 1985; Umebachi and Osanai, 2003). The reddish-brown scales of papilionids contain papiliochrome R, which is composed of kynurenine, β-alanine and dopamine (Umebachi, 1985).

In all these cases, the pigments act as long-pass filters, absorbing the short wavelengths, so that long-wavelength scattered light colours the scales and wings. Rare exceptions to this general rule are the bile pigments phorcabilin, pterobilin and sarpedobilin, which absorb prominently in the blue and red wavelength range, but not in between, thus yielding blue-green wing colours (Barbier, 1981; Stavenga et al., 2010), which are found in the papilionids *Papilio phorcas*, *Graphium sarpedon* and a number of other *Graphium* species, and also in some nymphalids (Choussy and Barbier, 1973).

In addition to the pigmentary or chemical colouration, butterfly wing scales often exhibit structural or physical colouration. This occurs when the scale structures are arranged regularly with a periodicity in the nanometre range and is because the structures enhance light reflection in specific wavelength bands and suppress the reflection at adjacent wavelengths by light interference (Vukusic and Sambles, 2003; Kinoshita et al., 2008; Kinoshita, 2008; Biró and Vigneron, 2010). The best-studied example is that of *Morpho* butterflies, which have scale ridges elaborated into multilayers, causing a striking, metallic-blue colour (Vukusic et al., 1999; Kinoshita et al., 2002). Many lycaenids (Polyommatinae and Theclinae) exhibit metallic blue and green structural colours owing to multilayers in the wing scale interior (Biró et al., 2007; Wilts et al., 2009), whereas other lycaenids (e.g. *Callophrys rubi*) and also papilionids (*Papilio sesostris*) create a diffuse green colouration with gyroid scale structures (Vukusic and Sambles, 2003; Michielsen and Stavenga, 2008; Poladian et al., 2009).

Pigmentary and structural colouration can act complementarily and/or constructively. In *Morpho* butterflies, melanin pigment below the multilayered ridges enhances the saturation of the colour signal, because transmitted light which potentially can be scattered back by the wing or other scale structures is effectively absorbed (Mason, 1926; Kinoshita and Yoshioka, 2006). In pierid butterflies, the interference reflectors act in the wavelength range where the wing pigments strongly absorb and thus create a chromatic colour signal, which increases contrast and/or visibility (Morehouse et al., 2007; Wijnen et al., 2007; Wilts et al., 2011; Pirih et al., 2011).

Here, we investigate the interplay of pigmentary and structural colouration in four papilionid species, *Papilio bromius*, *P. epiphorbas*, *P. nireus* and *P. oribazus* (note: the nomenclature is not unambiguous; we here follow the WorldFieldGuide.com: http://www.worldfieldguide.com/wfg-species-detail.php?taxno=8552&gr=world). We discovered that this group of papilionids apply a novel colouration mechanism, namely by tuning the structural colouration with a UV-absorbing pigment, acting as a spectral filter. The upper side of both the forewings and the hindwings of these species is marked by brilliant blue-green bands surrounded by black margins. The blue-green colouration is localized in cover scales (Ghiradella, 1998) that are nanostructured in a quite complex way (Vukusic and Hooper, 2005) (T.M.T., B.D.W., D.G.S. and P.V., unpublished data). The scales comprise a thin layered lower lamina and a thick upper lamina, which consists of a quasi-ordered lattice of cylindrical air-filled cavities. Variations in the local structural parameters of the air cavity lattice smooth the reflectance spectrum of the photonic structure of the scale (T.M.T., B.D.W., D.G.S. and P.V., unpublished data). In the present study, we investigate the distinctly fluorescing pigment in the scales (Vukusic and Hooper, 2005). To unravel the spectral and spatial reflection properties of the papilionid scales, we apply a variety of optical methods, among others (micro)spectrophotometry, imaging scatterometry and fluorometry. We find that the fluorescing pigment specifically functions in curtailing the short-wavelength reflectance of the *nireus* group scales.

MATERIALS AND METHODS
Animals
The investigated papilionids – *Papilio bromius* Doubleday 1845, *P. epiphorbas* Boisduval 1833, *P. nireus* Linnaeus 1758 and *P. oribazus* Boisduval 1836 – were obtained from World Wide Butterflies (Dorset, UK). The Japanese yellow swallowtail, *P. xuthus*, was obtained from K. Arikawa (Sokendai, Hayama, Japan).

Photography
Specimens, illuminated with a Nikon SB-800 flash, were photographed using a Canon EOS 30D camera equipped with a 50 mm macro-objective. For fluorescence pictures of the whole butterflies, the excitation light source was a blacklight (UV) bulb, and the emission was filtered by a 465 nm high-pass filter in front of the camera. Details of the scale arrangement on the wings were photographed with a Zeiss Axioskop microscope (Carl Zeiss, Oberkochen, Germany), applying white-light epi-illumination and using an Olympus DP-70 digital camera. For fluorescence pictures, 365 nm excitation light was used, and the emission was filtered by a 400 nm high-pass filter.

Fluorometry
Fluorescence excitation and emission spectra of the wings were measured with a Varian Cary Eclipse fluorometer (Varian, VIC, Australia).

Spectrophotometry
The absorbance spectrum of the papiliochrome II pigment contained by the pale-yellow wing scales of *Papilio xuthus* was determined by measuring transmittance spectra of single wing scales immersed in a fluid with refractive index 1.56 (Cargille Labs, Cedar Grove, NJ, USA) with a microspectrophotometer – a Leitz Otholux microscope connected to an AvaSpec-2048-2 photodiode array spectrometer (Avantes, Eerbeck, The Netherlands). Reflectance spectra of intact wings were measured with an integrating sphere (Avantes Avasphere-50-Refl) connected to the AvaSpec-2048-2 spectrometer. The light source was a xenon or a deuterium-halogen lamp [Avantes D(H)-S], and the angle of illumination was approximately 8 deg with respect to the normal to the wing surface. The angular distribution of the light scattered by the intact wings was measured with a set-up consisting of two optical fibres, one for illumination and the other for light detection, attached to two goniometers with the same rotation axis. A UV/VIS-polarizer (HNPB; Polaroid Corporation, Cambridge, MA, USA) was mounted in front of the detection fibre. The wings were placed on a black cardboard and positioned at the rotation axis of the goniometers. For all reflectance measurements, a white diffuse reflectance tile (Avantes WS-2) served as a reference.

Imaging scatterometry
The far-field angular distribution of the light scattered from single scales and wing patches, glued to the end of pulled micropipettes (Wilts et al., 2009), was visualized with an imaging scatterometer (ISM) (Stavenga et al., 2009; Vukusic and Stavenga, 2009). The scatterometer is built around an ellipsoidal mirror, which collects light from a full hemisphere around its first focal point, where the sample is positioned. A small piece of magnesium oxide served as a white diffuse reference object. Images were acquired with an Olympus DP-70 camera and were subsequently corrected for geometrical distortions using a MATLAB routine. For imaging fluorometry, the spatial distribution of the excited fluorescence was imaged using appropriate spectral filters.

Electron microscopy
The anatomical structure of the wing scales was investigated using scanning and transmission electron microscopy (SEM and TEM). A Philips XL30-ESEM instrument was used for SEM after samples had been sputtered with palladium. TEM was carried out using a JEOL TEM 1400 instrument after samples had been prepared using the protocol described by Vukusic and colleagues (Vukusic et al., 1999).

RESULTS
Wing colouration
The upper side of the wings of the papilionid butterfly species *Papilio bromius*, *P. epiphorbas*, *P. nireus* and *P. oribazus* is marked by blue-green coloured bands, which differ slightly in colour, hue and pattern, depending on the species (Fig. 1A–D, left side). The coloured bands appear to exhibit a distinct blue-green fluorescence when illuminated with ultraviolet light, indicating the presence of a short-wavelength-absorbing pigment (Fig. 1A–D, right side). Because blue-green wing fluorescence has been intensively studied in a related butterfly species, *Papilio xuthus*, we have included this papilionid in the present study (Fig. 1E). Although the colouration of the wings of *P. xuthus* in reflection is mainly pale white-yellow, very different from the *nireus* group wings (Fig. 1E, left), the fluorescence signal is quite similar (Fig. 1E, right).

Fig. 1. Photographs of the upper side of the wings of the five investigated papilionid butterflies. (A) *Papilio bromius*, (B) *P. epiphorbas*, (C) *P. nireus*, (D) *P. oribazus* and (E) *P. xuthus*. The left side of each specimen represents an RGB image (photograph with white light), and the right side is a fluorescence photograph made with ultraviolet (<395 nm) illumination, with the emission filtered by a 465 nm high-pass filter. Bar, 1 cm.

Observing the wings with an epi-illumination microscope demonstrated that the coloured bands consist of arrays of coloured cover scales (Fig. 2A). Below the cover scales, darkly coloured
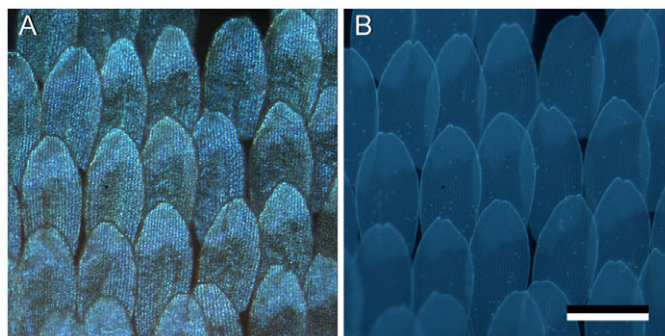

Fig. 2. Light microscopy images of cover scales of *P. oribazus*. (A) White-light epi-illumination reveals the distinctly blue reflecting scales. (B) Blue-green fluorescence induced by UV excitation (365 nm). Bar, 100 μm.

ground scales exist, which resemble the black scales of the wing margins. The black colour is most likely due to highly concentrated melanin. Using the fluorescence attachment with UV excitation light revealed that the wing fluorescence originates from the cover scales (Fig. 2B). Where the cover scales overlap, the intensity of both the reflection (Fig. 2A) and fluorescence (Fig. 2B) is more intense, indicating that the scales are somewhat transparent.

Identification of the fluorescent pigment

To identify the fluorescing pigment of the coloured cover scales, we measured the excitation and emission spectra of wing patches in the coloured bands of *P. xuthus*, *P. bromius*, *P. nireus* and *P. oribazus* with a fluorometer, using 395 nm excitation light (Fig. 3A). The obtained emission spectra all peaked in the blue-green wavelength range, at ~480 nm, and had a 100–120 nm bandwidth (FWHM). The excitation spectra, obtained by using 480 nm as the detection wavelength, peaked in the ultraviolet, at 390–410 nm (Fig. 3A). Trzeciak and colleagues (T.M.T., B.D.W., D.G.S. and P.V., unpublished data) determined the absorbance spectra of the wing scale pigments of the *nireus* group papilionids by measuring transmittance spectra of single scales. In Fig. 3B, we compare the normalized averaged absorbance spectrum of that study with the normalized averaged absorbance spectrum of single pale-yellow scales of *P. xuthus*, also obtained by measuring the transmittance spectrum. From the close correspondence of the spectra, we infer that the wing scales of the *nireus* group papilionids and *P. xuthus* contain the same pigment. The wavelength of maximal absorbance, 393 nm, agrees well with the peak at 390–410 nm of the measured excitation spectra (Fig. 3A).

The absorbance spectrum of the fluorescing pigment of *P. xuthus*, called papiliochrome II, measured from wing extracts peaked at ~380 nm (Umebachi, 1985) (Fig. 3B). The bathochromic shift of the papiliochrome II absorbance spectrum measured *in situ* with respect to the spectrum measured *in vitro* is most likely due to the different chemical environment of the pigment molecules. We therefore assume that the papiliochrome II absorbance spectrum *in situ* equals that of the extract spectrum, with the 380 nm peaking absorbance band shifted by 13 nm (Fig. 3B). We conclude, owing to the high overlap of the excitation and absorbance spectra, that all papilionids of the *nireus* group of Fig. 1 contain the same pigment as *P. xuthus* – papiliochrome II. The amount of pigment in the scales differs considerably, however. The peak absorbance of single scales at 393 nm for the *nireus* group butterflies was 0.60±0.05, whereas the peak absorbance for *P. xuthus* was approximately half that value: 0.35±0.03.
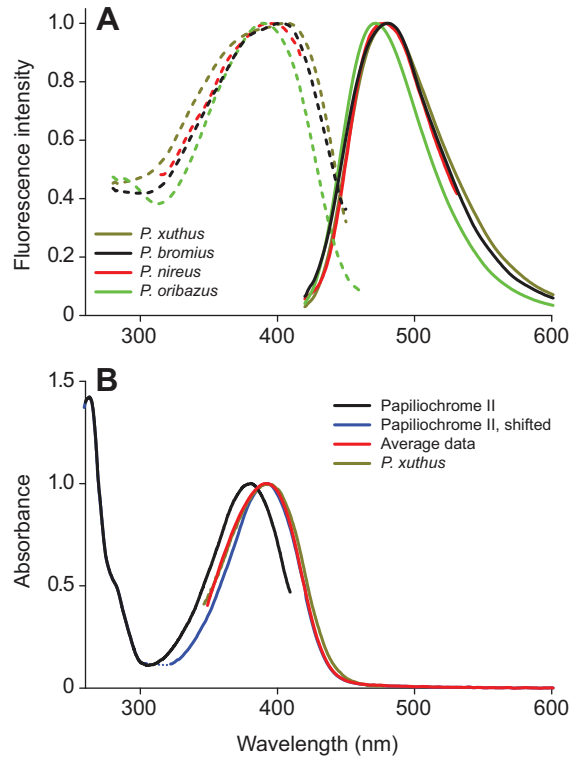
Fig. 3. Fluorescence and absorbance spectra of papilionid wing scales. (A) Fluorescence spectra of the wing scales of *P. xuthus*, *P. bromius*, *P. nireus* and *P. oribazus*. The excitation spectra (broken lines) were measured with a fixed detection wavelength of 480 nm, and, for the emission spectra (solid lines), the excitation wavelength was 395 nm. (B) Normalized absorbance spectra of refractive-index-matched single scales of the papilionids of the *nireus* group [red; average of single-scale absorbance spectra (T.M.T., B.D.W., D.G.S. and P.V., unpublished data) and *P. xuthus* compared with the absorbance spectrum of papiliochrome II (normalized at the 380 nm peak) measured *in vitro* [black (from Umebachi, 1985)]. The blue curve was obtained by a 13 nm bathochromic shift of the near-UV band of papiliochrome II to fit the *in situ* spectrum.
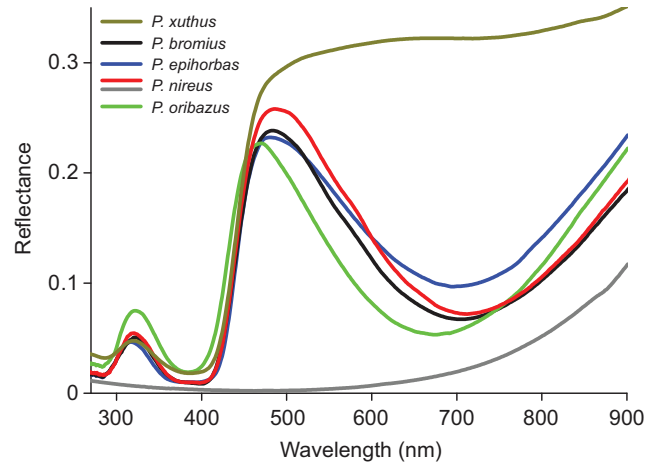

Fig. 4. Reflectance spectra of the blue-green bands in the dorsal wings of *P. xuthus* and the four *nireus* group papilionids and the reflectance spectrum of the black wing margin of *P. nireus* measured at near-normal illumination (~8 deg) with an integrating sphere.

Reflectance spectra of the wings

To investigate the effect of the papiliochrome II pigment on the wing appearance, we measured reflectance spectra from intact wings with an integrating sphere (Fig. 4). The reflectance spectrum of the pale-yellow wings of *P. xuthus* is low in the ultraviolet wavelength range (except for a minor reflectance band peaking at 320 nm) and high above 500 nm. Such a spectrum can be readily understood to result from virtually wavelength-independent scattering filtered by the papiliochrome II pigment.

The prominent, broad valley in the range 350–420 nm and the minor valley below 300 nm in the reflectance spectrum of *P. xuthus* are also seen in the reflectance spectra of the blue-green bands in the wings of *P. bromius*, *P. epiphorbas*, *P. nireus* and *P. oribazus*. However, in the longer wavelengths, the latter spectra strongly deviate from the *P. xuthus* spectrum. The reflectance spectra of the four *nireus* group species show a slightly asymmetric reflectance band peaking at ~480–510 nm, a pronounced minimum near 700 nm, and a rising reflectance in the long-wavelength range (Fig. 4). The reflectance minimum at ~700 nm cannot be attributed to an absorbing pigment in the cover scales because refractive-index-matched cover scales are transparent in the long-wavelength range (Fig. 3B). The observed differences in the reflectance spectra of the *nireus* group papilionids and *P. xuthus* therefore must be due to the differently structured scales.

Anatomy of the wing scales

We investigated the anatomical structure of the cover scales of the *nireus* group papilionids and *P. xuthus* by scanning electron microscopy (Fig. 5A,B). Trzeciak and colleagues (T.M.T., B.D.W., D.G.S. and P.V., unpublished data) showed that the wing scales of the *nireus* group have a ~1 μm thick upper lamina situated ~1.5 μm above a thin lower lamina that is layered (Fig. 5C) (Vukusic and Hooper, 2005). In top-view, the thick upper lamina of *P. nireus* shows a disordered array of air holes of diameter ~280 nm. The thin upper lamina of *P. xuthus* cover scales also has air holes, classically called windows, of size ~800 nm, significantly larger than those of the *nireus* group papilionids. This suggests that the distinct size differences of the air holes in the cover scales of the *P. xuthus* and the *nireus* group are related to the different reflectance spectra.

Imaging scatterometry and fluorometry

To determine the spatial reflection properties of the scale structures and especially the spatial distribution of the fluorescent signal, we mounted small clippings of the coloured wing areas of each species in our imaging scatterometer. An area with a diameter of ~100 μm, covering about three scales, was illuminated, and the resulting far-field scattering pattern was recorded with a digital camera.

Fig. 6A presents a scatterogram of *P. epiphorbas* created by about-normal illumination of the scales with a narrow aperture (5 deg) white-light beam. The blue-green light scattering appears to be very directional: its spatial distribution has a half-width of 10–15 deg. The scale optics thus approximates that of a mirror. Adjacent to the main reflection spot, in a plane perpendicular to the vertically oriented scales, additional spots are visible (Fig. 6A). The spatial distances of the additional spots to the central reflection maximum are ~25 deg and 35–40 deg. For other butterfly scales, similar patterns are known to be caused by diffraction at the grating created by the scale ridges (Vukusic et al., 1999; Kinoshita, 2008). In the cover scales of the papilionids, the ridge distance is $d \approx 2.5$ μm (Fig. 5), and hence, for a wavelength value of $\lambda = 500$ nm, the diffraction-grating formula $d \sin\theta = m\lambda$ predicts reflection maxima at scattering angles $\theta \approx 11$ deg, 24 deg, 37 deg, ... for orders m=1, 2, 3, ... Clearly, the additional spots in the scatterogram correspond to the second and third diffraction order, respectively. The first order, at 11 deg,
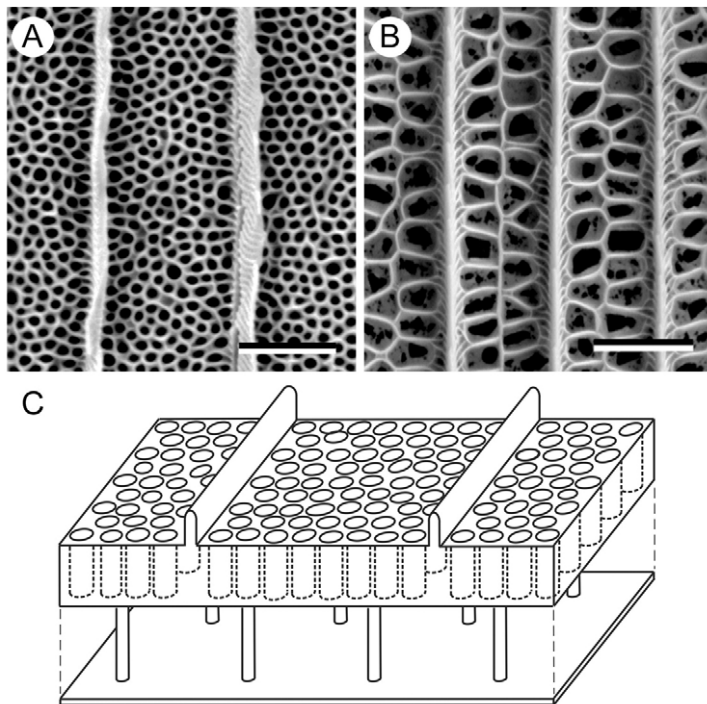
Fig. 5. Scanning electron micrographs of scales from *P. nireus* and *P. xuthus*, and a diagram of the *nireus* group cover scales. (A) Top view of a cover scale of *P. nireus*, showing a dense array of quasi-ordered air holes, with an average hole size of ~280 nm. (B) Top view of a cover scale of *P. xuthus*, with an average air hole size of ~800 nm. Bars, 2 μm. (C) Diagram of a cover scale of the four *nireus* group papilionid species.

is screened, together with the zeroth order, by the main 10–15 deg wide reflection spot that is due to the mirror properties of the scale.

Fig. 6B shows the scatterogram of the wing scales of *P. xuthus* illuminated with the same light beam of Fig. 6A. The scattered pale-yellow light now fills virtually the full hemisphere. Presumably, the upper lamina of the *P. xuthus* scales, with the large, randomly ordered windows (Fig. 5B), effectively acts as a diffuser – in sharp contrast

with the case of the *nireus* group papilionids (Fig. 6A), where the scale has small windows and acts as a mirror (Fig. 5B).

To measure the spatial profile of the fluorescence emerging from the pigment embedded in the scale structure, a 400 nm narrow-band filter was inserted into the illuminating beam of the scatterometer, and a 465 nm long-pass filter was put in front of the camera. Fig. 6C shows the spatial distribution of the fluorescence excited in the
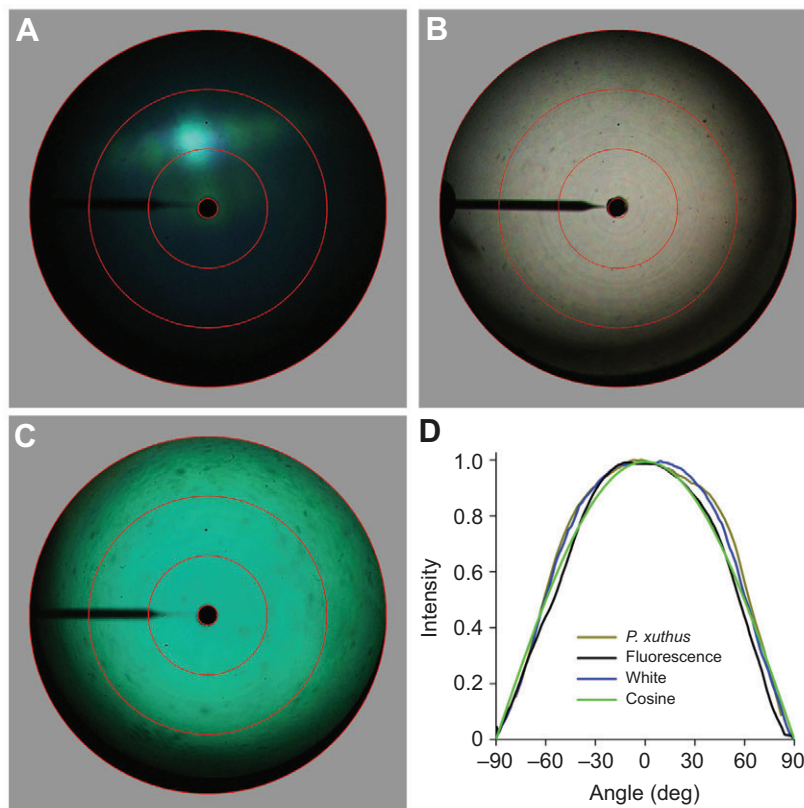

Fig. 6. Spatial profiles of the scattering and fluorescence of papilionid wing scales. (A) Scatterogram of a single wing scale of *P. epiphorbas* using white-light illumination, showing a single reflection spot with diffraction side-maxima. The glass-pipette holding the scale is visible at the '9 o'clock' position as a black bar (see also B,C). The red circles indicate scattering angles of 5, 30, 60 and 90 deg. Reflection spots occur at ~30 deg because the scale was rotated by ~15 deg with respect to the normal to the scale plane in order to avoid blocking of the reflections by the glass pipette. (B) Scatterogram of a single wing scale of *P. xuthus* showing strongly diffuse scattering. (C) Spatial distribution of the fluorescence of the *P. epiphorbas* scale measured by applying 400 nm excitation light and using a 465 nm high-pass filter in front of the camera. (D) Spatial profiles of the scattering by the *P. xuthus* scales, of the *P. epiphorbas* scale fluorescence, of a white standard (scatterogram not shown) and of an ideal, Lambertian cosine diffuser.
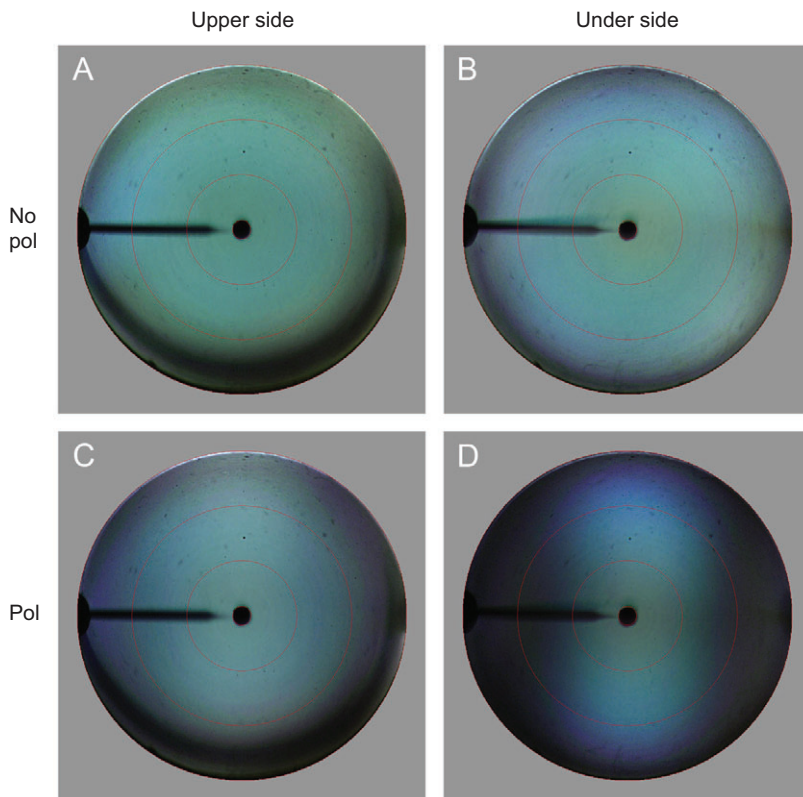
Upper side   Under side

Fig. 7. Imaging scatterometry of the upper side and the under side of a single scale of *P. nireus* applying a non-polarized and polarized wide-aperture, white-light beam. (A) Illumination of the upper side of the scale with unpolarized light ('no pol') yields a scatterogram with a virtually uniform distribution of yellow-green light. (B) Illumination of the under side, also with unpolarized light, shows a distinct angle dependence of the colour of the scattered light. For small angles of incidence, green-yellow light is reflected, and, for large angles of incidence, the reflection is bluish. (C) Illumination of the upper side with horizontally (linear-) polarized ('pol') light results again in a more-or-less diffuse pattern. (D) Upon illumination of the under side at large angles of incidence (~60 deg) with horizontally polarized light, TE-polarized light is strongly reflected, but the reflection of TM-polarized light is minimal.

P. epiphorbas scale. The fluorescence signal appears to be highly diffuse.

The intensity distribution along a line through the centre of the scatterogram of *P. xuthus* (Fig. 6B) shows a profile very similar to that of a white standard and the cosine profile of a Lambertian diffuser (Fig. 6D). This demonstrates that the wing scales of *P. xuthus* indeed act as effective diffusers. The fluorescence signal of the *P. epiphorbas* scale (Fig. 6C) appears to have a virtually identical spatial profile (Fig. 6D). Clearly, the spatial distribution of the emitted fluorescence into the dorsal hemisphere is independent of the mirror properties of the *P. epiphorbas* scale.

The *nireus* group wing scales act approximately as coherent scattering mirrors, not only when illuminated from the upper side (Fig. 6A) but even more so when illuminated from the scale under side (T.M.T., B.D.W., D.G.S. and P.V., unpublished data). In the case of specular objects, we can study the scattering properties as a function of incident angle with an equally simple and powerful approach, namely by using the scatterometer and applying wide-aperture, hemispherical illumination. For a specular object, incident light will be reflected in the mirror-symmetrical direction. The *nireus* group wing scales are slightly imperfect mirrors, and therefore, with wide-aperture illumination, the reflection pattern will be a convolution of the incident light distribution and the local point-spread function. When the latter function is very narrow, like that of Fig. 6A, the reflection pattern will be only slightly broadened.

In the experiments of Fig. 7, we illuminated single scales of *P. nireus*, both from the upper side and from the under side, using a wide-aperture white-light beam. With unpolarized light, the upper side of the scale scatters green-yellow light more or less uniformly for all angles of incidence (Fig. 7A). The same green-yellow colour is observed in the centre of the scatterogram of the under side of the scale (Fig. 7B); that is for about-normal illumination and reflection. With an increasing angle of illumination of the scale under side, the colour of the reflected light shifts to shorter wavelengths

(Fig. 7B). The under side shows iridescent properties, indicating that the layered lower lamina of the scale acts as an optical thin-film multilayer.

The reflectance peak wavelength and amplitude of a thin film or multilayer depends on the angle of incidence (and reflection). The angle dependence of the amplitude also strongly depends on the degree of polarization (Yeh, 2005). We therefore investigated the polarization dependence of the scattered or reflected light by inserting a horizontally polarizing filter into the illumination beam (Fig. 7C,D), causing transverse electric (TE- or s-) polarized light along the vertical plane and transverse magnetic (TM- or p-) polarized light along the horizontal plane of the scatterograms. The resulting scatterogram of the scale upper side had virtually the same colour for all angles of light incidence and reflection (Fig. 7C). However, for an increasing angle of incidence, the scatterogram of the under side showed a strong blue-shift of the TE-reflected light and a strong decrease in reflected TM-polarized light.

It is well known for thin films and multilayers that TM-polarized light becomes extinct at a certain illumination angle – the generalized Brewster angle (Mahlein, 1974). The scatterograms of Fig. 7C,D show a strong polarization dependence of the reflected light only with illumination from the under side but not from the upper side, which means that the upper lamina of the *P. nireus* scale is effective at depolarizing light.

Angle- and polarization-dependent reflectance spectra

In a more detailed angle- and polarization-dependent analysis of the reflectance of the whole wing, we measured the angle-dependent reflectance of blue-green wing areas of *P. epiphorbas* with a setup consisting of two rotating optical fibres. The wings were positioned in the rotation centre of two goniometers and were illuminated with focused white light from the fibre on one goniometer. The second fibre, on the other goniometer, equipped with a polarizer, collected the reflected light (Stavenga et al., 2011; Pirih et al., 2011). We
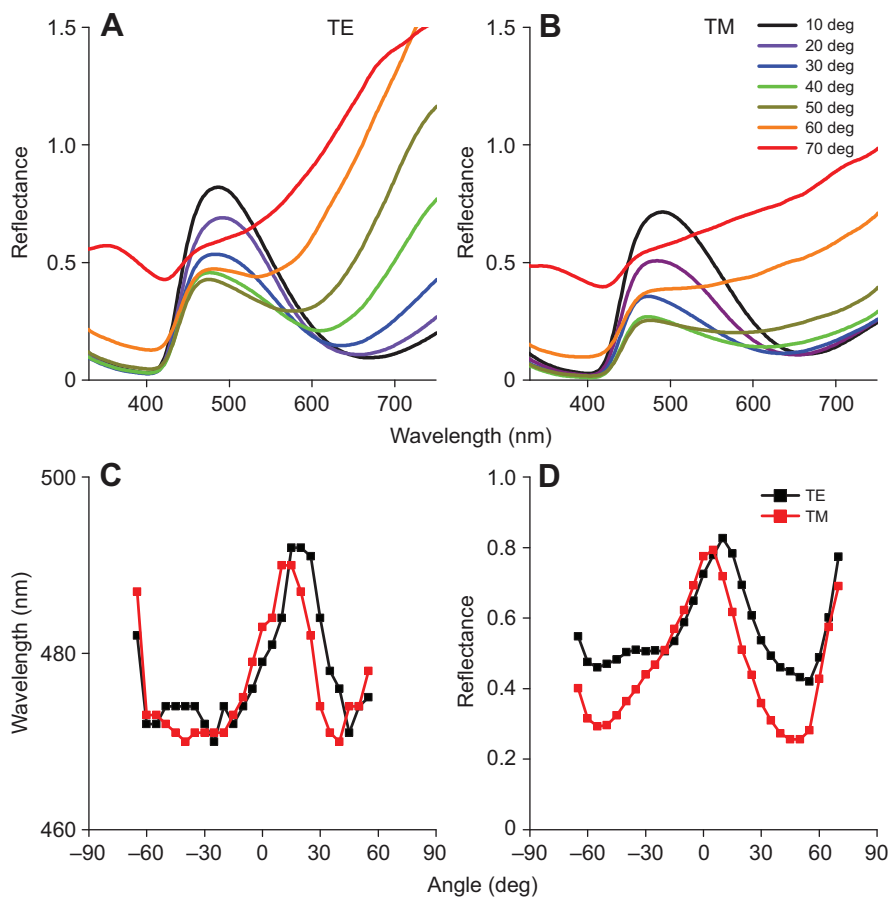
Fig. 8. Angle- and polarization-dependent reflectance measurements of a blue-green wing area of *P. epiphorbas*. (A,B) Reflectance spectra for TE- and TM- polarized light as a function of the angle of light incidence, measured in steps of 5 deg. (C,D) Angle dependence of the peak wavelength and peak reflectance.

varied the angle of illumination in steps of 5 deg in the range of −70 deg to +70 deg. Simultaneously, we changed the angle of detection of the measurement fibre symmetrically with respect to the normal at the wing surface. Fig. 8A,B shows the resulting angle-dependent reflectance spectra for TE- and TM-polarized light. With about-normal illumination, the reflectance spectra peaked at ~490 nm (Fig. 8C), and this value slightly decreased with increasing angle of incidence. For angles above 60 deg, the clear peak in the blue-green wavelength range vanished. The peak amplitude changed by no more than a factor of two with increasing angle of incidence (Fig. 8D). For increasing angles, the side band observable at >750 nm rapidly increased. The angle dependence of the peak wavelength and the peak amplitude was more or less symmetrical, around an angle of incidence of ~20 deg, indicating an angle of ~10 deg between the plane of the wing and the scales. The 10 deg curves in Fig. 8A,B therefore represent about-normal illumination of the scales.

DISCUSSION
Fluorescent pigments in papilionid butterflies
We investigated the wing colouration of four similarly coloured papilionid butterfly species of the *nireus* group. The upper side of the forewing and hindwing of the investigated butterflies all had prominently coloured bands due to a dense lattice of blue-green cover scales. The scales exhibited a distinct fluorescence, revealing the presence of a strongly UV–violet absorbing pigment. Its absorbance maximum was determined to be 393 nm. The fluorescence spectra appeared to be very similar to those of the extensively studied Japanese yellow swallowtail, *P. xuthus* (Fig. 3). In this butterfly, the fluorescing pigment was demonstrated to be papiliochrome II, a combination of N-β-alanyldopamine and L-

kynurenine, which is a characteristic fluorescent pigment for papilionid butterflies, as it is also found in, for instance, *P. demoleus*, *P. protenor* and *P. dardanus* (Umebachi, 1985; Umebachi and Osanai, 2003). The maximal excitation and emission wavelengths of papiliochrome II are approximately 390 nm and 470 nm, respectively (Kumazawa et al., 1994; Kumazawa and Tabata, 2001). Thus, based on the very close correspondence of the fluorescence spectra of the pigment encountered in the wings of *P. xuthus* and the other four papilionid species, we concluded that papiliochrome II is the fluorescent pigment of the studied species. We have considered that the wing scales of the Postman, *Heliconius erato*, and related heliconiine butterflies have yellow-coloured wing scales, similar to those of *P. xuthus*. The pigment in *Heliconius* is 3-hydroxy-DL-kynurenine (Gilbert et al., 1988; Reed et al., 2008; Briscoe et al., 2010), and its absorption spectrum and fluorescence properties approximately resemble those of papiliochrome II (Umebachi and Yoshida, 1970; Gilbert et al., 1988). However, measurements on a few *Heliconius* species (data not shown) showed that the reflectance spectra and the fluorescence intensity deviate noticeably from those of *P. xuthus*, and we therefore conclude that kynurenine derivatives other than papiliochrome II do not contribute to the colouration of the investigated papilionids.

Structural colouration
Contrary to *P. xuthus*, the wing areas of the investigated papilionids with papiliochrome II are not pale-yellow but blue-green. Imaging scatterometry showed that both the fluorescence and the reflection of the scales of *P. xuthus* are diffuse – that is, the spatial distribution of the fluorescence and scattering is Lambertian (Fig. 6). The scales of the four papilionids of the *nireus* group also fluoresce diffusely,
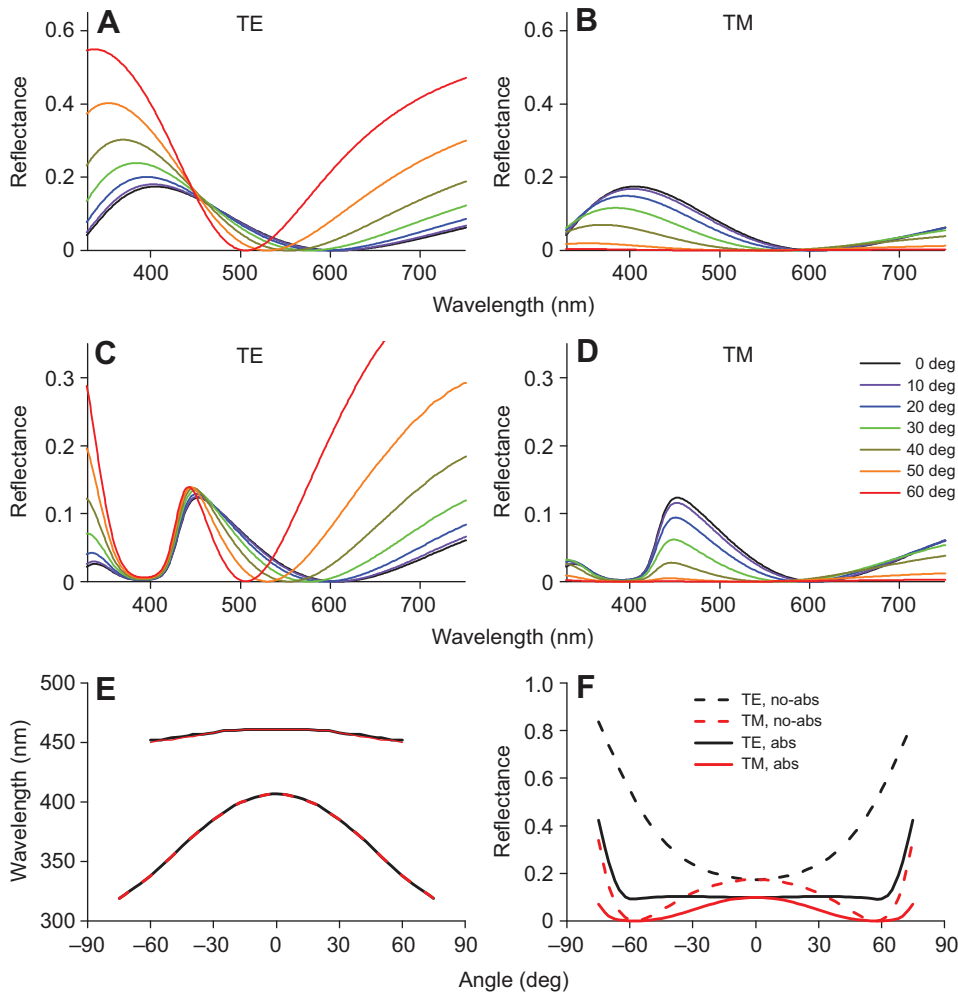
Fig. 9. Modelling of the angle- and polarization-dependent reflectance of a 200 nm thin film of chitin. An absorbing filter, based on the absorbance of papiliochrome II (Fig. 3B) was modelled in front of the thin film. (A,B) Reflectance spectra calculated in steps of 10 deg for incident light with TE- and TM-polarization in the absence of the filter, respectively. A strong peak and polarization dependency are seen. (C,D) Reflectance spectra calculated in the presence of a frontal absorbing filter with an optical density 1.87 at 390 nm. The peak wavelength shift is now masked by the absorption of the pigment, and a stable peak arises. (E,F) Angle- and polarization-dependency of the peak wavelength and the peak reflectance. The presence of the absorbing filter suppresses the polarization- and angle-dependency intrinsic to the structure.

but the reflection is instead strongly directional, indicating that the reflection properties of the scales are approximately specular.

Anatomical investigations have shown that the scales comprise two main layers, a lower lamina and an upper lamina (Vukusic and Hooper, 2005; Ingram and Parker, 2008) (T.M.T., B.D.W., D.G.S. and P.V., unpublished data). The lower lamina is a 200 nm layered thin-film structure (Vukusic and Hooper, 2005). The upper lamina, with a layer thickness of ~1 μm, consists of small cylindrical air cavities in a cuticular medium. The diameter of the cavities is ~200 nm, and the average distance between the cylinder axes is ~300 nm (T.M.T., B.D.W., D.G.S. and P.V., unpublished data). The air gap between the upper and lower lamina is ~1.5 μm. The anatomy thus suggests that the scale structure can be treated as a multilayer. Theoretical modelling indicated that the experimental reflectance spectra are the average of local spectra for multilayers with slightly different structural parameters and that the average spectrum approximates the spectrum of a thin-film reflector with a frontal filter (T.M.T., B.D.W., D.G.S. and P.V., unpublished data).

Modelling

To improve our insight into the measured polarization- and angle-dependence of the scale reflectance spectra, we have calculated reflectance spectra for a chitinous thin film with refractive index 1.56 (Vukusic et al., 1999) and thickness 200 nm. The effect of the papiliochrome II pigment, which mainly acts as a frontal long-pass

filter (T.M.T., B.D.W., D.G.S. and P.V., unpublished data), is assessed by modelling the photonic response of the scale without and with the frontal filter.

The non-filtered thin film has a broad reflectance spectrum, which shifts towards shorter wavelengths when the angle of illumination increases (Fig. 9A,B). The amplitude of the reflectance spectra for TE-polarized light increases with increasing angle of incidence, but the amplitude of the spectra for TM-polarized light decreases for the shown angles of incidence (from 0 to 60 deg).

The reflectance spectra of the filtered thin film show a somewhat different angle dependence. A narrow reflectance band, peaking at ~470 nm, decreases in amplitude and wavelength when the angle of incidence increases, for both TE- and TM-polarized light (Fig. 9C,D). In the case of TE-polarized light, the long-wavelength reflectance band becomes more prominent with increasing angle of incidence (Fig. 9C). At angles greater than 70 deg (data not shown), the blue-green peak diminishes and the long-wavelength side band becomes more prominent.

The filtering severely affects the reflectance peak wavelength (Fig. 9E). With normal illumination of the non-filtered layer, the peak wavelength is ~400 nm. This value decreases with increasing angle of incidence towards 300 nm. In the presence of the pigment filter, the peak wavelength is strongly shifted to longer wavelengths. For normal illumination, the peak wavelength is ~470 nm. This value only slightly decreases when the angle of incidence increases, leveling at ~455 nm for angles above 40 deg.

The filtering also affects the reflectance amplitude (Fig. 9F). Without the pigment filter, the amplitude of the TE-spectra increases with an increasing angle of incidence, but the amplitude of the TM-spectra decreases until the generalized Brewster angle ($\theta_B<60$ deg) – above this angle, the amplitude increases. With the pigment filter in place, the amplitude of the TE-spectra remains virtually constant for angles of incidence up to ~40 deg, but the angle dependence of the amplitude of the TM-spectra is more prominent. A broad reflectance minimum occurs at ~60 deg.

The modelled reflectance spectra (Fig. 9) are qualitatively in good agreement with the scatterometry (Fig. 7C,D) and the measured spectra (Fig. 8A,B). The filter strongly suppresses the shift in the reflectance peak wavelength, characteristically occurring when thin films or multilayers are illuminated from different angles, and thus the filter suppresses the scale iridescence. The absorption of the pigment filter reduces the reflectance for normal illumination by no more than a factor of approximately two (Fig. 9F). We conclude that the pigment in the upper lamina of the wing scales of the *nireus* group papilionids functions to achieve a more-or-less angle- and polarization-independent signal.

Contribution of the fluorescence

Previous measurements by Vukusic and Hooper (Vukusic and Hooper, 2005) suggested that both the fluorescence of the wing scales of *P. nireus* and the coherent scattering from the layered lower lamina of the scales are responsible for the directional colouration of butterflies and that the fluorescence emission is controlled by both the upper slab-like lamina and lower layered lamina. The scatterometry data presented here, however, demonstrate that, although the lower lamina does limit fluorescence emission into one hemisphere, it is only the back-scattering from the lower lamina that is very directional and that contributes most strongly to the appearance of the butterflies. The fluorescent spatial signal behaves like that of an ideal Lambertian diffuser. We have assessed the contribution of the fluorescence emission to the reflectance peak by measuring the reflectance with an integrating sphere in two ways, namely without, and with, a long-pass filter inserted between the xenon lamp and the illumination fibre. A filter with a cut-off wavelength of 465 nm was chosen so that it inhibits the excitation of fluorescence by short-wavelength light. The fluorescence contribution appeared to be minor, ~3–5%. The emission of a xenon light source approximates that of daylight, and we therefore conclude that, in natural conditions, the contribution of the fluorescence to visual signalling compared with that of the reflection is relatively negligible and that the principal function of the fluorescent pigment is to act as a light absorber. However, wings illuminated with a bright and very directional light source (for instance, the sun) will emit fluorescent light in all directions, mostly differing from the direction of the reflected light. In such cases, the fluorescence might enhance the visibility of the butterflies (see Vigneron et al., 2008).

Patterning of the wing

In the investigated papilionids, the blue-green-coloured wing areas are framed by black wing areas. The reflectance spectrum of the margin of *P. nireus*, measured with an integrating sphere, is shown in Fig. 4. The scales in the black marginal areas are structured in a manner similar to that of the extremely black scales of the blue mountain swallowtail, *P. ulysses*. The blackness of the latter scales is principally due to a high concentration of melanin pigment, but the structuring of the scale enhances the black appearance (Vukusic et al., 2004). As we have shown above, the colour of the green wing areas of the papilionids of the *nireus* group is nearly angle

independent. Therefore, the framing of the structurally coloured green wing areas with the deep-black margins will create an angle-independent, stable pattern contrast.

Concluding remarks

This study of the *nireus* group of papilionids has revealed a novel technique to create roughly angle- and polarization-independent blue-green wing colouration. Iridescence suppression in papilionids has been discussed before in the context of morphological changes (Wickham et al., 2006). Here, the highly angle- and polarization-dependent reflectance of the photonic structure of the scales is reduced by a strongly absorbing frontal filter.

So far, a number of methods have been discovered that produce a green wing colouration. This is achieved, for instance, with sculpted multilayers in *P. palinurus* and *P. blumei* scales (Vukusic et al., 2001; Kolle et al., 2010), with perforated multilayers in lycaenids (Wilts et al., 2009) or with gyroid three-dimensional photonic crystals in the lycaenid *Callophrys rubi* and the papilionid *Parides sesostris* (Michielsen and Stavenga, 2008; Michielsen et al., 2010). Butterflies can clearly employ a rich variety of optical tools to tune their colours.

REFERENCES

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

Biró, L. P. and Vigneron, J.-P. (2010). Photonic nanoarchitectures in butterflies and beetles: valuable sources for bioinspiration. *Laser Photon. Rev.* **4**, 1-26.

Biró, L. P., Kertész, K., Vertésy, Z., Mark, G. I., Bálint, Z., Lousse, V. and Vigneron, J. P. (2007). Living photonic crystals: Butterfly scales – Nanostructure and optical properties. *Mat. Sci. Eng. C* **27**, 941-946.

Briscoe, A. D., Bybee, S. M., Bernard, G. D., Yuan, F., Sison-Mangus, M. P., Reed, R. D., Warren, A. D., Llorente-Bousquets, J. and Chiao, C.-C. (2010). Positive selection of a duplicated UV-sensitive visual pigment coincides with wing pigment evolution in *Heliconius* butterflies. *Proc. Natl. Acad. Sci. USA* **107**, 3628-3633.

Choussy, M. and Barbier, M. (1973). Pigment biliaires des lépidoptères: identification de la phorcabiline I et de la sarpédobiline chez diverses espèces. *Biochem. Syst.* **1**, 199-201.

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.

Gilbert, L. E., Forrest, H. S., Schultz, T. D. and Harvey, D. J. (1988). Correlations of ultrastructure and pigmentation suggest how genes control development of wing scales of *Heliconius* butterflies. *J. Res. Lepid.* **26**, 141-160.

Ingram, A. L. and Parker, A. R. (2008). A review of the diversity and evolution of photonic structures in butterflies, incorporating the work of John Huxley (The Natural History Museum, London from 1961 to 1990). *Philos. Trans. R. Soc. B* **363**, 2465-2480.

Kinoshita, S. (2008). *Structural Colors in the Realm of Nature.* Singapore: World Scientific.

Kinoshita, S. and Yoshioka, S. (2006). Structural or pigmentary? Origin of the distinctive white stripe on the blue wing of a *Morpho* butterfly. *Proc. R. Soc. Lond. B* **273**, 129-134.

Kinoshita, S., Yoshioka, S. and Kawagoe, K. (2002). Mechanisms of structural colour in the *Morpho* butterfly: cooperation of regularity and irregularity in an iridescent scale. *Proc. R. Soc. Lond. B* **269**, 1417-1421.

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

Kolle, M., Salgard-Cunha, P. M., Scherer, M. R. J., Huang, F., Vukusic, P., Mahajan, S., Baumberg, J. J. and Steiner, U. (2010). Mimicking the colourful wing scale structure of the *Papilio blumei* butterfly. *Nat. Nanotech.* **5**, 511-515.

Kumazawa, K. and Tabata, H. (2001). A three-dimensional fluorescence analysis of the wings of male *Morpho sulkowskyi* and *Papilio xuthus* butterflies. *Zool. Sci.* **18**, 1073-1079.

Kumazawa, K., Tanaka, S., Negita, K. and Tabata, H. (1994). Fluorescence from wing of *Morpho sulkowskyi* butterfly. *Jpn. J. Appl. Phys.* **33**, 2119-2122.

Luke, S. M., Vukusic, P. and Hallam, B. (2009). Measuring and modelling optical scattering and the colour quality of white pierid butterfly scales. *Opt. Express* **17**, 14729-14743.

Mahlein, H. F. (1974). Generalized Brewster-angle conditions for quarter-wave multilayers at non-normal incidence. *J. Opt. Soc. Am.* **64**, 647-653.

Mason, C. W. (1926). Structural colors in insects. *I. J. Phys. Chem.* **30**, 383-395.

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., De Raedt, 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.

Morehouse, N. I., Vukusic, P. and Rutowski, R. (2007). Pterin pigment granules are responsible for both broadband light scattering and wavelength selective absorption in the wing scales of pierid butterflies. *Proc. R. Soc. Lond. B* **274**, 359-366.

Nijhout, H. F. (1991). *The Development and Evolution of Butterfly Wing Patterns.* Washington: Smithsonian Institution Press.

Pirih, P., Wilts, B. D. and Stavenga, D. G. (2011). Spatial reflection patterns of iridescent pierid butterfly wings and the dependence of visibility on scale curvature. *J. Comp. Physiol. A* **197**, 987-997.

Poladian, L., Wickham, S., Lee, K. and Large, M. C. J. (2009). Iridescence from photonic crystals and its suppression in butterfly scales. *J. R. Soc. Interface* **6**, S233-S242.

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. Lond. B* **275**, 37-45.

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

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 colouration of the wing membrane. *J. Exp. Biol.* **213**, 1731-1739.

Stavenga, D. G., Wilts, B. D., Leertouwer, H. L. and Hariyama, T. (2011). Polarized iridescence of the multilayered elytra of the Japanese jewel beetle, *Chrysochroa fulgidissima*. *Philos. Trans. R. Soc. B* **366**, 709-723.

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

Umebachi, Y. and Osanai, M. (2003). Perturbation of the wing color pattern of a swallowtail butterfly, *Papilio xuthus*, induced by acid carboxypeptidase. *Zool. Sci.* **20**, 325-331.

Umebachi, Y. and Yoshida, K. (1970). Some chemical and physical properties of papiliochrome II in the wings of *Papilio xuthus*. *J. Insect Physiol.* **16**, 1203-1228.

Vigneron, J.-P., Kertész, K., Vértesy, Z., Rassart, M., Lousse, V., Bálint, Z. and Biró, L. P. (2008). Correlated diffraction and fluorescence in the backscattering iridescence of the male butterfly *Troides magellanus* (Papilionidae). *Phys. Rev. E* **78**, 021903.

Vukusic, P. and Hooper, I. (2005). Directionally controlled fluorescence emission in butterflies. *Science* **310**, 1151.

Vukusic, P. and Sambles, J. R. (2003). Photonic structures in biology. *Nature* **424**, 852-855.

Vukusic, P. and Stavenga, D. G. (2009). Physical methods for investigating structural colours in biological systems. *J. R. Soc. Interface* **6**, S133-S148.

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., Sambles, J. R. and Ghiradella, H. (2000). Optical classification of microstructure in butterfly wing-scales. *Phot. Sci. News* **6**, 61-66.

Vukusic, P., Sambles, R., Lawrence, C. and Wakely, G. (2001). Sculpted-multilayer optical effects in two species of *Papilio* butterfly. *Appl. Optics* **40**, 1116-1125.

Vukusic, P., Sambles, J. R. and Lawrence, C. R. (2004). Structurally assisted blackness in butterfly scales. *Proc. R. Soc. Lond. B* **271 Suppl. 4**, S237-S239.

Wickham, S., Large, M. C. J., Poladian, L. and Jermiin, L. S. (2006). Exaggeration and suppression of iridescence: the evolution of two-dimensional butterfly structural colours. *J. R. Soc. Interface* **3**, 99-109.

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**, S193-S202.

Wilts, B. D., Pirih, P. and Stavenga, D. G. (2011). Spectral reflectance properties of iridescent pierid butterfly wings. *J. Comp. Physiol. A* **197**, 693-702.

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, USA: Wiley-Interscience.