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Butterfly wing colours: scale beads make white pierid wings brighter

D. G. Stavenga[1*], S. Stowe[2], K. Siebke[3,4], J. Zeil[4] and K. Arikawa[5]

[1]*Department of Neurobiophysics, University of Groningen, Nijenborgh 4, NL-9747 AG Groningen, The Netherlands*
[2]*ANU Electron Microscopy Unit,* [3]*Ecosystem Dynamics, and* [4]*Visual Sciences, Research School of Biological Sciences, Australian National University, Canberra, ACT, Australia*
[5]*Graduate School of Integrated Science, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama 236-0027, Japan*

The wing-scale morphologies of the pierid butterflies *Pieris rapae* (small white) and *Delias nigrina* (common jezabel), and the heliconine *Heliconius melpomene* are compared and related to the wing-reflectance spectra. Light scattering at the wing scales determines the wing reflectance, but when the scales contain an absorbing pigment, reflectance is suppressed in the absorption wavelength range of the pigment. The reflectance of the white wing areas of *P. rapae*, where the scales are studded with beads, is considerably higher than that of the white wing areas of *H. melpomene*, which has scales lacking beads. The beads presumably cause the distinct matt-white colour of the wings of pierids and function to increase the reflectance amplitude. This will improve the visual discrimination between conspecific males and females.

Keywords: Pieridae; *Heliconius*; wing pigmentation; reflectance; colour contrast

1. INTRODUCTION

Butterflies are universally attractive because of their often striking wing colours. Research into the optical basis of the wing colours has so far mainly focused on cases where the wings display a bright directional iridescence, attributed to wave optical phenomena in the wing scales: multilayer interference (little yellow, *Eurema lisa* (Ghiradella *et al.* 1972)); diffraction (shining blue nymph, *Cynandra opis* (Brink & Lee 1999) and siva hairstreak, *Callophrys siva* (Allyn & Downey 1976)); interference and diffraction (*Morpho* (Vukusic *et al.* 1999; Gralak *et al.* 2001; Kinoshita *et al.* 2002) and *Ancyluris meliboeus* (Vukusic *et al.* 2002)); or photonic crystal effects (Meleager's blue, *Polyommatus daphnis* (Biro *et al.* 2003; review Vukusic & Sambles 2003)). Here, we investigate the seemingly simple case of the wing colours of pierids.

The colour of objects, including living creatures, is determined by the wavelength-dependent interaction of incident light with the object. The resulting coloration can be fully structural, i.e. it can arise from interference, diffraction and/or scattering of incident light as a result of structural variations of high and low refractive index materials (Land 1972). Pigments can strongly modulate the spectral distribution of the scattered and/or reflected light when the absorption of these substances is only substantial in a restricted wavelength range. Accordingly, colours are commonly distinguished as either physical or chemical colours.

Butterfly wing colours originate from the cover of scales, which are arranged in a more or less regular pattern of rows. The scales are constructed according to a general blueprint. The upper surface of the scales bears longitudinal ridges roofed with overlapping lamellae, which cover a series of folds, perpendicular to the lamellae, the microribs. The longitudinal ridges are connected by crossribs, and together they frame so-called windows to the scale interior (Ghiradella 1998). This construction is highly malleable, allowing extravagantly varying shapes, and evolutionary experimentation has, in several cases, produced highly aesthetic, regular landscapes that can give rise to spectacular iridescence (Ghiradella 1984; Nijhout 1991; Vukusic & Sambles 2003). When the wing coloration is a result of pigments, the scale cover paints a colourful pointillistic pattern on the wings, because each scale generally expresses only one type of pigment (Nijhout 1991).

Many butterfly species of the family Pieridae exhibit a marked sexual dichroism (Eisner *et al.* 1969; Mazokhin-Porshnyakov 1969; Kolb & Scholz 1985). The wings of female and male little yellow, *E. lisa*, and orange sulphur butterflies, *Colias eurytheme* (Coliadinae, sulphurs), are yellow to orange, as a result of pterin pigments absorbing at short (i.e. blue to yellow) wavelengths (Watt 1964). The males display, in addition, a strong ultraviolet (UV) iridescence, produced by layered lamellae in the ridges, which plays a role in mate selection (Ghiradella *et al.* 1972; Silberglied & Taylor 1973; Rutowski 1977). The dominant wing colour of the small white, *Pieris rapae* (Pierinae, whites), is a bright matt-white, indicating the absence of absorbing pigments. Female Japanese small white, *P. rapae crucivora*, exhibit a considerable wing reflectance throughout the visible wavelength range, including the UV (Obara 1970). The wing reflectance of the male is minimal in the UV, however (Obara 1970), as a result of UV-absorbing pterin (Makino *et al.* 1952). The resulting strong contrast between the UV and the (human) visible wavelength range plays a crucial role in initial mate recognition (Obara 1970; Obara & Majerus 2000).

The white and yellow wing scales of pierids characteristically differ from other butterfly scales. Electron microscopic studies have shown that the crossribs are densely decorated with ovoid beads (Yagi 1954; Ghiradella *et al.*

*Author for correspondence (stavenga@phys.rug.nl).

Figure 1. The wings of pierid butterflies are white as a result of their strongly light-scattering scales. (*a*) A large white, *Pieris brassicae*, on a butterfly bush (*Buddleia*). (*b*) The scales, here of the small white, *P. rapae*, are generally scalloped, and are locally interspersed with plume scales. Scale bars: (*a*) 1 cm and (*b*) 40 μm.

1972; Ghiradella 1998). To gain insight into the function of the beads, we have measured wing reflectances of a number of butterfly species. The data suggest that the beads enhance the wing reflectance, i.e. make the wings brighter at wavelengths where the pigment absorption is negligible. The enhanced brightness yields an increase in colour contrast with wavelengths where pigments absorb strongly.

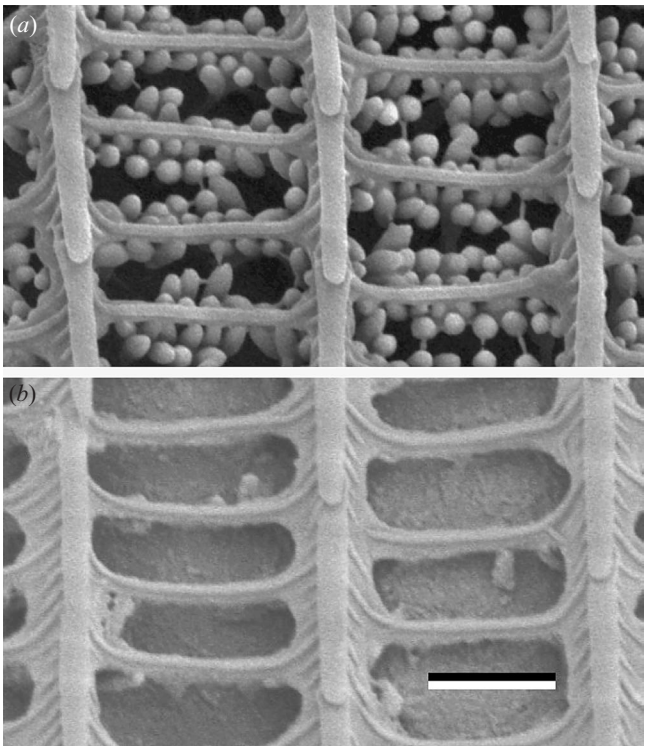

Figure 2. SEMs of scales of the small white, *Pieris rapae*. (*a*) A white scale. The ridges have only slightly overlapping lamellae, the crossribs are densely adorned with beads. (*b*) A black scale, lacking beads. Scale bar, 1 μm.

2. MATERIAL AND METHODS

(a) *Animals*

Small whites, *P. r. rapae*, were captured in Canberra. Specimens of the Japanese subspecies *P. r. crucivora* were obtained from a culture maintained by K.A. in Yokohama. Common jezabel butterflies, *Delias nigrina*, were obtained from Dr M. F. Braby, Australian National University, Canberra, and captured near Bateman's Bay, Australia. The heliconine, *Heliconius melpomene*, came from a culture maintained by Dr P. M. Brakefield, Leiden University.

(b) *Scanning electron microscopy*

Wing pieces were gold sputtered and the scale structure was investigated with a field-emission scanning electron microscope (FESEM) (Hitachi S-4500). For focused ion beam scanning electron microscope (FIBSEM) work, small wing fragments or clusters of scales were mounted on conductive carbon tape (ProSciTech, Thuringowa, QLD, Australia), and viewed usually without a coating. The FIBSEM was a Camion 31 gallium ion gun (Orsay Physics, Aix-en-Provence, France) mounted on a tungsten-filament scanning electron microscope (SEM) (JSM 6460LV, JEOL, Tokyo, Japan). Ion beams of between 0.5 and 10 pA were used to etch lines 5–20 μm long on the scales. The samples were then removed from the FIBSEM, lightly gold sputter-coated and the cut surfaces viewed with the FESEM.

(c) *Transmission electron microscopy*

To stabilize scales during processing for transmission electron microscopy (TEM), wing pieces were coated with fresh egg-white before fixation in Karnovsky's (2.5% glutaraldehyde, 3.7% formaldehyde in 0.1 M of sodium cacodylate with 0.1 M of sucrose at pH 7.2) for 2–4 h, osmication in 1% osmium

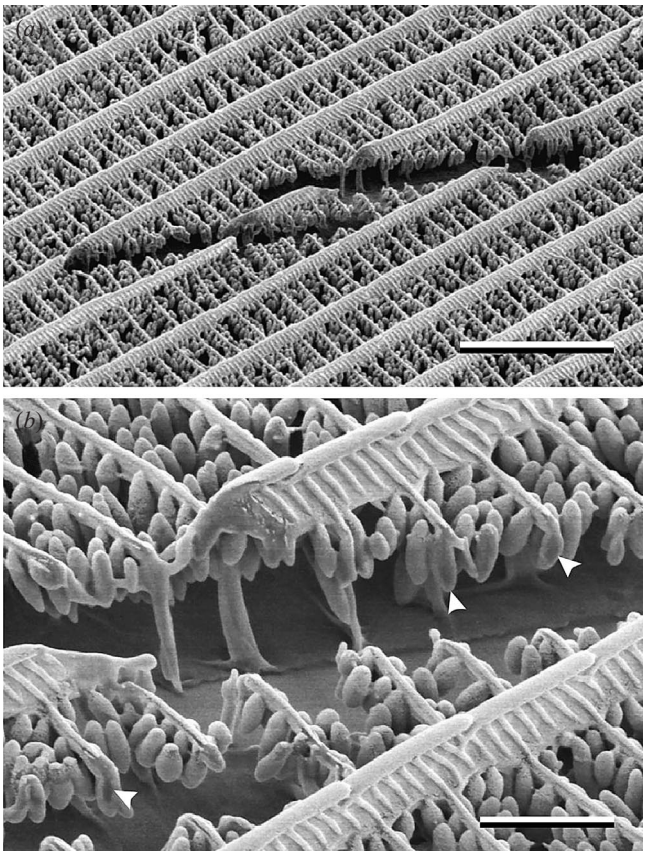

Figure 3. FESEM of a *Pieris rapae rapae* wing scale prepared with a focused ion beam (FIB). (*a*) Cuts made with the FIB, intersecting several longitudinal ridges. (*b*) Enlargement showing the elongated ovoid beads attached to the crossribs and microribs. Sectioned beads appear to be hollow (arrowheads). Scale bars: (*a*) 5 μm and (*b*) 1 μm.

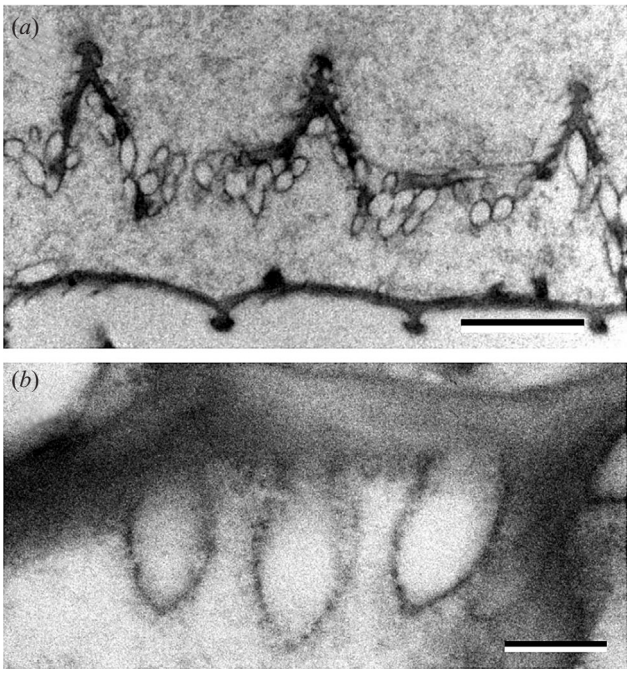

Figure 4. TEM of a white scale. (*a*) Cross-section through three longitudinal ridges, showing lamellae on the ridges and beads on the cross-ribs. The lower surface is virtually flat, but near the scale tip, as here, small ridges are apparent on the lower surface. (*b*) Whereas the upper surface sheet of the scale contains stained material, the beads do not stain, indicating that they are hollow. Scale bars: (*a*) 1 μm and (*b*) 0.1 μm.

tetroxide in the same buffer, dehydration through ethanol and propylene oxide and embedding in Epon/Araldite. Sections stained with uranyl acetate were observed on a Hitachi 7100FA TEM at 75 kV and photographed with a Megaview digital camera (Soft Imaging System, Münster, Germany).

(d) *Reflection spectrophotometry*

(i) *Spectrographic imaging*

Wing-reflectance spectra were obtained under identical conditions from the dorsal wings of both *P. rapae* and *H. melpomene* with a spectrographic imager (CASI, ITRES, Calgary, Alberta, Canada), using diffuse white light from halogen lamps, with the wing surface perpendicular to the optical axis of the measuring instrument. The CASI measures reflectance spectra simultaneously from a row of pixels while scanning the image (for a detailed description, see Zeil & Hofmann (2001)). The effective pixel size at the level of the wings was $200 \times 200\ \mu m^2$, an area that is covered by five to six scales. The reflectance spectra were calibrated against a white reflectance standard (Spectralon, Labsphere, North Sutton, NH, USA).

(ii) *Microspectrophotometry*

Wing-scale reflectance spectra were also measured with a microspectrophotometer, consisting of an epi-illumination microscope (Leitz Ortholux; objective Zeiss 5×, 0.15 Neofluar), equipped with a photodiode-microarray spectrophotometer (SD2000, Avantes, Eerbeek, The Netherlands), and a xenon light source. The reflection of a patch of *ca.* $100 \times 200\ \mu m^2$ was measured. The reflectance spectra, calibrated with a diffuse reflectance standard (Labsphere, USA), were measured immediately before the application of a drop of immersion fluid (xylene, refractive index 1.494) as well as during the few minutes taken by the drop to evaporate.

3. RESULTS

The wings of the small white, *P. rapae*, are covered with scales, of which a vast majority are spatulate and scalloped; occasionally plume scales (androconia; Downey & Allyn 1975) can be seen (figure 1). The distances of longitudinal ridges and crossribs in the scales of *P. rapae* are *ca.* 2.0 μm and 0.7 μm, respectively (figure 2). In the white wing scales, the crossribs, and to a lesser extent the ridges, are decorated by ellipsoidal beads (figure 2*a*). The scales from the black wing areas are completely free from beads (figure 2*b*). These scales show a similar unspecialized structure to that seen in many butterfly species, with ridges, crossribs and scattered trabeculae between the upper and lower scale surface (Ghiradella 1998).

With conventional SEM it is difficult to obtain a detailed three-dimensional view of the scale structures. Figure 3 shows that this limitation can be overcome with a FIBSEM, which allows cutting away of material in a controlled manner (figure 3*a*). The beads are then revealed as strongly elongated ovoids with dimensions *ca.* 100–500 nm (figure 3*b*). Sectioned beads appear hollow (figure 3*b*, arrowheads).

TEM sections (figure 4) show that the upper and lower scale surfaces are thin sheets of electron dense material,

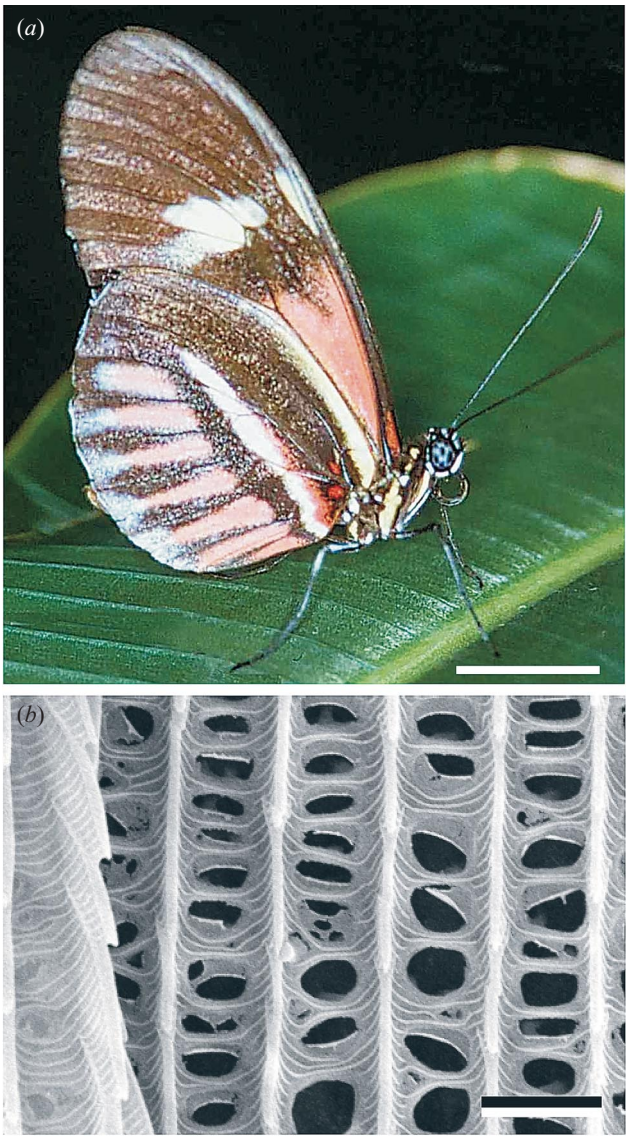

Figure 5. The heliconine *Heliconius melpomene* (*a*) has wing scales without beads and with variably sized windows (*b*). Scale bars: (*a*) 1 cm and (*b*) 2 µm.

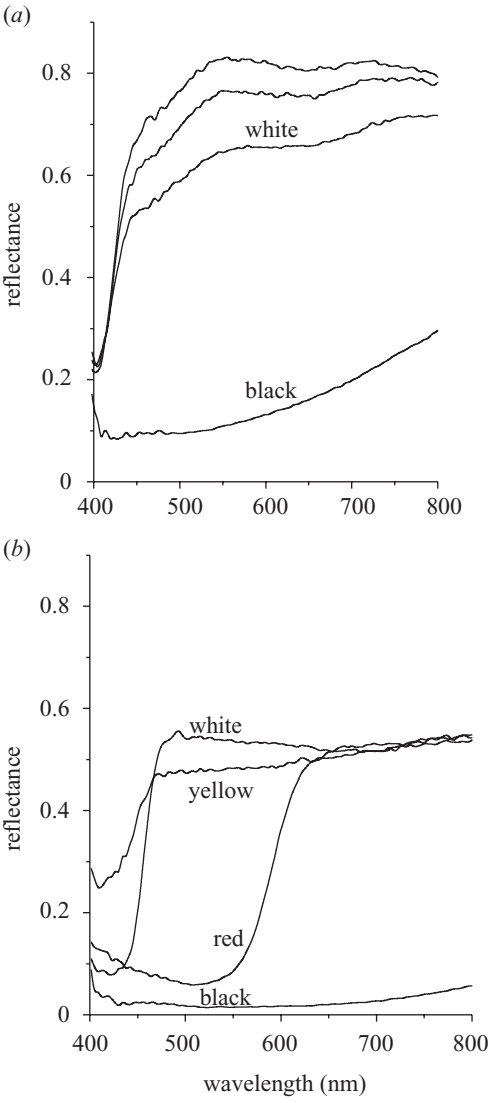

Figure 6. Reflectance spectra of the dorsal wings of (*a*) *Pieris rapae rapae* and (*b*) *Heliconius melpomene*, determined with a spectrographic imager (CASI). For *P. r. rapae*, the spectra of three different locations on the white wing are shown, together with the reflectance spectrum of one of the black spots. For *H. melpomene* the reflectance spectra of one of the white chevrons and of a yellow, red, and black area at the dorsal wings are shown. The reflectance amplitude is more or less constant at the longer wavelengths, where pigment absorption is negligible. The amplitudes of the white areas of *P. r. rapae* at the long wavelengths are distinctly higher than the amplitudes of the spectra for the white, yellow and red scales of *H. melpomene*.

with a thickness of *ca*. 60 nm. The beads appear as unstained ovoids with a 5–8 nm thick membrane, in agreement with the FIBSEM images, which suggest that the beads are empty (figures 3*b* and 4). In contrast with the sculpted upper surface, almost the whole lower surface of the scales is virtually flat, although ridges are seen at the lower surface near the scale tips (figure 4*a*).

The structural differences between the white and black scales (figure 2) suggest an optical function for the beads of the white scales. We therefore compared the wing reflectance of *P. r. rapae* (male) with that of the heliconine *H. melpomene* (figure 5), which has beadless scales in a variety of colours, including white and black.

Figure 6*a* presents the reflectance of three white areas, together with that in a black spot, of *P. r. rapae*. The three white areas all reflect little UV, because of a UV-absorbing pigment; the reflectance amplitude in the long-wavelength range varies somewhat. The reflectance of the black area is low throughout the visible wavelength range. Figure 6*b* shows the reflectance spectra of four wing areas of *H. melpomene*, with white, yellow, red and black scales. The reflectance spectra of all scale classes, except the black class, coincide above 650 nm. This suggests that the scale structures scatter incident light in the same way, with the only difference being that each scale class has its specific pigment. The reflectance amplitudes of white, yellow and red *H. melpomene* scales are the same above 650 nm, indicating that there is no differential pigment absorption at long wavelengths. The observation that *P. rapae* scales have much higher reflectance amplitudes above 650 nm compared with *H. melpomene* scales suggests that beads play a role in amplifying scale reflectance.

The scale structures act as light scatterers, owing to the difference in refractive index with air, giving unpigmented

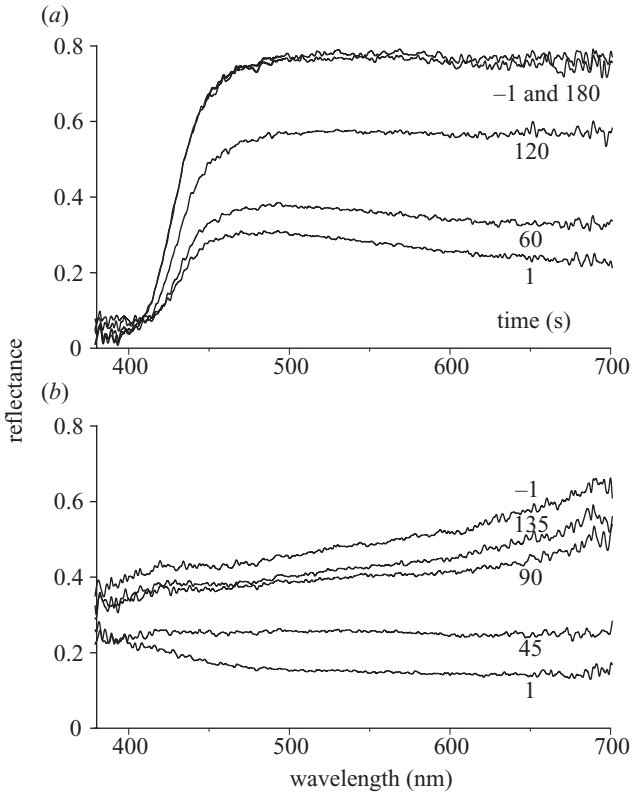

Figure 7. Reflectance changes induced by a drop of immersion fluid, xylene, on the wings of *Pieris rapae crucivora*. (*a*) The initial reflectance (−1 s) of the white wing of the male is high at wavelengths greater than 450 nm, but low in the UV (see figure 5*a*). Xylene causes an instant decrease in the reflectance, but the reflectance recovers within a few minutes upon evaporation of the xylene. (*b*) The initial reflectance of the white wing of the female (−1 s) only slightly decreases in the UV. Recovery of reflectance is similar to that in (*a*).

scales a white colour. This was already demonstrated early in the twentieth century (Mason 1926). Application of volatile immersion fluids reduced the white colour, which was regained after drying. We have repeated this experiment with the dorsal wings of both the male small white, *P. r. crucivora* (figure 7*a*) and the female (figure 7*b*). The reason for also investigating female wings is their substantial UV reflectance (Obara 1970). Figure 7 shows how the reflectance changes as a result of the application of a drop of xylene, which strongly reduces the refractive index difference between the scale material and the surrounding medium (Mason 1926; Gilbert *et al.* 1988; Nijhout 1991). The reflectance is high immediately before application of the xylene (–1 s), but immediately (1 s) afterwards the reflectance is reduced by 50–75%. The initial reflectance fully recovers after evaporation of the xylene, which occurs within minutes. We may therefore conclude that the whiteness of butterfly wings is purely a physical colour, where the reflectance amplitude depends on the number and precise dimensions of scattering structures. In males, reflectance at wavelengths below 420 nm is only insignificantly affected by the xylene treatment, indicating that the scales carry a UV-absorbing pigment.

A UV-absorbing pigment does not affect the white colour as seen by humans. However, when the scales exhibit a non-white colour, pigments absorbing at visible wavelengths are involved, as is shown by immersion experiments on *D. nigrina* (common jezabel, Pieridae). This striking animal has brown–black coloured ventral wings, with yellow and red stripes (figure 8*a*). The male's dorsal wings are bright white, with black tips (figure 8*b*), and the scales feature dense arrays of beads, similar to *P. rapae* (figure 8*c*). Before application of xylene (−1 s), the reflectance of the white dorsal wings is low in the UV (figure

Figure 8. Wing colour and beads on the wing scales of the common jezabel, *Delias nigrina*. (*a*) The ventral wings are darkly coloured, except for a few patches with red and yellow scales. (*b*) The dorsal wings are white, with black tips. (*c*) The scales have a high concentration of beads. Scale bars: (*a*,*b*) 1 cm and (*c*) 1 μm.

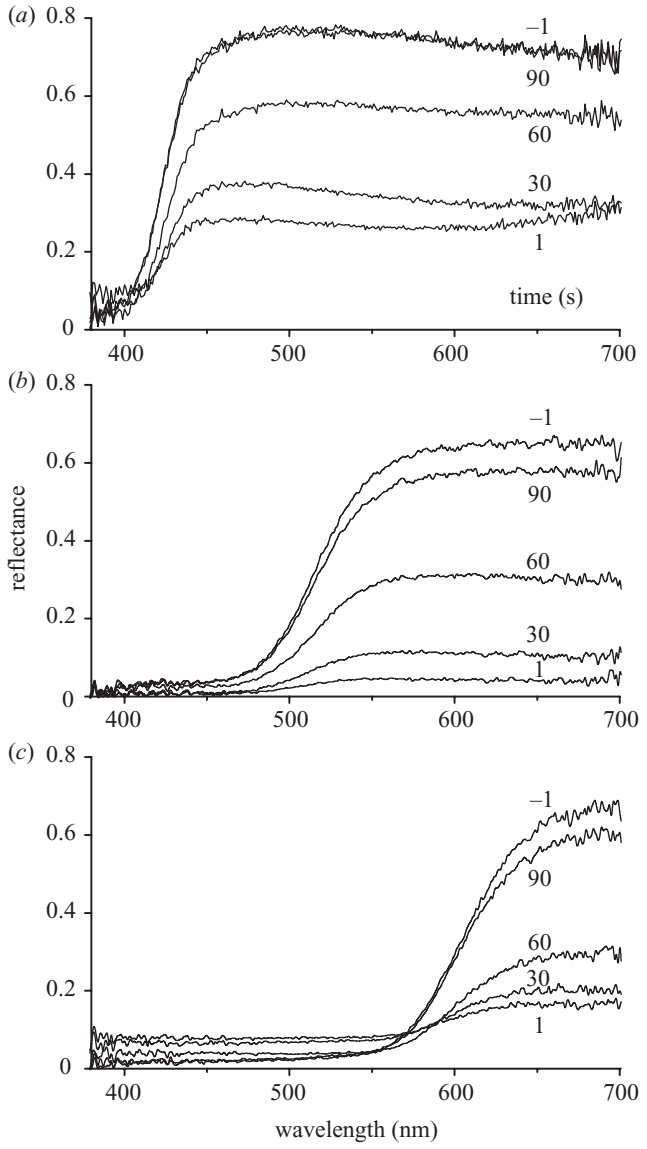

Figure 9. Reflectance changes induced by xylene in the wings of *Delias nigrina*. (*a*) A white area in the dorsal wings. (*b*) A yellow area of the ventral forewings. (*c*) A red area of the male ventral hindwings. A drop of xylene induces a reflectance decrease that is roughly wavelength independent. The reflectance recovers upon the evaporation of the xylene. The (*a*) white,(*b*) yellow and (*c*) red wing scales contain pigments absorbing UV, blue, and blue plus yellow, respectively. The reflectance amplitude is comparable at the longer wavelengths where the pigment absorption is negligible.

9*a*), demonstrating the ample presence of a UV-absorbing pigment, while the yellow and red wing areas contain pigments that absorb up to *ca*. 550 nm and 650 nm, respectively (figure 9*b*,*c*). The application of a drop of xylene yields very similar effects to those seen with the small white butterfly (compare figures 7 and 9). Xylene causes a sharp fall in the reflectance amplitude at long wavelengths, and at the shorter wavelengths, where pigment absorption occurs, reflection is reduced proportionally. The reflectance of the red scales seems to increase at shorter wavelengths, but this is a result of spurious reflections by the xylene film.

4. DISCUSSION

Butterfly wing colours depend on the structure and optical properties of the scales. Treatment with an immersion fluid such as xylene, which equilibrates the refractive index, reduces the lustre but not the colour of the scales (Gilbert *et al.* 1988; Nijhout 1991), which is a result of absorbing pigment filtering the scattered light. Gilbert *et al.* (1988) investigated the chemical nature of the absorbing wing-scale pigments in various *Heliconius* species as well as in crosses of *H. cydno* and *H. melpomene*. They found that the yellow scales contain alkaline 3-hydroxykynurenine, the brown and red scales contain xanthommatins in different oxidized forms, and the black scales contain melanin. White *Heliconius* scales were stated to have no pigment (Gilbert *et al.* 1988), but our measurements show a UV-absorbing pigment (figure 6*b*). Leucopterin and xanthopterin *a* were identified as UV-absorbing pigments in the wings of male *P. rapae* (Makino *et al.* 1952). (The term 'pterin' was originally the generic term for wing pigments (Kayser 1985).)

The reflectance spectra of figure 2 were measured with the wing surface perpendicular to the axis of the spectrographic imager, using a wide-angle light source and a diffusing reference standard. The spectra obtained from the same locations, but with the wing plane rotated, showed that the reflectance is quite independent of the direction of observation. Only at angles larger than 45° was the reflectance reduced by more than 20% for both *P. rapae* and *H. melpomene* (data not shown). How well the wings approximate a diffuser deserves further detailed study, because the directional dependence of the reflectance is an important ecological factor.

A general theory of butterfly wing reflectance from scattering on the scale structures and pigment absorption does not yet exist. The reflectance cannot be understood from the optics of single scales, because the scales are shingled in overlapping rows on a rather smooth, reflecting wing. The measured light reflection consists of light redirected by any structure where the refractive index changes, i.e. at the upper and lower sheets of both cover and ground scales at both faces of the wing, as well as at the wing surface proper. If present in the scales, absorbing pigments modulate the resulting light flux. Iridescent wings have a high reflectance which can be quite directional. The different cover and ground scales of *Morpho didius* yield in concert a wide angle of iridescence (Vukusic *et al.* 1999; Kinoshita *et al.* 2002).

In *Pieris*, cover and ground scales together produce wide-angle scattered light. The reflectance of *H. melpomene* wings is, at long wavelengths, lower than that of *P. r. rapae* and the scales do not have beads. This suggests a causal relationship between the beads and the high reflectance of the *P. r. rapae* scales (figure 6). Scale structure generally correlates with pigmentation (figure 2), as has been demonstrated for *Heliconius* (Gilbert *et al.* 1988; Janssen *et al.* 2001). The difference in the sheen of the white scales in *P. rapae* and *H. melpomene* (Gilbert *et al.* 1988) is probably primarily a result of the beaded scales of *Pieris*, which are much more differentiated than the scales of *Heliconius* (figures 2–5).

The beads are a general characteristic of the scales of pierid butterflies. They have been classified as pigment

granules (Yagi 1954; Kolyer & Reimschuessel 1970; Ghiradella *et al.* 1972; Ghiradella 1984, 1998) or pterinosomes (Descimon 1975). We suggest that this nomenclature should be avoided because there is, at present, no evidence that the beads do contain pigment. A white colour in fact suggests that the material is non-absorbing or pigmentless (Gilbert *et al.* 1988). If a material contains a UV-absorbing pigment, its subjective whiteness is determined only by the refractive indices and structural details and is independent of the pigment concentration. Therefore, to define a UV-absorbing pigment as a white pigment (Descimon 1975) is at least ambiguous and most probably confusing. The beads have been stated to be solid without further evidence (Vukusic & Sambles 2003), whereas our FIBSEM and TEM images suggest that the beads are hollow (figures 3 and 4). The bead size, in the order of a few hundred nanometres, is possibly optimized for light scattering (Johnsen & Widder 1999).

Pigment deposited in the scales filters the reflected light depending on pigment density. Inspection of isolated pigmented scales with a light microscope shows that the pigment is more or less diffusely dispersed throughout the scale (Köhler & Feldotto 1935; Ghiradella 1998). A very broadband absorbing pigment, presumably melanin, is present in the black spots on the wings of *P. rapae* and the black wing areas of *H. melpomene* (figure 6). The low reflectance of the black scales of *H. melpomene* indicates a high pigment density. The absence of beads in black, low-reflecting scales as opposed to the presence of beads in white or well-reflecting scales (figure 2) may be a general property of pierids (Kolyer & Reimschuessel 1970). The variable reflectance amplitude of the white wing areas of *P. rapae* (figure 6*a*) is presumably related to the density of beads, which varies considerably among the scales (not shown). The reflectances of the white, yellow and red coloured scales of *D. nigrina* have a very similar magnitude at those long wavelengths where the pigments no longer absorb (figure 9). The only difference between the various scales of *Delias* presumably is that they contain different absorbing pigments.

The dorsal wings of female Pierinae (whites) commonly have a high reflectance in the UV (Mazokhin-Porshnyakov 1969; Obara 1970; Kolb & Scholz 1985), but the wings of the female large white, *P. brassicae* (figure 1*a*), reflect little UV, and in only the visible wavelength range do the reflectance amplitudes differ somewhat from those of the male (Kolb & Scholz 1985). The same holds for the green-veined white, *P. napi* (Kolb & Scholz 1985). British *P. r. rapae* females have low UV reflectance, quite different from their Japanese relatives, *P. r. crucivora*, where the females reflect UV light well. The sexual differences in wing coloration were found to correlate with local differences in sexual behaviour of the two subspecies (Obara & Majerus 2000). Variable UV reflectance, in this case correlated with geographical latitude, was also found in the Finnish *P. napi* (Meyer-Rochow & Järvilehto 1997).

It is interesting (or more confusing) that the visual system of *P. rapae* is also not without variability. For example, the eyes of male *P. r. crucivora* contain a fluorescent pigment (Qiu *et al.* 2002), which acts as a specialized UV filter, shaping a narrow-band violet receptor (Qiu & Arikawa 2003) that presumably improves discrimination of UV contrast. The fluorescent pigment does not exist in the eyes of male *P. r. rapae* (D. G. Stavenga, unpublished data). We therefore hypothesize that the Japanese *P. rapae* subspecies *crucivora* has evolved improved discrimination of UV-visible patterns in the wings, the contrast of which is enhanced by scattering beads.

Other pierids take a different approach to the problem of displaying a colourful pattern. Coliadinae (sulphurs) are butterflies forming a subfamily that together with the Pierinae (whites) belongs to the family of Pieridae. They are often strikingly yellow or orange coloured, meaning that the scales contain pigments absorbing blue to blue–green, short-wavelength light. Males of several species of Colianidae have strongly UV-reflecting wings (Eisner *et al.* 1969; Mazokhin-Porshnyakov 1969; Ghiradella *et al.* 1972; Silberglied & Taylor 1973; Wehner 1981; Kolb & Scholz 1985). Compared with the male whites, this appears to be an alternative strategy to display a colourful pattern. Whereas male whites display wings with a strong contrast between UV and (human) white, male sulphurs have wings with contrasts between UV and blue as well as between blue and yellow. The distinction between the Pierinae and Coliadinae is not sharp, however, as male orange tips, characterized as Pierinae, combine orange markings with UV reflection in their dorsal forewings (*Eroessa cilensis* (Eisner *et al.* 1969) and yellow orange tip *Ixias pyrene* (Eguchi & Meyer-Rochow 1983)).

We conclude that wing colours play a dominant role in sexual recognition in many butterfly species. The visual systems of male butterflies are probably often tuned to discriminate potential sexual partners, as is known for many insect species (Wehner 1981; Zeil 1983; Hardie 1986; Bernard & Remington 1991; Stavenga 1992). In butterflies, the visual system presumably coevolves with the colour contrast in the wings.

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