MARCO ANTONIO GIRALDO

Butterfly wing scales

pigmentation and structural properties

To life.

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Butterfly wing scales

Pigmentation and structural properties

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Chapter 1
Introduction

Colour plays an important role in nature. Evolution has favoured the production of visual organs and, consequently, systems that display colour have appeared. Natural and sexual selection have driven the production of such systems that possess a diversity of physico-chemical properties. For the study of colour in nature, the insect order Lepidoptera is perhaps the most attractive since there we encounter a large diversity of colour producing mechanisms. For the research on butterfly wing colouration presented in this thesis, a number of accessible butterfly species have been investigated. Particularly, species belonging to the family Pieridae constituted a rich source for this research, since their wings possess multiple, both chemical and physical strategies to produce colour.

Interesting optical effects and patterns are present in various butterflies, serving different purposes. To illustrate this, Fig. 1 presents several examples. The pupae of some species have a metallic appearance, either gold or silver, as in *Euploea core* (Fig. 1a). This is achieved with a large number of layers, up to 250 pairs, with different refractive indices, in the endocuticle of the pupa (Steinbrecht *et al.* 1985). The shiny cover thus acts as a mirror, and in that way protects the pupa from predators during the most vulnerable stage of the butterfly. Pretending being part of the foliage, the pupa goes unnoticed.

A quite different optical protection mechanism is the coloration of adult Monarch butterflies. The caterpillar's diet contains a poison that makes the adult unpalatable. A predator with a first bad experience will in a next encounter recognize and avoid the distinctive orange-black pattern of the Monarch (Fig. 1b) (Brower *et al.* 1968). Some other butterflies have "eyes" on their wings. A striking example is the Owl butterfly, *Caligo memnon*. Big circular patterns on its ventral hindwings resemble the eyes of an owl (Fig. 1c). Hesitant predators will think twice before attacking the butterfly, and that precious extra time is what gives to the butterfly the possibility to escape. Another way of colouring butterfly wings is iridescence, a reflection phenomenon, which changes in hue and intensity with angle. This optical effect is especially prominent in *Morpho*

Fig. 1. Use of colour and patterns that butterflies apply. **a** Shiny pupae of *Euploea core*. **b** Male Monarch butterfly, *Danaus plexippus*. **c** Wing eye pattern of the Owl butterfly, *Caligo memnon* (right) compared to an actual owl eye (left). **d** *Morpho didius* photographs with normal (left) and oblique (right) illumination. The silver colour of the pupae in (a) is due to a graded system of multilayers in the cuticle that efficiently reflects light. Due to the mirror effect, the pupae resemble their surroundings, and thus they can go unnoticed. The similarity of the eye pattern of the Owl butterfly and the actual owl eye in (c) is amazing. The wing colour of the majority of *Morphos* is highly dependent on the angle; changing from blue (d, left) to violet, until disappearing (d, right), leaving only the pigmentary brown colour of melanin, located behind the iridescent scales.

butterflies, whose intense blue colour covers a large area of their wings (Fig. 1d). During flight in a sunny day, flashes are produced, which dazzle a persecuting predator. They will also permit recognition of conspecifics at long distances.

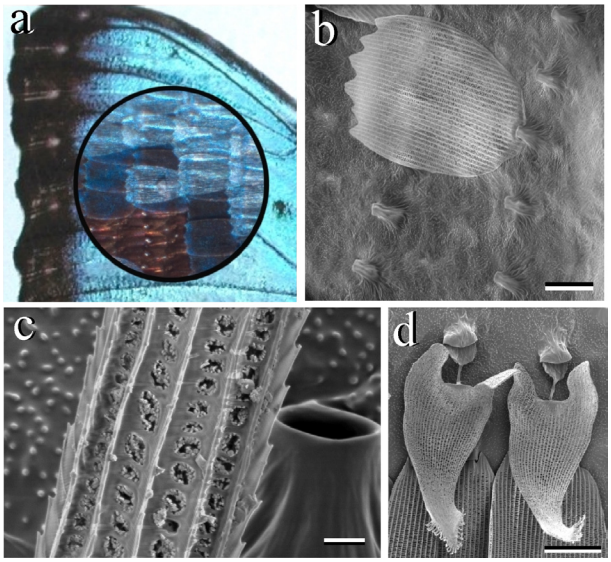

Fig. 2. **a** Photograph of part of a dorsal forewing of *Morpho peleides*. Inserted in a circle is a microphotograph that shows the arrangement of scales on the wing. **b** SEM image of a wing substrate with the arrangement of the sockets and a single scale that was not loose. **c** Bristle and socket of a ventral hindwing of *Pieris brassicae*. **d** Two androconia scales of a dorsal wing of *P. brassicae*. Scales are encrusted in the sockets on the wing substrate. Bars: 20 µm (b, d) and 2 µm (c).

The previous examples show the two forms of producing colour in butterfly wings; pigmentary and structural. To have a better understanding of these two processes it is necessary to analyze the wing anatomy. By observing with a light microscope, we see that a butterfly wing is composed of an aggregation of tiny structures; the scales (Fig. 2a). Electron microscopy allows studying the superficial structure as well as the inner structure of single scales. Scales thus appear to be attached to the wing surface by sockets that are organized in rows (Fig. 2b). Adjacent scales overlap and thus are distinguished as cover and ground scales, usually each with different optical properties (Figs. 2a, b) (Ghiradella 1998; Vukusic *et al.* 1999; Vukusic *et al.* 2000). Other structures are also part of the wing. Hairs and bristles are often much longer than scales and are highly structured too (Fig. 2c). Androconia scales are modified scales present in males, which liberate chemicals that have a role in courtship (Fig. 2d).

Although the elements that compose the scale depend upon the species, we can identify general structures (Fig. 3). Typically, ridges compose the upper layer of the scale, running along its longer axis, which are usually connected by crossribs (inset of Fig. 3a, and Fig. 3b). The ridges are structured, to a certain degree that depends on the species, forming the lamellae (white arrows in Figs. 3b and 3c).

At both sides of the ridges are microribs (black arrow in Fig. 3c), which sometimes specifically scatter light. Pillars, or trabeculae, join the upper and lower laminae of the scale (Fig. 3d, white arrowhead). The lower lamina is smooth, and sometimes producing thin film effects.

Fig. 4 shows transverse sections of the wing of a Brimstone, *Gonepteryx rhamni*. In the scanning electron microscopical image (Fig. 4a), the longer axes of the

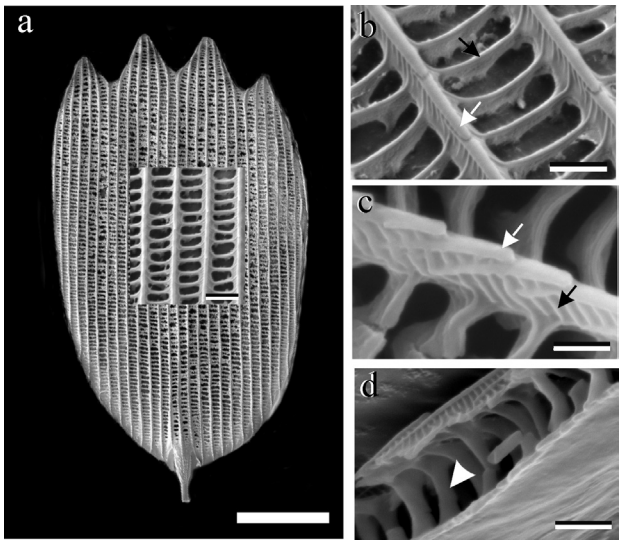

Fig. 3. SEM images of an isolated ground scale of *P. r. rapae*. **a** The scale with an inserted a magnified image of the structure that shows clearly the ridges and crossribs. **(b-d)** General scale structures: **b** Ridges (white arrow), and crossribs (black arrow) on the upper lamina. **c** Lamellae (white arrow) and microribs (black arrow) forming the ridges. **d** Pillars, or trabeculae (white arrowhead), that join the upper and lower laminae. Bars: 20 µm (a), 2 µm (inset), 1 µm (b, d) and 0.5 µm (c).

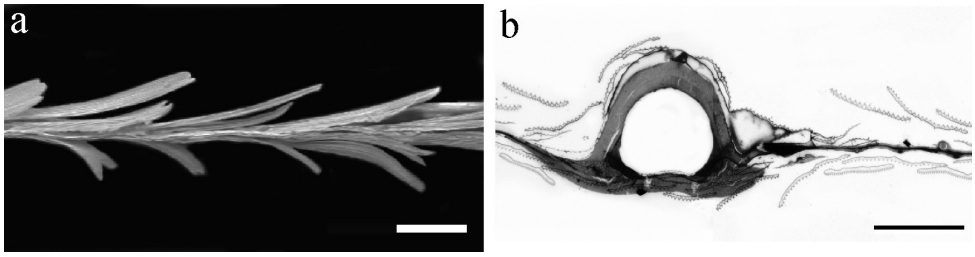

Fig. 4. a SEM image and **b** microtomed light- microscopical image of the side view of the wing of Brimstone. The longer axes of the scales are parallel to the plane of the image in (a) but perpendicular in (b). Cover and ground scales are easily distinguished in both images. The cross section of a vein can be seen in (b). Scales cover also the vein. Bars: 50 µm.

scales are parallel to the plane of the image. The light microscopic section of Fig. 4b is perpendicular to a vein and also to the longer axes of the scales. The scales on the wing thus overlap, and therefore the final colour of a butterfly wing is due to a combination of optical effects of the overlapping scales. Two scales on each side (dorsal and ventral) together with the wing substrate form a system of five reflecting layers, which together can achieve a reflectance of up to 70%. Considering the small thickness of a typical scale (~1 µm), as it is the case for the wings of some *Pierids*, this is a remarkable achievement.

The general structures of Fig. 3 are usually modified in a more or less extended manner in order to produce different optical effects. The lamellae, for example, can become elaborated to produce an interference multilayer system that scatters light coherently; that is, iridesces, as in the *Morpho* scale type (Fig. 1d) (Vukusic *et al.* 2000). Pigments, contained in the cuticular material and absorbing in a certain wavelength range, also colour the wing. As an example, Fig. 5 shows a reflectance spectrum of the tip of the dorsal forewing of a male Purple Tip, *Colotis regina*. The figure also shows diagrammatically the experimental setup. The male Purple Tip is especially interesting because it displays the three basic physico-chemical phenomena that produce the variety of colours in butterfly wings. A white light beam is focused on the sample. The reflected light is collected and transmitted to the spectrometer. A blue reflectance peak, around 500 nm, is due to the constructive interference created by multilayers in the ridges. The pigments contained in the scale beads absorb light in a broad wavelength range: UV-blue-green. The remaining part of the spectrum (red light) is scattered incoherently, thus causing, together with the blue iridescence, purple as the resulting colour.

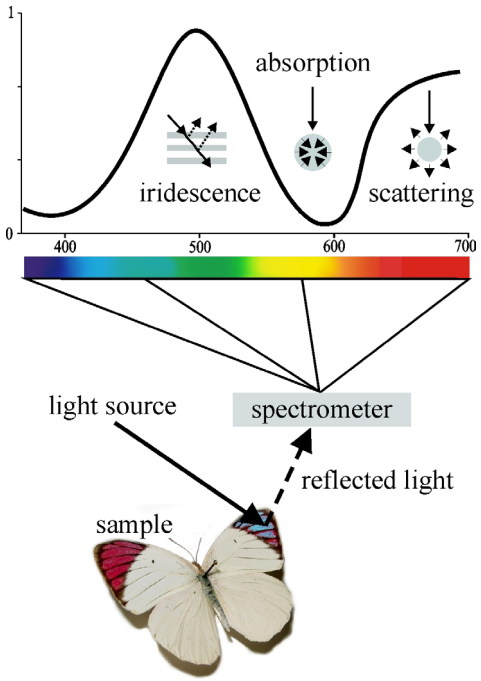

Fig. 5. Diagram explaining the way reflectance spectra are measured using a spectrophotometer, and the three physico-chemical phenomena that produce the variety of colours in butterfly wings. Bright white light is emitted by a light source and focused on the sample. The reflected light is collected and guided to a spectrometer, which splits it in the different wavelengths that compose it and displays a spectrum that shows the relative intensity of each wavelength. For the particular case of *C. regina*, the multilayers produce a peak due to the constructive interference of certain wavelengths (blue); the pigment contained in the scale structures absorbs light in a given range of wavelengths (UV-blue-green) and the rest is scattered incoherently (orange-red).

1.1 Outline

In Chapter 2, the methods used during the research are described. Necessary for spectrophotometric studies, is the ability to manipulate single scales. A procedure to catch single scales is therefore detailed. The equipment and optical setups used for spectrophotometry are also described here. The anatomy of scales has been investigated with electron microscopy; the preparation of the samples is explained.

Chapter 3 presents a recurrent model using the multiple reflection of light in a stack of scales to explain the colour of the scales *in situ*. Denuded and semi-denuded wings were studied using an integrating sphere. Experimental data are compared to theoretical predictions.

Chapter 4 presents a study of the two subspecies of the Small White, *Pieris rapae*. The sexual dichromatism observed in the Japanese subspecies, which is not present in the European one, shines new light on the discussion about the role of pigment granules in the scales of *Pieridae*.

Chapter 5 presents a study of the gradual development of the structure in single scales of pierids that causes also a gradient in optical properties. Artificial gradients have been created by the extraction of pigments with aqueous ammonia.

Spectrophotometric analyses of manipulated overlapping scales show that this gradient has functional consequences for the wing reflectance.

Chapter 6 is a study of the spatial distribution of the light reflected by five different types of scales. It explores the optical and structural properties of single scales, which combine absorbing pigments with coherent and/or incoherent scattering. The assumption of Chapters 3 and 4, which states that white beaded scales of pierids behave as an almost perfect diffusing reflector, is positively evaluated.

In Chapter 7 a special case of wing coloration is described. The optical properties and anatomy of the silvery scales on the ventral wings of the Silverspot, *Dione juno*, were studied. The light reflected and transmitted by single scales produces in the far field a linear pattern perpendicular to the direction of the ridges. The transmitted light shows a strong diffraction pattern. The optics of intact silver areas was also studied. The pattern broadens and becomes white due to the superposition of single scale scattering patterns.

Chapter 2
Methods used to study butterfly wings and wing scales

The methods and systems used during the present investigation are described. An innovative method to catch single scales with a microelectrode-like glass micropipette is presented. The equipment and techniques used to study the optical properties of butterfly wings as well as that of single wing scales are described. The back- and forward-scattering of intact and denuded wings were measured with an integrating sphere. For single scale analysis, a microspectrophotometer was used to study the transmitted as well as reflected light. Furthermore, a special optical set-up for studying the angular distribution of reflected and transmitted light by single scales is described. The anatomical structure of butterfly wing scales was studied with scanning and transmission electron microscopy.

2.1 Catching scales

Measuring the optical properties of single butterfly scales requires the possibility to accurately position the sample in the optical set-ups. We therefore developed a method to catch single scales, which includes the following steps:

1. *Isolation of scales from the wing substrate.* As described in Chapter 1, butterfly wing scales are sustained from their base to the remaining cell in the wing substrate. To separate the scales, the wing is gently pressed upon a glass microscope slide.
2. *Production of micropipettes.* Using a microelectrode puller, a glass tube is heated in its centre and pulled apart, which yields two usable micropipettes. The puller variables (heat, velocity, pull strength) have been set so that a long tip with a diameter of approximately 5 µm results. One micropipette is put in a micromanipulator and its tip smeared with glue (not fast drying).

3. *Positioning of micropipette and glass slide under the stereoscope.* The glass slide with scales is placed obliquely under the stereoscope (Fig. 1a) and the pipette is moved slowly, approaching the scales (Fig. 1b).
4. *Selection of scale suitable to catch.* Most of the times it is useful to have the scale with its ridges either perpendicular or parallel to the pipette. A scale that approximates one of the two ideal situations is selected (Fig. 1c). The scale is approached until it sticks to the pipette with glue, so that it can be removed from the slide.
5. *Use of a second pipette to manipulate the scale.* Even though the best scale has been selected, it often needs adjustment. This is done with the aid of a second (clean) pipette, which is kept vertical and stable.

The final result is a single scale (or two, as in Fig. 1d) conveniently sustained by a very thin holder that can be translated and rotated. Different types of holders were used (e.g. polished needles and eyelashes), but a microelectrode-like glass pipette showed the best results, because a very thin tip, which is still flexible and stable, is easy to produce. Furthermore, the electrostatic charge created by touching the tip lightly with the fingers is very helpful during the preparation of samples for scanning electron microscopy (Section 2.3).

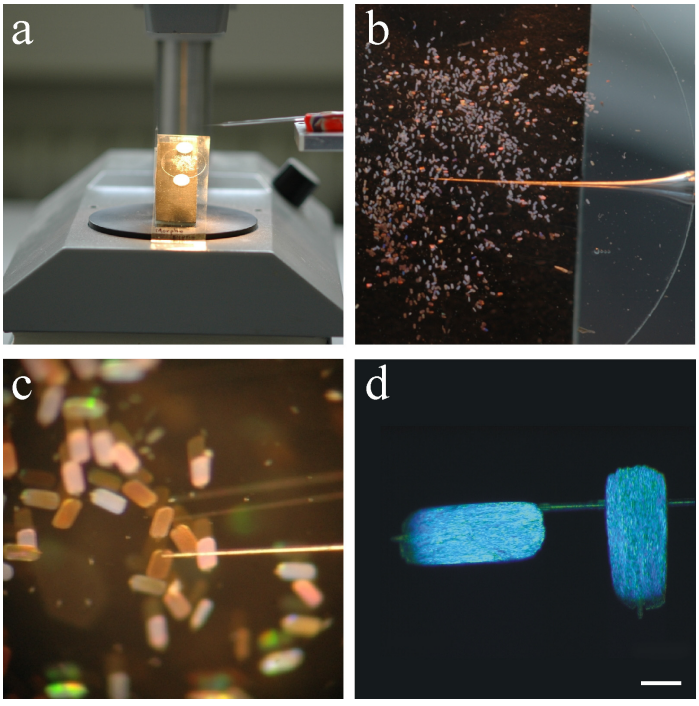

Fig. 1. Sequence of the procedure to catch single scales. **a** The micropipette and the glass cover are positioned under the stereoscope. **b** The micropipette's tip, smeared with the glue, approaches the selected scale. **c** The selected scale(s) is (are) caught and positioned in the desired manner with the second perpendicular pipette. **d** Scales are ready to use. Bar: 50 µm.

The scales in Fig. 1d are conveniently placed in such a way that rotation of the pipette results in rotation around the longer and the shorter axis of the scale, respectively. Those directions are convenient in optical measurements, because the ridges, which run along the longer axis, frequently cause diffraction of light.

This can result in a linear pattern of the reflected light (e.g. *Morpho aega*) or in a transmitted diffraction pattern (e.g. *Dione juno*). In order to study these optical effects, an accurate adjustment method is necessary.

2.2 Spectrophotometry

Spectrophotometric measurements are performed on a given sample in order to know how it interacts with light. To study the spectral properties of single scales, a microspectrophotometer was used. For larger samples (a few millimetres of diameter) a fibre optic connected to a probe was used. Most of the samples do not behave as good diffusers and thus light is partially specularly reflected. To collect all the light reflected (back-scattered) or transmitted (forward-scattered) an integrating sphere can be used. To know precisely the angular distribution of the scattered light, an optical setup which consists of aligned lenses and a goniometer was used. In the following, this equipment is briefly described.

2.2.1 The microspectrophotometer

A microspectrophotometer (MSP) is fundamentally a microscope combined with a spectrophotometer. Its advantage is that microscopical areas can be accurately

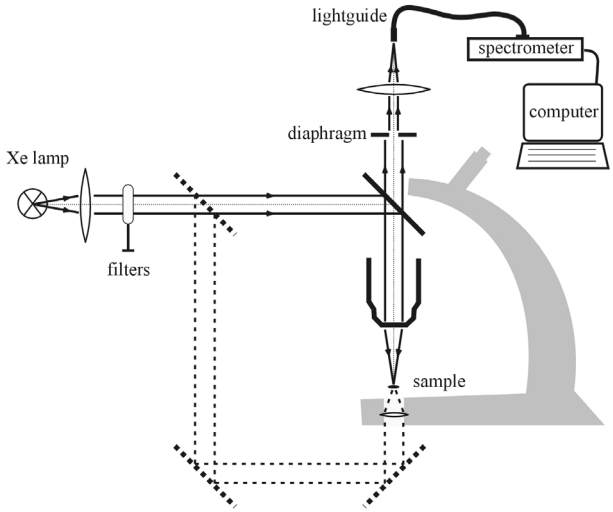

Fig. 2. Microspectrophotometer diagram. The light is emitted by a Xenon lamp and passes through a diaphragm and selected filters. The beam is then focused on the sample by a microscope objective (reflectance mode) or a lens after being redirected by a system of mirrors (transmittance mode). The reflected (or transmitted) light is then collected by the objective and the part that comes from the area to analyze is selected by a diaphragm and focused on a fibre optic connected to a spectrophotometer, which in turn stores the data in a computer.

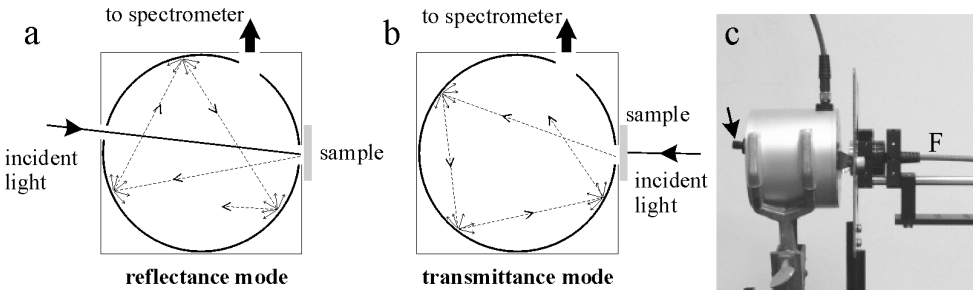

Fig. 3. Integrating sphere. Diagram of **a** reflectance and **b** transmittance modes. **c** Photograph of the integrating sphere, set in transmittance mode. The incident light is focused on the sample with a microlens. After multiple scattering on the inner surface of the sphere, the reflected (transmitted) light is collected with a fibre optic and conducted to the spectrometer. The arrow in (c) shows the socket where the fibre, F, is screwed on for measurements in reflectance mode.

selected. Furthermore, transmittance as well as reflectance by epi-illumination can be studied. The latter means that incident light as well as reflected light pass through the objective of the microscope. Fig. 2 shows a diagram of the MSP. Light emitted from a broad band Xenon lamp is collected by a lens and filtered if necessary. A half-mirror deviates the beam, which then passes through the objective to be focused on the sample. After the area of interest has been selected by a diaphragm, the reflected light is collected by the objective and focused on a fibre optic. Transmitted light can also be studied by redirecting the light with mirrors and focusing it on the sample (dashed lines in Fig. 2). The path is then the same as for the reflected light.

2.2.2 The integrating sphere

Independently of their characteristics, scattered light can be effectively collected by using this equipment. It consists of a sphere, the inner surface of which is coated with a highly diffusing reflective material (e.g. spectralon). Three small holes exist for placement of the sample and two fibre optics; one for the incident light and one for the collector (Fig. 3a). Transmitted light can also be measured by changing the incident light fibre to the position indicated in Fig. 3b. The integrating sphere is particularly useful for large samples. Single scales, however, require a very long measuring time.

2.2.3 The angular-distribution setup

The angular distribution of the reflected (and transmitted) light reveals the diffuser and specular characteristics of butterfly wing scales. Experiments can be performed for single scales as well as for scales *in situ*. By using this setup, reflectance and transmittance spectra in a plane over a 360° angle can be measured.

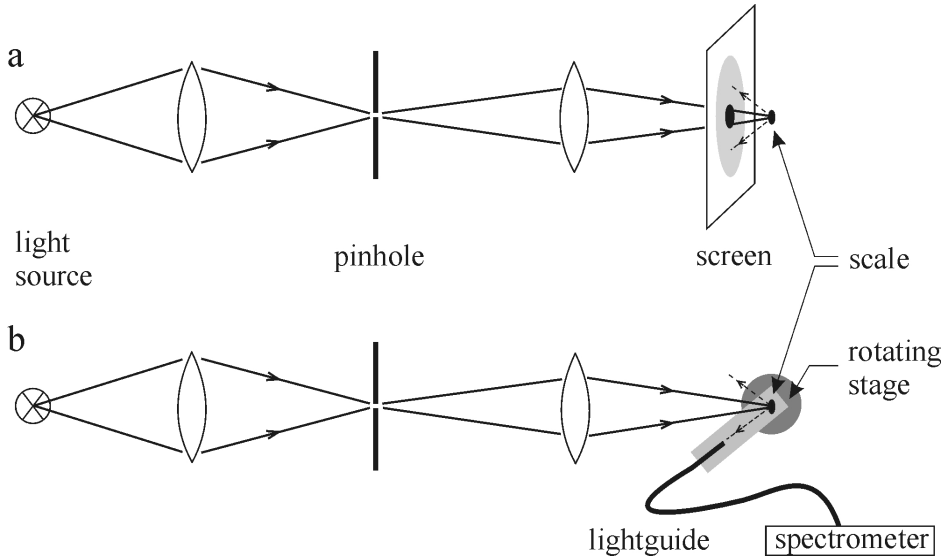

Fig. 4 Diagram of the optical system used for measuring the angular distribution of the scale reflectance. Light from a light source is focused on a pinhole, which is imaged on the scale. **a** A white screen with a small hole is placed in between the imaging lens and the scale. The light reflected by the scale causes a light pattern on the screen, which is photographed. **b** The light reflected by the scale is collected by a lightguide, which relays the captured light to a spectrometer. The lightguide is mounted on a stage rotating in the horizontal plane (top view drawing).

Fig. 4 shows a diagram of the angular-distribution setup. Light from a light source is focused on a very small pinhole (typically 30 μm) and imaged on the scale. In order to know the spatial distribution of the light reflected by the scale, a white screen with a small hole (to let the incident beam pass through) is placed between the imaging lens and the scale (Fig. 4a). For transmitted light, a semitransparent paper is placed behind the scale. Light is reflected (transmitted) by the scale on the screen and its pattern can be photographed. To measure the reflectance spectra as a function of angle, the reflected light is collected with a fibre optic, which is mounted on a rotating stage and then is relayed to a spectrometer.

2.3 Scanning electron microscopy

For scanning electron microscopy (SEM) of butterfly wing scales, small pieces of wing were cut and placed on a conductive carbon-made sticker, on a SEM holder. Before putting the samples inside the microscope's chamber, they were sputtered with palladium, to minimize the charging and consequently improve the quality of the images. The layer of palladium had a thickness of a few nanometer that did

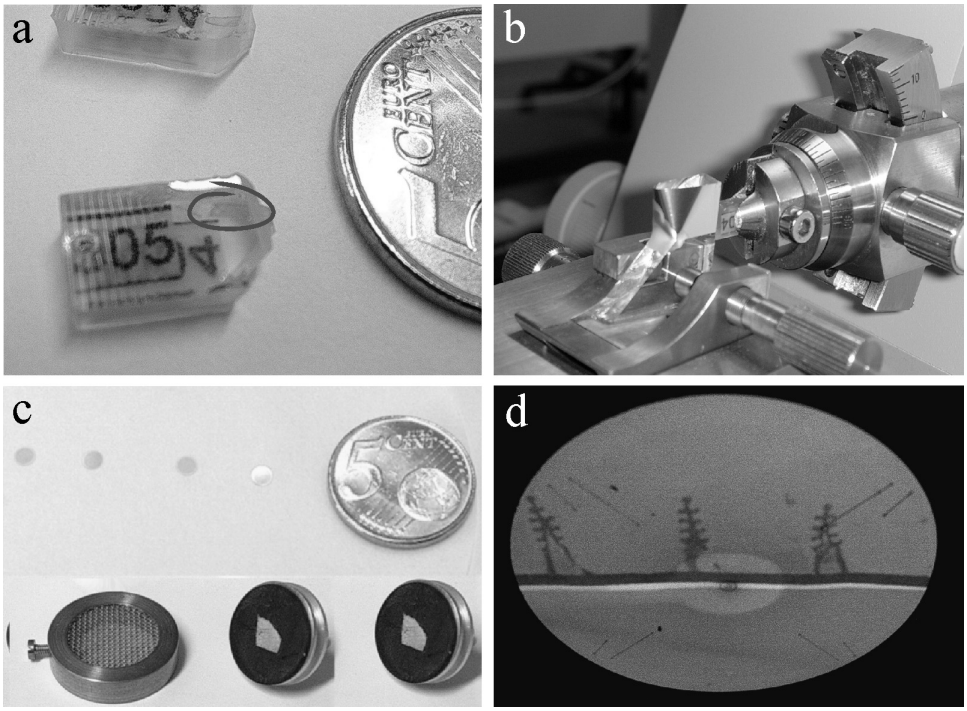

Fig. 5. a Wing pieces embedded in Epon ready for the microtome. **b** Microtome cutting a thin layer of the sample by using a glass knife. **c** Sample holders for TEM (up) and SEM (down). **d** Screen at the bottom of the TE microscope, showing the light transmitted through the microtomed sample of a M*orpho peleides* cover scale.

not perturb the much larger sized butterfly structures, which range from hundred nanometer to a few micrometer. The voltage used in the microscope for biological tissues (2-12 kV) is much lower than that for metallic and solid samples (30-60 kV). Large voltages will cause contraction of the structures and deformation. It is preferable to use a low voltage of 3 kV and a small size spot, although higher voltages usually give better resolution.

The way of cutting and positioning the samples is also important when exploring the inner structure of the scales by SEM. Making an image of the cross section of a scale will require cutting the scale, perpendicular to the ridges, by using a razor blade or eye surgical scissors. By putting the sample almost vertically on the holder and using the tilting stage of the microscope, a good image can be achieved, as long as there is good support for the scale. When the scale is still attached to its socket on the wing, the electron beam momentum usually moves it during the slow scanning period necessary for making an image. A good solution is to glue single, isolated scales to the sticker on the holder. The method described above for attaching single scales to the glass pipette can be used for that; but

instead of glue, electrostatic forces will help to keep the scale attached to the pipette while positioning on the SEM holder. A ridge in the carbon sticker, made previously with a razor blade, can be useful to introduce an enhanced tilt, so that better images of cross sections can be obtained.

2.4 Transmission electron microscopy

For transmission electron microscopy (TEM) of butterfly wing scales, the samples were processed and embedded in Epon, using standard protocols.

After embedding, sections ~ 80 nm thick are cut with an ultramicrotome (Fig. 4b) and put on copper grids previously covered with paraffin. The contrast of the samples for the microscope is made with uranyl acetate in methanol during 2 min and lead in water during 1 min. The preparation of the samples takes about a week but the procedure to make the photos in the microscope is rather simple.

Chapter 3
Reflectance and transmittance of light scattering scales stacked on the wings of pierid butterflies

The colours of butterfly wings are determined by the structural as well as pigmentary properties of the wing scales. Reflectance spectra of the wings of a number of pierid butterfly species, specifically the Small White, *Pieris rapae*, show that the long-wavelength reflectance of the scales in situ, on the wing, is distinctly higher than that of single, isolated scales. An optical model explains that this is due to multiple scattering on overlapping scales by treating the layers of scales on both sides of the wing as a stack of incoherently scattering plates. The model sheds new light on the adaptive significance and evolution of butterfly wing patterns.

3.1 Introduction

Butterflies are generally strikingly coloured, due to light reflected by the wing scales, which are arranged on the wing surface like shingles on a roof. The reflected light commonly results from scattering on the scale structures, the prominent longitudinal ridges and the connecting crossribs. A distinct colour is observed when the scales contain a pigment that absorbs part of the scattered light in a restricted spectral band. Butterflies thus combine physical and chemical colouration methods. The scattered light usually is random and spectrally indistinct, but interference can create striking, intense iridescent colours, as occurs in the multilayers in the scales of the blue *Morpho* and other nymphalid butterflies (Nijhout 1991; Brink & Lee 1999; Vukusic & Sambles 2003; Kinoshita & Yoshioka 2005). Similar multilayers in the wing scales of male sulphurs cause a strong UV reflectance (Ghiradella *et al.* 1972; Silberglied & Taylor 1973;

Rutowski 1977). Photonic crystals in the scales of lycaenids (blues and hairstreaks) result in a blue or green colour (Biro *et al.* 2003).

Many pierids apply a specific method of structural colouration in order to achieve brightly-coloured wings. The scales are studded with beads (Yagi 1954; Ghiradella 1998) that act as strong scatterers, which enhance the reflectance (Stavenga *et al.* 2004; Rutowski *et al.* 2005). The wing scales of the yellow sulphurs (Coliadinae) contain pigments that absorb in the ultraviolet as well as blue wavelength ranges. The distinct yellow wing colour thus is due to the combined effect of strong scattering in the long wavelength range and strong absorption at the shorter wavelengths. The scales of male whites (Pierinae) contain an ultraviolet-absorbing pigment, causing a low UV-reflectance, which is well recognized by butterflies, but not by humans (Obara 1970; Obara & Majerus 2000).

The scales on each side of the wing are usually arranged in two layers, a layer of ground scales and an overlapping layer of cover scales. In order to clarify the optical consequences of the scale layering and their biological significance, we have studied the optics of the butterfly wing scale assembly by measuring reflectance and transmittance spectra of intact wings, of partially denuded wings, and of single, isolated scales of pierid butterflies, and we have analyzed the results with a simple model that treats the scales on the wing as a stack of layers, assuming random scattering. We conclude that pierid butterflies realize their bright wing colourations with stacks of strongly scattering scales, which contain short wavelength absorbing pigments.

3.2 Materials and methods

Animals

All investigated butterflies belong to the family Pieridae. The Small White, *Pieris rapae*, was obtained from a culture maintained by Dr J.J. van Loon, Entomology department of the Agricultural University in Wageningen, the Netherlands. The Autumn Leaf Vagrant, *Eronia leda*, was studied in the butterfly collection of the latter department and in the Royal Museum of Central Africa, Brussels (curator Dr U. Dall'Asta). The Common Jezabel, *Delias nigrina*, was obtained from Dr M. Braby, Australian National University, Canberra.

Anatomy

The wing anatomy was investigated with standard scanning-electron-microscopical methods.

Spectrophotometry

Reflectance spectra of intact wings were measured with a reflection probe connected to a fibre optic spectrometer (SD2000, Avantes, Eerbeek, the Netherlands). Reflectance and transmittance spectra of intact as well as denuded wings were measured with an integrating sphere (AvaSphere-50-Refl), connected to the fibre optic spectrometer. Spectra of single scales were measured with a home-built microspectrophotometer consisting of a Leitz Ortholux microscope and the fibre optic spectrometer. A white reflectance standard (Spectralon, Labsphere, North Sutton, NH, USA) served as a reference.

Modelling

The optical model that describes light propagation in a stack of layers assumes non-coherent scattering. Each layer is characterized by reflectances r and s, and transmittances t and u, depending on the direction of the incident light (Fig. 1). The reflected and transmitted light fluxes in a two-layer stack with incident light flux I_1 from one side and I_6 from the opposite side are then described by the following set of equations (Fig. 1a):

$$I_2 = r_1 I_1 + u_1 I_4; \quad I_3 = t_1 I_1 + s_1 I_4;$$
$$I_4 = r_2 I_3 + u_2 I_6; \quad I_5 = t_2 I_3 + s_2 I_6 \qquad (1)$$

When the incident light flux is only from medium 1, then $I_6 = 0$, or $I_4 = r_2 I_3$, so that $I_3 = t_1 I_1 /(1 - s_1 r_2)$ and $I_4 = t_1 r_2 I_1 /(1 - s_1 r_2)$. The transmittance, $T = I_5/I_1$, and reflectance, $R = I_2/I_1$, of the two layer stack then are:

$$T = \frac{t_2 t_1}{1 - s_1 r_2} \quad \text{and} \quad R = r_1 + \frac{t_1 u_1 r_2}{1 - s_1 r_2}. \qquad (2)$$

A stack consists in general of n layers. Equation (1) (where $n = 2$) can be generalized as:

$$\begin{bmatrix} I_{2i} \\ I_{2i+1} \end{bmatrix} = A_i \begin{bmatrix} I_{2i-1} \\ I_{2i+2} \end{bmatrix}, \quad \text{with} \quad A_i = \begin{bmatrix} r_i & u_i \\ t_i & s_i \end{bmatrix}, \quad \text{for} \quad i = 1...n, \qquad (3)$$

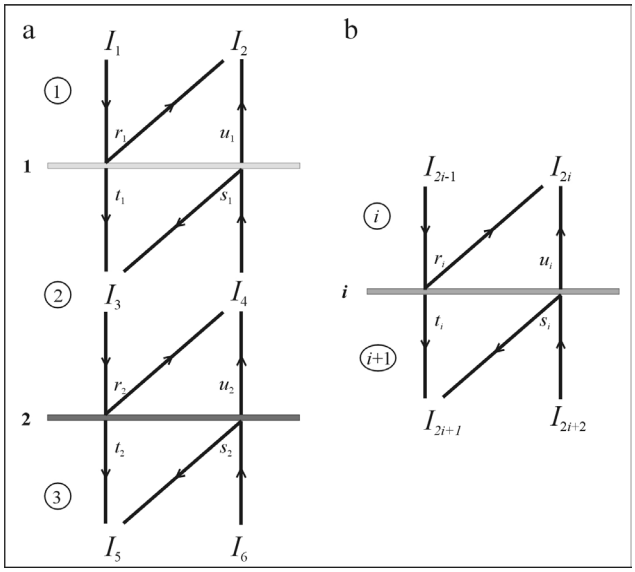

Fig. 1. Diagrams of the light flux in a pile of plates. **a** Two rough layers, 1 and 2, each with reflectances r and s and transmittances t and u, separate three media, 1 – 3. **b** Layer i of a stack of n layers separates media i and i+1.

where layer i is characterized by matrix A_i (Fig. 1b). By introducing $\tau_i = I_{2i+1} / I_{2i-1}$ (for $i = 1 \ldots n$) and $\rho_i = I_{2i} / I_{2i-1}$ (for $i = 1 \ldots n + 1$), Eq. (3) is equivalent to:

$$\tau_i = \frac{t_i}{1 - s_i \rho_{i+1}} \quad \text{and} \quad \rho_i = r_i + u_i \rho_{i+1} \tau_i = r_i + \frac{t_i u_i \rho_{i+1}}{1 - s_i \rho_{i+1}} . \qquad (4)$$

With only incident light I_1, $I_{2n+2} = 0$ or $\rho_{n+1} = 0$, which allows calculation of the values of ρ_i and τ_i by recursion. Starting from $i = n$, it follows that $\tau_n = t_n$, $\rho_n = r_n$, $\tau_{n-1} = t_{n-1} / (1 - s_{n-1} \rho_n)$, etc. The multilayer transmittance and reflectance are thus directly derived from:

$$T = \prod_{i=1}^{n} \tau_i \quad \text{and} \quad R = \rho_1 . \qquad (5)$$

It is easy to see that Eq. (5) is identical to Eq. (2) for $n = 2$.

3.3 Results

3.3.1 Reflectance spectra of intact wings of two pierids

The male Autumn Leaf Vagrant, *Eronia leda*, provides a characteristic example of the colouration of pierid butterflies (Fig. 2). The dorsal forewings are marked by a prominent orange tip, which highly reflects ultraviolet light (Fig. 2, location

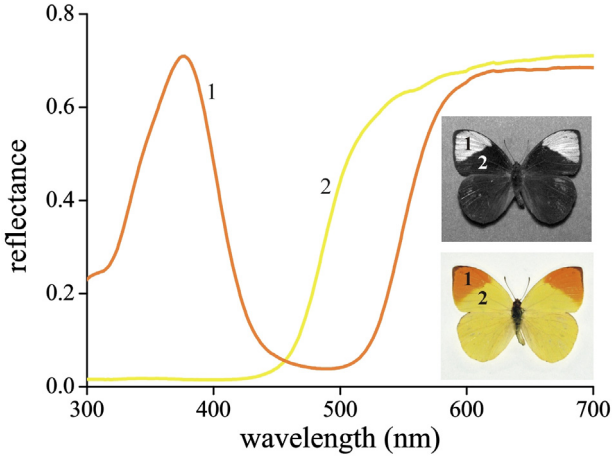

Fig. 2. Reflectance spectra of the dorsal forewing of the male Autumn Leaf Vagrant, *Eronia leda* (inset: upper – UV, lower – RGB), measured with a fibre optic spectrometer. Spectrum 1 is from the orange tip, which exhibits a pronounced UV band, due to interference reflectors in the scale ridges. The orange colour results from scattered light, filtered by a pigment absorbing in the UV, blue and green wavelength range. Spectrum 2 is from the dorsal forewing area outside the orange tip. The yellow colour results from scattered light filtered by a pigment absorbing in the UV and blue wavelength range.

and spectrum 1). The remaining parts of the dorsal forewings and hindwings have yellow colour (Fig. 2, location and spectrum 2). The orange as well as yellow colours are due to pigments that selectively absorb short-wavelength light scattered at the scale structures, the ridges, crossribs as well as the beads that adorn the scales (Ghiradella 1998; Stavenga *et al.* 2004; Rutowski *et al.* 2005). The high reflectance of the tips in the UV results from iridescence in the ridge lamellae (Ghiradella *et al.* 1972; Silberglied & Taylor 1973; Rutowski 1977). The ventral wings of the male *Eronia leda* are overall yellow, except for a minute UV-reflecting spot in the ventral forewing. *E. leda* has a distinct sexual dichromatism, as the female has pale yellow dorsal and ventral wings and only ventrally a few scattered UV-reflecting spots.

The dorsal wings of the male Common Jezabel, *Delias nigrina*, have a simple colour pattern (Fig. 3). The tips of the forewings are black, characteristic of a melanin pigment, and they lack iridescence. The remaining parts of the dorsal forewings and hindwings are white, due to strongly scattering scales. The reflectance is low in the ultraviolet, due to a pigment that absorbs exclusively in the UV (Fig. 3, spectra and locations 1-3). The ventral forewings and hindwings are marked by yellow and red bands (spectra and locations 4 and 5) in a mainly brown-black background (spectrum and location 6). Interestingly, the red bands can be seen at the dorsal side as a slight red sheen, which is reflected in the increase in reflectance in the wavelength range above 560 nm (spectra and locations 1 and 3), corresponding to the wavelength range where the reflectance of the red bands increases (spectrum and location 5).

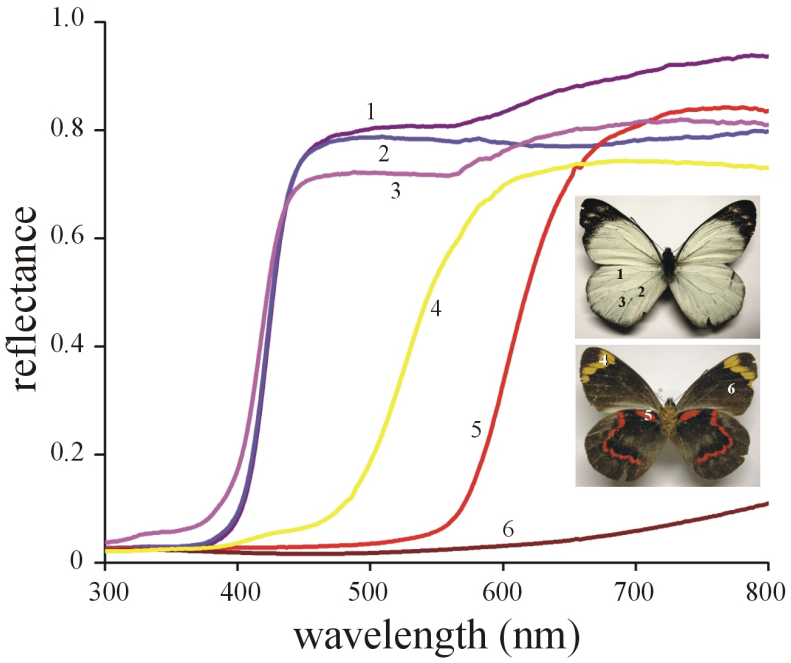

Fig. 3. Reflectance spectra of various parts of the wings of the male Common Jezabel, *Delias nigrina* (inset: upper – dorsal, and lower – ventral), measured with a fibre optic spectrometer. The reflectance of white areas is high and constant in the visible region (2). The low reflectance below 400 nm demonstrates that the white scales contain a UV absorbing pigment. The red sheen in 1 and 3 is due to bands of red scales at the ventral hindwing (5), which contain pterin pigments absorbing throughout the visible wavelength region, except the red. The yellow bands in the ventral forewing (4) contain UV and blue absorbing pterins. Spectrum 4 has a small foot between 400 and 470 nm due to the contribution of white scales that occur in white spots at the dorsal side. The ventral wings are mainly covered by brown-black scales, due to strongly absorbing melanin pigment (6).

This observation demonstrates that the ventral scales contribute to the dorsal wing reflectance. The opposite also holds, namely the white spots in the tip of the dorsal scales contribute to the ventral wing reflectance, as can be seen from the slight foot at wavelengths above 400 nm in spectrum 4 of Fig. 3. The scales in the white spots at the dorsal side add to the reflectance of the scales in the yellow bands.

3.3.2 Reflectance and transmittance spectra of intact and denuded wings of the Small White, *Pieris rapae*

We have attempted to assess the relative contributions of the scales to the reflectance on both wing sides by considering the butterfly wing as a stack of plates (Fig. 4), using the formalism described in the Methods. The reflectance was

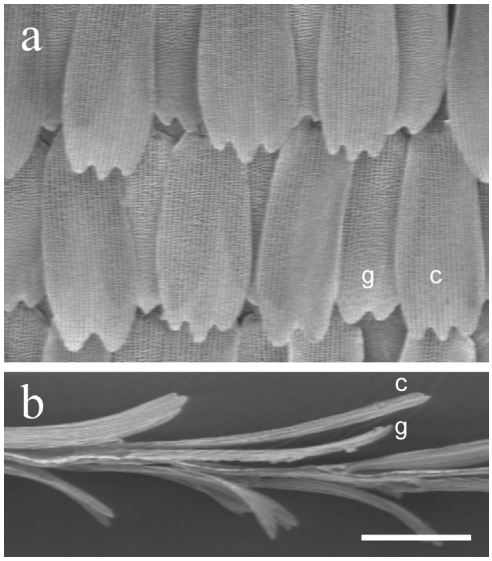

Fig. 4. Scanning electron microscopy of wings of the Small White *Pieris rapae*. **a** Rows of cover (c) and ground (g) scales are stacked on the wing substrate. **b** Side view of a cut wing, showing cover and ground scales on both dorsal and ventral sides of the wing. Bar: 50 µm.

measured with an integrating sphere from both sides of intact wings (DWV, VWD; Fig. 5a), from wings with scales removed on one side (DW, WD, WV, VW; Fig. 5a, b) or from fully denuded wings (Wd, Wv; Fig. 5b). The reflectance of the wings with scales is low in the UV and high at wavelengths above 450 nm. The peak reflectance, around 500 nm, is about 0.6 for the intact wing, but the reflectance of the scaleless wing is about 0.1, virtually independent of wavelength (Fig. 5b). The overall spectral dependence of the transmittance, $T(\lambda)$, resembles that of the reflectance, $R(\lambda)$; the transmittance is low in the UV and high in the visible wavelength range (Fig. 5c, d).

Accordingly the absorptance, $A(\lambda) = 1 - R(\lambda) - T(\lambda)$, is high in the ultraviolet and negligible at the visible wavelengths (Fig. 5e, f). The scaleless wing absorbs only slightly in the ultraviolet (Fig. 5f). The absorptance spectra of denuded wing and wings with scales differ in shape, indicating that the pigments in wing substrate and scales differ.

The wing covered by scales can be considered as a stack of layers, each with a specific reflectance and transmittance, depending on the direction of the incident light. The intact wing thus is a three layer stack, consisting of a layer of (partly overlapping) scales at the dorsal side, D, the wing substrate, W, and the layer of scales at the ventral side of the wing, V (Fig. 5). The reflectances r and s and the transmittances t and u (Fig. 1) can be calculated for each of the three layers with the formalism of the Methods. The wing reflectances r_W and s_W (Fig. 5b) as well as the transmittances t_W and u_W (Fig. 5d) were measured directly, and further spectral data were obtained for various two layer combinations, DW, WD, VW, or VW, where only one layer of scales was present.

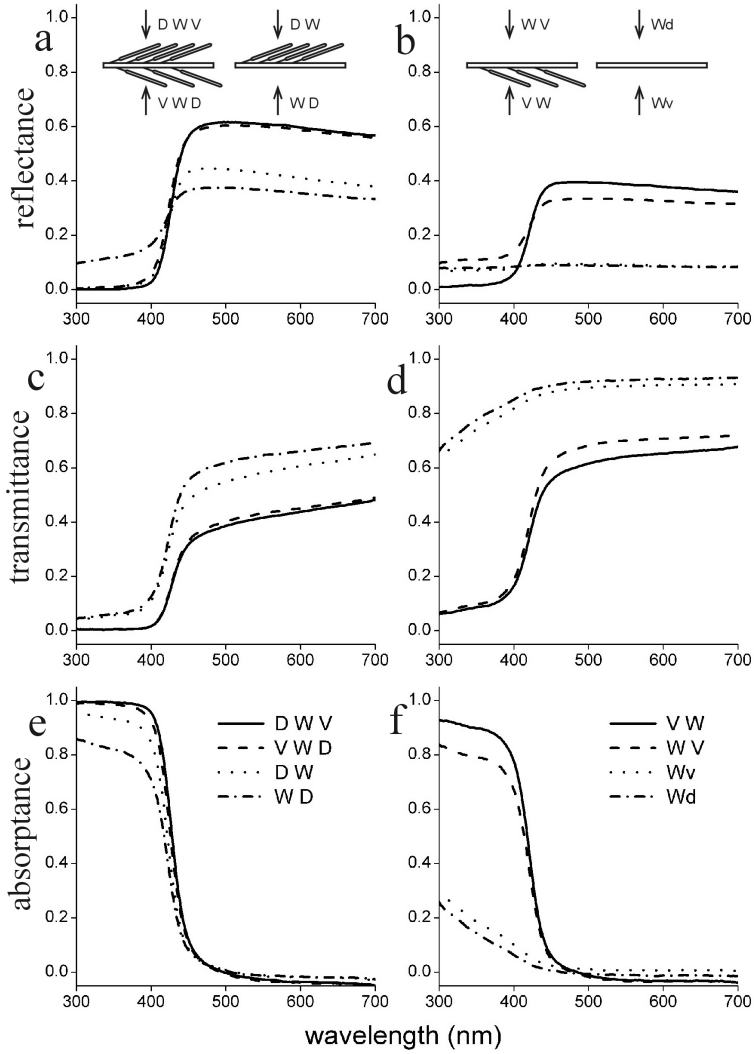

Fig. 5. Reflectance (**a**, **b**) and transmittance (**c**, **d**) spectra measured from wings of the Small White butterfly, *Pieris rapae*, in various conditions, together with the resulting absorptance spectra (**e**, **f**). The measurements were performed with an integrating sphere. The wing was intact for the conditions DWV and VWD, where D indicates the dorsal side of the wing, W is the wing substrate, and V is the ventral side. The order of the letters indicates the direction of the incident light. For DW and WD, the wing scales were removed at the ventral side, and for VW and WV, the wing scales were removed at the dorsal side. For Wv and Wd, both dorsal and ventral scales were removed, and the incident light came from the ventral (v) and dorsal side (d), respectively. The scales contain a strongly UV absorbing pigment, resulting in a very low transmittance, or, a very high absorptance, in the ultraviolet. The wing scales strongly scatter in the visible wavelength range. The wing reflectance is virtually constant throughout the whole spectral range, with amplitude about 0.11 (b), and the wing substrate contains a small amount of pigment that absorbs in the UV (f).

The results for the dorsal scales are given in Fig. 6a and those for the ventral scales in Fig. 6b. The absorptance for the D-scales with illumination from the dorsal (d) side was calculated from $a_{Dd} = 1 - r_D - t_D$, and the absorptance with illumination from the ventral (v) side from $a_{Dv} = 1 - s_D - u_D$ (Fig. 6a). Similar expressions hold for the V-scales (Fig. 6b).

Knowing the spectral data for all three layers (D, W, and V) allows calculation of the reflectance and transmittance spectra of the intact wing, for example the three layer stack DWV. This produced the calculated reflectance $R_c = R_{DWV}$ and transmittance $T_c = T_{DWV}$ (Fig. 6c). The calculated spectra have a similar shape as the measured spectra, R_m and T_m (Fig. 5a, c), but the calculated reflectance is too small, and the calculated transmittance is too high (Fig. 6c). The corresponding absorptances, A_m and A_c, calculated from $A_{m,c} = 1 - R_{m,c} - T_{m,c}$, closely agree.

3.3.3 Reflectance and transmittance spectra of single scales

Butterfly scales are more or less flat structures (Fig. 4), but they are quite asymmetrical. The face toward the side of the wing (adwing) is rather smooth, but the opposite side (abwing) is highly structured (inset Fig. 7b). We have measured the reflectance of single scales, glued to the tip of a thin glass rod, with a microspectrophotometer (Fig. 7a, b). We investigated both faces of ground and cover scales from both the dorsal and ventral side of the scale. The reflectances of both scale faces slightly differ, and the reflectance spectrum also depends

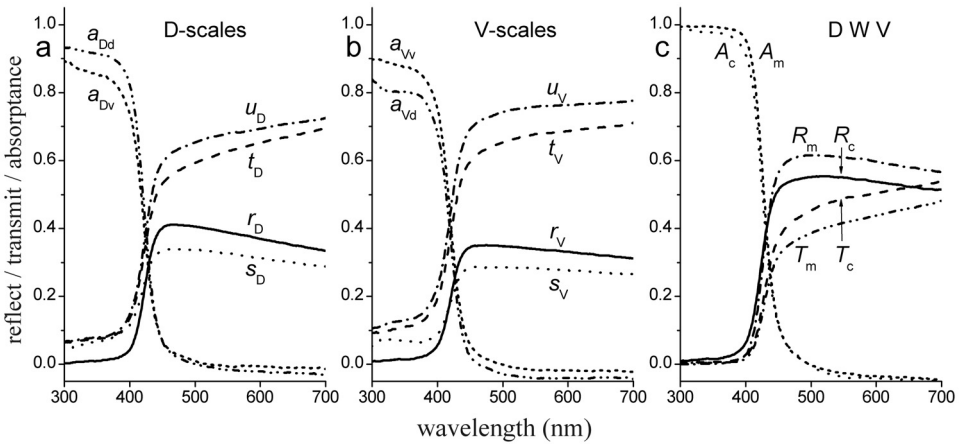

Fig. 6. Calculations of the reflectance, transmittance and absorptance of the dorsal (**a**) and ventral (**b**) scale layers, using the data of Fig. 5 and the formalism described in the Methods. **c** The reflectance and transmittance spectra measured from the intact wing (Fig. 5a and 5c, DWV), R_m and T_m, are compared with the calculated spectra, R_c and T_c. The calculated reflectance (transmittance) is somewhat smaller (larger) than the measured reflectance (transmittance). The absorptances are calculated with $A_{m,c} = 1 - R_{m,c} - T_{m,c}$.

somewhat on the type of scale. The peak reflectance is roughly 0.3 in both cases, at about 500 nm, but the reflectance in the UV is lower for the scale surface not facing the wing (Fig. 7a).

The reflectance clearly depends on the scattering on the scale structures as well as on the ultraviolet-absorbing pigment. To assess the amount of absorption in a single scale, we measured the transmittance of various scales embedded in xylene, an immersion fluid with a refractive index approximating that of insect cuticle (Stavenga *et al.* 2004) (Fig. 7c). Fig. 7c also contains a transmittance spectrum calculated from the absorptance spectra of Figs. 5 and 6. The calculation was performed as follows. It was assumed that the absorption process in the layers can be approximated with Lambert-Beer's law. The absorbance then is $D(\lambda) = -\log_{10}[1 - A(\lambda)]$.

The absorptance spectra of Figs. 5e and 5f thus yielded absorbance spectra, which after normalization appeared to be very similar. The mean of the normalized absorbance spectra was therefore used to calculate a transmittance spectrum (Fig. 7c, bold curve, LB) with an amplitude in the ultraviolet of 0.15, about equal to the transmittance in the UV of the immersed scales. The calculated transmittance spectrum deviates from the measured transmittance spectra. Presumably xylene does not fully annihilate the difference in refractive index of the scale structures with that of the surrounding medium, which can be understood, because probably the refractive index of pierid scales is not constant. The transmittance then remains affected by scattering. Another reason for the deviation may be the limited applicability of the model, which assumes perfectly diffuse light (see Chapter 6).

3.3.4 Reflectance spectra of a stack of scales

We have used the model for a stack of reflecting and absorbing plates (Section 3.2) to calculate the reflectance of a multilayer of butterfly wing scales on the wing. The measurements of Fig. 7 demonstrate that the reflectance slightly depends on the type of scale (cover or ground). The reflectance also depends on which side of the scales faces the incident beam. We nevertheless assumed a stack of identical scales, with abwing (r) and adwing (s) reflectances equal to the mean of the measured spectra (bold curves of Figs. 7a and 7b, respectively). The scale transmittances t and u (Fig. 1) were calculated from $t = 1 - r - a$ and $u = 1 - s - a$, where the absorptance is $a = 1 - t_{LB}$, with t_{LB} the transmittance given by the bold curve in Fig. 7c.

The resulting reflectance spectra of various stacks of scales on the wing are given by Fig. 8. The reflectances of both sides of the denuded wing were assumed to be equal to the average (W) of the two wing reflectance spectra given in Fig. 5b. Spectrum SW of Fig. 8 presents the reflectance spectrum of a wing with one layer

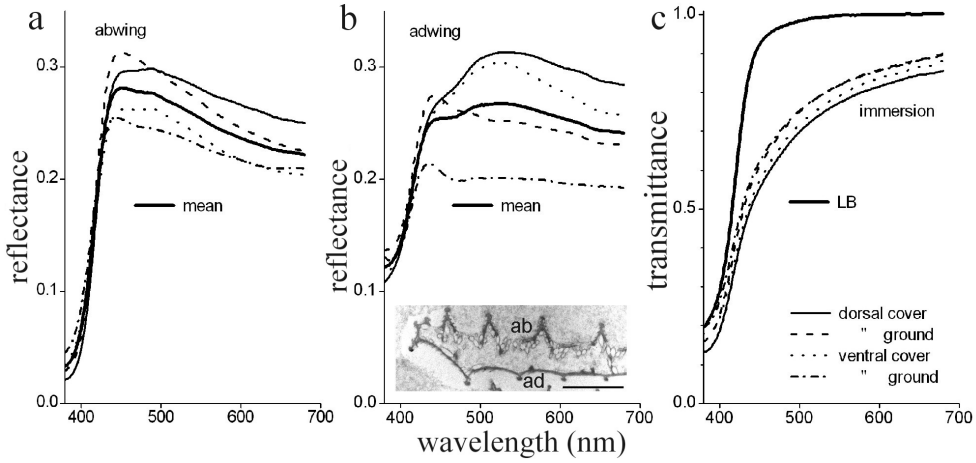

Fig. 7. Reflectance and transmittance spectra measured with a microspectrophotometer of single cover and ground scales isolated from the dorsal as well as the ventral side of the forewing of a male *Pieris rapae*. **a** Reflectance spectra measured abwing, that is, with the illumination coming from the side of the ridges, which do not face the wing (see inset in **b**). The bold curve is the mean of the measured curves. **b** Reflectance spectra measured adwing, that is, with the illumination coming from the side of the scale that does not have ridges. The bold curve is the mean of the measured curves. The maximum scale reflectance, both abwing and adwing, is roughly 0.3. In the short wavelength range, the abwing reflectance (a) is more affected by the absorbing pterin pigment than the adwing reflectance (b). **c** Transmittance spectra measured with the scales immersed in xylene, to reduce scattering. The transmittance is low due to pterin pigment that absorbs strongly in the UV. The transmittance at 375 nm is about 0.15, meaning a peak absorbance of about 0.8. The bold curve (LB) is the transmittance spectrum calculated assuming Lambert-Beer's law, taking the absorption spectrum derived from the average normalized absorptance curves of Fig. 5e and f. The distinct difference between the measured curves and the bold curve demonstrates that the immersion fluid did not fully annihilate the refractive index differences. Inset **b** Transmission electron microscopic image of a wing scale of *Pieris rapae*. The abwing side has prominent ridges that are connected by crossribs. Beads adorn the latter structures. The adwing side is rather smooth, although regularly spaced protrusions exist, resembling minor ridges (see Stavenga *et al.* 2004).

of scales on the side of the incident beam. At a glance it may seem strange that the reflectance spectrum SW is lower than reflectance spectrum W in the UV. The reason is the low reflectance as well as low transmittance of a single scale (S) at short wavelengths (Fig. 7a), resulting in a low reflectance of the assembly, SW.

Spectrum SWS presents the case with one layer of scales on each side of the wing, etc. The reflectance in the visible wavelength range steadily increases with an increase in the number of scale layers, but the increment in reflectance gradually decreases. The reflectance in the ultraviolet is completely governed by the reflectance of the first layer of scales.

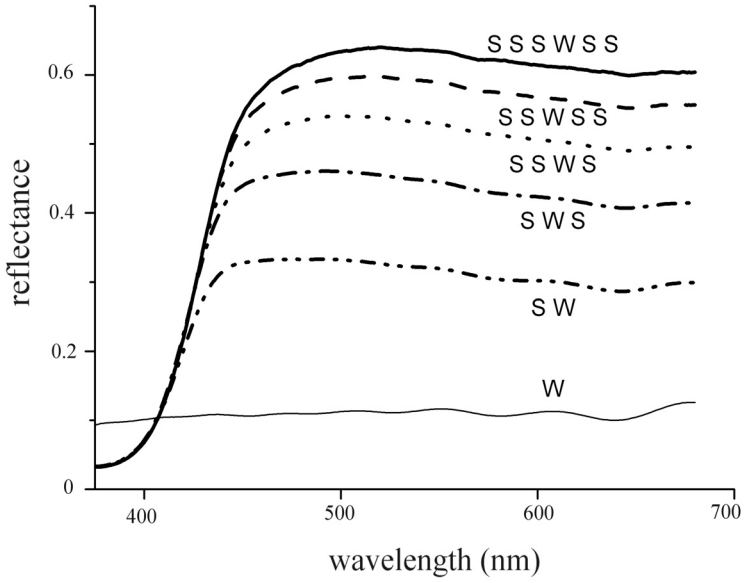

Fig. 8. Reflectance spectra calculated for a set of identical scales stacked on the wing. The reflectance of the scaleless wing (W) is the average of the spectra Wd and Wv of Fig. 5b. SW is the reflectance calculated for a single layer of scales at the wing, with the reflectance *r* taken to be the mean abwing reflectance of Fig. 5b and *s* is the mean adwing reflectance of Fig. 5b. The illumination is from the side of the scale, S. SWS is the reflectance when a single scale exists on both sides of the wing. SSWS has two layers of scales on the side of the wing from which the illumination comes, and one layer of scales on the other side of the wing; and so on.

3.4 Discussion

Butterfly scales are extremely thin, in the order of hundreds of nanometers. Not surprisingly, multilayer interference plays therefore a dominant role in many cases of butterfly colouration. This is specifically the case in the cover scales on the dorsal wings of male sulphur butterflies (Ghiradella *et al.* 1972; Silberglied & Taylor 1973; Rutowski 1977; Rutowski *et al.* 2005), and in the tips of male pierid butterflies of the *Colotis* group (Fig. 2).

Stokes (Stokes 1862) gave the first theoretical treatment of light reflected and transmitted by a pile of plates where interference effects are negligible, which was followed by a few more elaborate studies on stacks of thick layers (Smith 1926; Baumeister *et al.* 1972). We have presented here a simple recursion procedure for calculating the incoherent light flux in a stack of plates. For the modelling we have assumed that the layers are sufficiently rough so that interference effects are averaged out. The reflectance and transmittance spectra are then determined by light scattering on the scale structures as well as by the absorbing pigments. Using spectra measured on wings of the Small White butterfly (Fig. 5a-d) we have

derived the reflectance and transmittance of the scale layers on each side of the wing (Fig. 6a, b). The calculated spectra for the reflectance and transmittance of an intact wing did not completely match the measured spectra, however (Fig. 6c). This implies that the assumption of random scattering is not fully valid. In addition, coherence may be not fully negligible. The general features are nevertheless very clear. The wing reflectance of the Small White is not exclusively determined by the scales on one side, but also the scales on the other side of the wing contribute.

The maximal wing reflectance of the Small White is about 0.6 (Fig. 5a). This corresponds to the reflectance value obtained for a stack of two scale layers on each side of the wing (Fig. 8, SSWSS). Light and scanning electron microscopical observations indeed show that in average about two scales overlap at the wings of the Small White (Fig. 4). The wing reflectance of the Large White, *Pieris brassicae*, can go up to 0.8, in agreement with our observation that stacks of effectively three to four scales overlap in this species. Similarly, the dorsal wings of the Common Jezabel (Fig. 3) have a high reflectance as well as several layers of overlapping scales. In the latter case, the white reflectance is mainly due to scales on the dorsal side of the wings, because most scales at the ventral side contain a dense brown-black pigment. The scales in the striking red bands at the ventral wing noticeably contribute to the dorsal reflectance in the long-wavelength region (Fig. 3, spectra 1 and 3).

Enhancement of reflectance by multilayers of scales has been recently reported for *Morpho* butterflies by Yoshioka and Kinoshita (2006b). They have used a three layer wing model similar to that used above, but the procedure presented here is easier and more generally applicable.

The wing reflectance is only a few percent in the ultraviolet for all cases presented here. This is due to pigments absorbing in the short-wavelength range, even in the white wings. Stacking several scales at the wing enhances the reflectance at the long-wavelengths; meanwhile the short-wavelength reflectance stays low. The pierid butterflies achieve a strong, saturated colour by combining a distinct short-wavelength absorption with strong long-wavelength scattering, which is realized by a multitude of nanometer-sized beads (Stavenga *et al.* 2004). The strong colouration plays an important role in mutual, intersexual recognition (Obara 1970). This will be especially the case where the male adds a strong direction-depending iridescence in the ultraviolet (Ghiradella 1998; Rutowski *et al.* 2005) to a yellow or orange pigment colour (Fig. 2).

In conclusion, we have demonstrated that the reflectance of butterfly wings results from the coordinated effect of reflectances of single scale that are arranged in stacks on the wings. This insight will be of considerable significance for biologists working to understand the adaptive significance and evolution of butterfly wing patterns.

Acknowledgments

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Chapter 4
Sexual dichromatism and pigment localization in the wing scales of *Pieris rapae* butterflies

The beads in the wing scales of pierid butterflies play a crucially important role in wing colouration as shown by spectrophotometry and scanning electron microscopy (Stavenga *et al.* 2004). The beads contain pterin pigments (Rutowski *et al.* 2005), which in *Pieris rapae* absorb predominantly in the ultraviolet. Scanning electron microscopy demonstrates that in the European subspecies *Pieris rapae rapae* both males and females have dorsal wing scales with a high concentration of beads. In the Japanese subspecies *Pieris rapae crucivora*, however, only the males have dorsal wing scales studded with beads, and the dorsal scales of females lack beads. Microspectrophotometry of single scales without beads yield reflectance spectra that increase slightly and monotonically with wavelength. With beads the reflectance is strongly reduced in the ultraviolet and enhanced at the longer wavelengths. By stacking several layers of beaded scales pierid butterflies achieve strong colour contrasts, which is not realized in the dorsal wings of female *P. r. crucivora*. Consequently, *P. r. crucivora* exhibits a strong sexual dichromatism that is absent in *P. r. rapae* (Obara & Majerus 2000).

4.1 Introduction

The colours of butterflies are usually determined by the scales that shingle the wings (Nijhout 1991). Numerous studies have been devoted to iridescent wings where coherent scattering occurs by regularly arranged, nanosized structures in the wing scales (e.g. (Ghiradella 1984; Vukusic *et al.* 1999; Kinoshita &

Yoshioka 2005)). However, the colour of most butterflies results from selective absorption of incoherently scattered light by pigments in the wing scales. Each wing scale is the cuticular product of a single cell, with a rather flat, unstructured lower scale leaf and a highly structured upper leaf, consisting of longitudinal ridges connected by crossribs, which in pierids are adorned with granules (Ghiradella 1998). Wavelength-dependent scattering by these structures, together with the spectral absorption by the pigments they may contain, result in the wing colours. Butterflies of the family Pieridae are divided into two broad groups based on their colour: the sulphurs (the Coliadinae, with predominantly yellow to orange wings) and the whites (the Pierinae, with mostly white wings).

The wing pigments of pierid butterflies, first analyzed in the Brimstone, *Gonepteryx rhamni*, (Hopkins 1895), were appropriately called pterins. Various ultraviolet and blue-green absorbing pterins were later characterized in the Orange Sulphur, *Colias eurytheme* (Watt 1964), and virtually exclusively ultraviolet absorbing pigments were encountered in the Small White, *Pieris rapae* (Makino *et al.* 1952). The pterins were concluded to be located in granules, studded at the scale crossribs (Yagi 1954). Transmission and scanning electron microscopical photographs of the granules indicated, however, that they were void (Stavenga *et al.* 2004), leading to the assumption that the pterin pigments were dispersed in the scale structures, as is the case for the pigments in the scales of other butterfly families, where granules are absent. Furthermore, spectrophotometric measurements demonstrated that the granules enhance the wing reflectance of pierids by scattering, and therefore the granules were called beads (Stavenga *et al.* 2004).

Chemical removal of the beads from the wing scales of *C. eurytheme* severely reduced the long-wavelength reflectance, confirming the general scattering function of the beads, but the bead removal also caused an increase in short-wavelength reflectance, indicating that the beads absorb short-wavelength light, by a pigment (Rutowski *et al.* 2005). This finding suggests a dual function for the beads, namely to enhance long-wavelength reflectance and simultaneously to suppress short-wavelength reflectance, thus creating colour contrast.

Here we investigate this hypothesis by relating the reflectance spectra to the density of beads in the Small White *Pieris rapae*, and we compare the European subspecies *P. r. rapae* with the Japanese subspecies *P. r. crucivora*. The general appearance of pierid wing reflectance spectra is low reflectance at short wavelengths and high reflectance at long wavelengths, which is a cumulative effect of multilayers of scales (Stavenga *et al.* 2006). In *P. r. rapae* both sexes conform to this rule, as both combine low reflectance in the ultraviolet with a brilliant diffuse white colouration, but in *P. r. crucivora* this is only the case for the male. The reflectance of the dorsal wings of female *P. r. crucivora* is substantial in the ultraviolet, but it gradually rises with longer wavelengths, where

it still remains much lower than the wing reflectance of the males (Obara & Majerus 2000). *P. r. crucivora* thus features a distinct sexual dichromatism (Obara 1970), which is absent in *P. r. rapae* (Obara & Majerus 2000). The bead density of the dorsal wing scales of female *P. r. crucivora* is low (Hidaka & Okada 1979) and correlates with the reflectance characteristics. Our findings provide further insight into the optical mechanisms of butterfly wing colouration, which will presumably be useful in enhancing our understanding of butterfly speciation and development.

4.2 Materials and methods

Animals

Specimens of the European Small White butterfly, subspecies *Pieris rapae rapae*, were obtained from a continuous culture (with non-diapausing pupae) maintained by Dr J.J. van Loon, Entomology department of the Agricultural University in Wageningen, The Netherlands (Hopkins and van Loon 2001). Japanese Small Whites, *Pieris rapae crucivora*, were obtained from Prof. K. Arikawa, University of Yokohama, Japan (Arikawa et al. 2005). Three males and females of each subspecies were investigated.

Spectrophotometry

Reflectance spectra of intact wings were measured with a reflection probe connected to a fibre optic spectrometer (SD2000, Avantes, Eerbeek, the Netherlands), using a deuterium/halogen light source. On the dorsal as well as ventral side of both forewings and hindwings three locally adjacent areas from each of four regions indicated with a number in the insets of Figs. 1a and 1b, were investigated. The three reflectance spectra were averaged. The fibre aperture (half-angle of the maximum cone of light) is about 12^{o}, and the sampled area had a diameter of about 1 mm. Spectra of the abwing (upper) side of single scales, taken from the same areas and glued to the tip of a micropipette, were measured with a microspectrophotometer (MSP), consisting of a xenon light source, a Leitz Ortholux microscope, and the fibre optic spectrometer. The microscope objective was an Olympus 20x, NA 0.25. A white reflectance standard (Spectralon, Labsphere, North Sutton, NH, USA) served as a reference.

Scanning electron microscopy

The wing areas from which reflectance spectra were measured were prepared for scanning electron microscopy (SEM) by sputtering the samples with palladium for 5 min at 800 V and 200 millitorr (Hummer, Technics, Alexandria, VA). The

scale anatomy was then investigated with a Philips XL-30, using a voltage of 3 kV.

4.3 Results

Small White butterflies, *Pieris rapae*, have white wings with a black dorsal wing tip and a few characteristic black dots, which are sex dependent (Obara & Majerus 2000). Especially male *Pieris rapae rapae* have very white dorsal wings, as is directly recognizable from the reflectance spectra (Fig. 1a). In the visible wavelength range the reflectance is high, ~70%, whilst the reflectance in the ultraviolet is not more than a few percent. The reflectance of the male ventral hind wings is depressed in the blue-green, which correlates with a higher, although still minor reflectance in the ultraviolet (Fig. 1a). For the female *P. r. rapae*, the reflectance spectra of both dorsal and ventral wings feature a blue-green depression (Fig. 1b), similar to that in the reflectance spectrum of the ventral hind wing of the male (Fig. 1a). The modest reflectances in the ultraviolet of the female wings are only slightly larger than the reflectances of the male dorsal wings.

The situation is different in the Japanese subspecies *P. r. crucivora*. For the human observer, the visual appearance of the male is similar to that of the male (and female) *P. r. rapae*, but the wings of the female *P. r. crucivora* are greyer and very slightly brownish (Obara & Majerus 2000). The reflectance spectra of the male *P. r. crucivora* are indeed very similar to those of the male *P. r. rapae* (Fig. 2a). The reflectance spectra of the female increase monotonically from 10-20% in the ultraviolet to about 20-50% in the visible wavelength range (Fig. 2b). *P. r. crucivora* hence features a marked sexual dichromatism (Obara 1970), certainly from the viewpoint of the butterflies, which detect ultraviolet light well (Arikawa et al. 2005). Compared to the sexual dichromatism of *P. r. crucivora*, that of *P. r. rapae* is much reduced.

To investigate the physical origin of the different colouration and reflectance spectra, we have examined the anatomical structure of scales in the same wing areas as were studied in Figs 1, 2. The reflectance measured from single scales is low in the ultraviolet, only a few percent, and higher in the visible wavelength range, with a maximum of about 30% (Fig. 3a). Scanning electron microscopy shows that the scales of both male and female *P. r. rapae* in the dorsal as well as ventral wing areas are densely packed with beads (Fig. 3c-j). Quite noticeably, however, is the slightly lower bead density of the scales of the ventral hind wings in both the male (Fig. 3f) and the female (Fig. 3j), which correlates with the lower reflectance of single scales from the hindwings (Fig. 3a,b). The depression of the

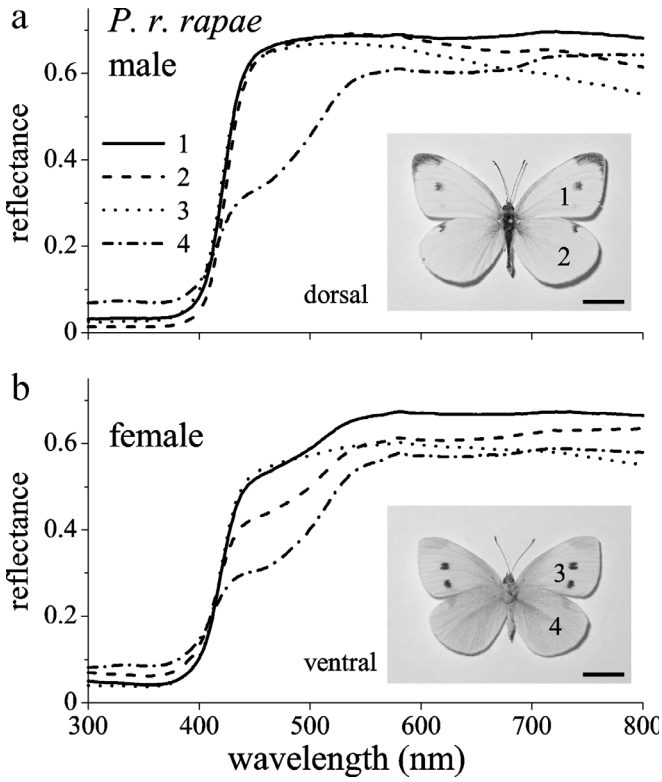

Fig. 1. Reflectance spectra of the wings of *Pieris rapae rapae* measured with a reflection probe connected to a fibre optic spectrometer from restricted areas (indicated by a number) of the dorsal forewing (1), dorsal hindwing (2), ventral forewing (3), and ventral hindwing (4) of a male (**a**) and a female (**b**). The inset in (a) shows the dorsal side of a male *P. r. rapae*, and the inset in (b) presents the ventral side of a female. The reflectance spectrum of the ventral hindwing of the male is lower than the other spectra in the blue-green wavelength range, resulting in a yellowish colour. The female wings are generally slightly yellower than those of the male. The indicated wing locations were the same for both male and female. Bars: 1 cm.

reflectance spectra of the latter area in the blue-green correlates with the yellowish colour (see Fig. 1).

The reflectance of single scales from the dorsal wings of male *P. r. crucivora* is also only a few percent in the ultraviolet and maximally about 30% in the visible wavelength range, as in *P. r. rapae* (Fig. 4a). Also, the scales from the ventral hind wing have a lower reflectance in the blue-green compared with scales from the other wing areas (Fig. 4a). The density of beads in the scales of male *P. r. crucivora* is high (Fig. 4c-f). For the female *P. r. crucivora*, the characteristics of scales from the dorsal forewing and hindwing dramatically differ. The single scale reflectance spectra rise almost linearly from 10% in the ultraviolet to about 20% in the red (Fig. 4b), and beads appear to be fully lacking dorsally (Fig. 4g, h). The ventral forewing and hindwing scales generally carry a low, although somewhat variable concentration of beads (Fig. 4i, j). The reflectance in the ultraviolet is about 5% and can rise to about 25% in the red (Fig. 4b).

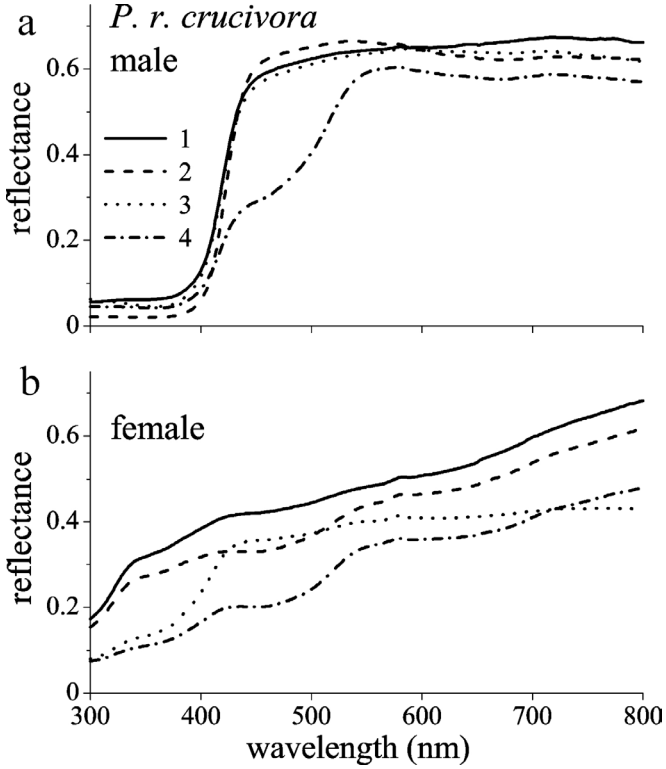

Fig. 2. Reflectance spectra of the wings of *Pieris rapae crucivora* measured from the dorsal forewing (1), dorsal hindwing (2), ventral forewing (3), and ventral hindwing (4) of a male (**a**) and a female (**b**); see insets in Fig. 1a and 1b. The reflectances in the ultraviolet of the dorsal forewing and hindwing of the male are considerably lower than those of the female, whilst the reflectances are higher at the longer wavelengths, yielding a distinct sexual dichromatism.

4.4 Discussion

The colour of butterfly wings commonly results from the combined optical effects of light scattering on the scales and wing substrate and absorption by pigments deposited in these structures. Coherent scattering on regularly arranged lamellae in the scale ridges is a dominant factor for the colouration of many male pierids of the subfamily Coliadinae, creating bright UV iridescence (Ghiradella *et al.* 1972), but this phenomenon is not featured by the Pierinae, the subfamily of *Pieris rapae*. In a comparative study on butterfly wing reflectance, we found that incoherent scattering on the scale beads substantially contributes to the brightness of the Small White's wings in the long wavelength range (Stavenga *et al.* 2004), a conclusion supported by Rutowski *et al.* (2005), who studied the wing reflectance of the Orange Sulphur, *Colias eurytheme*. We here demonstrate that the beads are absent in the dorsal wing scales of the female *Pieris rapae crucivora*, resulting in a lower reflectance at long-wavelengths, compared to the males (Fig. 2). The female wing reflectance is higher in the UV, however, providing strong evidence for the view that the beads contain a UV absorbing pigment. The present results show that an increased bead density is related to a decrease in UV reflectance and an increase in long-wavelength reflectance.

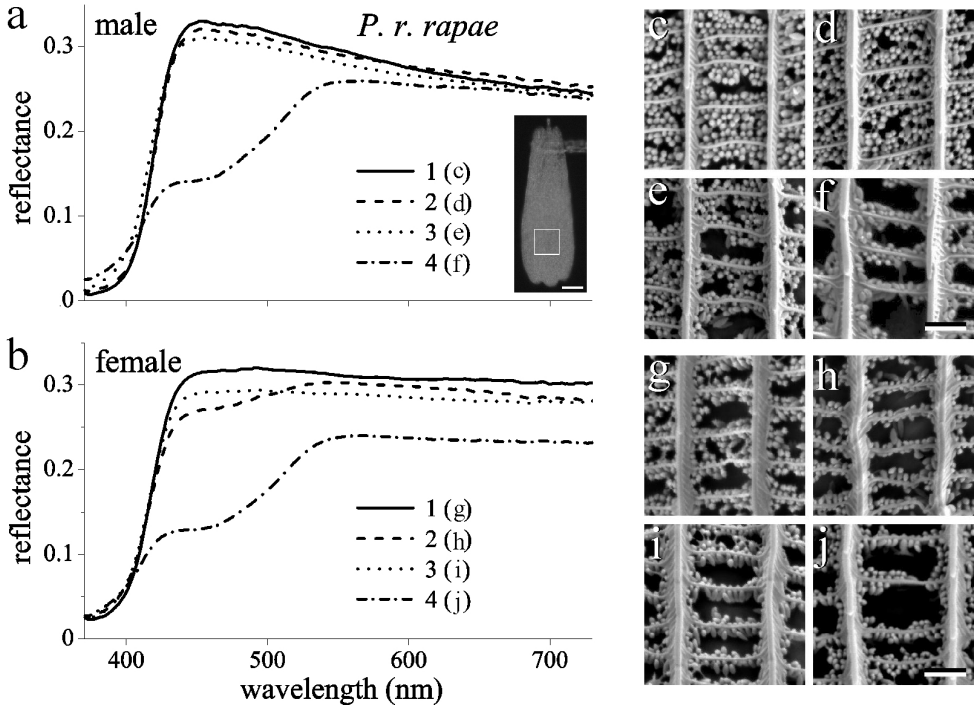

Fig. 3. Reflectance spectra of male (**a**) and female (**b**) *Pieris rapae rapae* and scanning electron microscopy (SEM) of single scales (**c-j**). The reflectance spectra 1-4 are from single scales, taken from wing locations shown by the numbers in the insets of Fig. 1a and 1b, of the dorsal forewing (1), dorsal hindwing (2), ventral forewing (3), and ventral hindwing (4). The single scale reflectance spectra were measured from an area indicated by the square in the inset in (a). The spectra of (a) correspond to the male SEM photographs (c-f), and the spectra of (b) correspond to the female SEM photographs (g-j), as indicated. Bars: 20 μm (inset in a) and 1 μm (c-j).

In previous work the beads were called pigment granules (Yagi 1954; Hidaka & Okada 1979; Waku & Kitagawa 1986; Ghiradella 1998), because they were assumed to contain the pterin pigments that cause the distinct yellow colour of sulphurs (Hopkins 1895; Watt 1964). The pterins of *Pieris rapae crucivora*, leucopterin and (iso)xanthopterin (Makino *et al.* 1952), absorb exclusively in the ultraviolet. Broad-band absorbing pigments, the melanins, are expressed in the darkly coloured scales (Nijhout 1991), which together create the black spots of *P. rapae* wings. The black scales do not contain beads (Hirata & Uehara 1959; Allyn & Downey 1977; Stavenga *et al.* 2004), meaning that the melanin pigment is distributed in the scale surfaces, ridges and/or crossribs. This must also hold for the pigments in the scales of butterflies from families other than the Pieridae, because they also do not have beads.

The beads of the white pierid scales appeared in transmission electron microscopical photographs as clear bodies (Stavenga *et al.* 2004), which seemed to indicate that they were empty, suggesting that the pterin pigments exist similarly distributed throughout the scales as the melanin. The optical function of the beads thus seemed to be exclusively to be that of strong scatterers. Rutowski *et al.* 2005 however showed that alkalic treatment of the scales simultaneously extracts pterins and removes beads, resulting in an increased reflectance in the short-wavelength range and a decreased reflectance at longer wavelengths. They hence concluded that the beads, in addition to being scatterers, contain the pterin pigments. Furthermore, Morehouse *et al.* (2007) found that the scale reflectance increases proportionally to the number of beads. The high reflectance in the ultraviolet of depigmented *C. eurytheme* wings (Fig. 1b of Rutowski *et al.* 2005 resembles that of the dorsal wings of female *P. r. crucivora* (Fig. 2b). The latter wings have beadless scales, and we thus conclude that the present results underscore the findings of Rutowski *et al.* 2005.

The reflectance spectra of female wings are rather featureless (Fig. 2b), rising slightly with increasing wavelength. The presence of beads strongly decreases the reflectance in the UV and enhances the longer-wavelength reflectance (Figs. 3, 4). The function of the beads thus appears to be twofold. By concentrating a pigment with an appropriate absorbance band in numerous small, nanosized granules, the scale reflectance at short wavelengths is reduced. Because the beads create an additional scattering medium with a refractive index distinctly higher than that of air, the reflectance at wavelengths outside the absorption band is increased.

Several components determine the reflectance of butterfly wings, including the assembly of scales together with the wing substrate (Stavenga *et al.* 2006). In the case of the female *P. r. crucivora*, the reflectance of the intact dorsal forewing is 40-50% between 400 and 600 nm (Fig. 2b), whereas the reflectance of single scales of the dorsal forewing is 15-20% in that wavelength range (Fig. 4b). The enhanced wing reflectance results from the presence of a stack of 2-3 scales on both sides of the intact wing. Studding a scale with pigmented beads, which heavily occurs in male dorsal scales, depresses the reflectance in the UV to a few percent and increases the reflectance in the red to about 30%. For a single scale these changes may still seem to be minor, but at the intact wing the effects are multiplied, resulting in maximum reflectances of about 70%. This high reflectance of the intact wings is predominantly determined by the scales on the illuminated side of the wing, but the scales on the opposite side can also contribute substantially, as has been analyzed in detail for *P. r. rapae* (Stavenga *et al.* 2006). Whereas the dorsal wing reflectance of female *P. r. crucivora*, with unpigmented scales, gradually and slightly increases from short to long wavelengths, the reflectance spectrum of the pigmented wings of male *P. r crucivora* changes abruptly at about 400 nm, and similar steep spectral changes

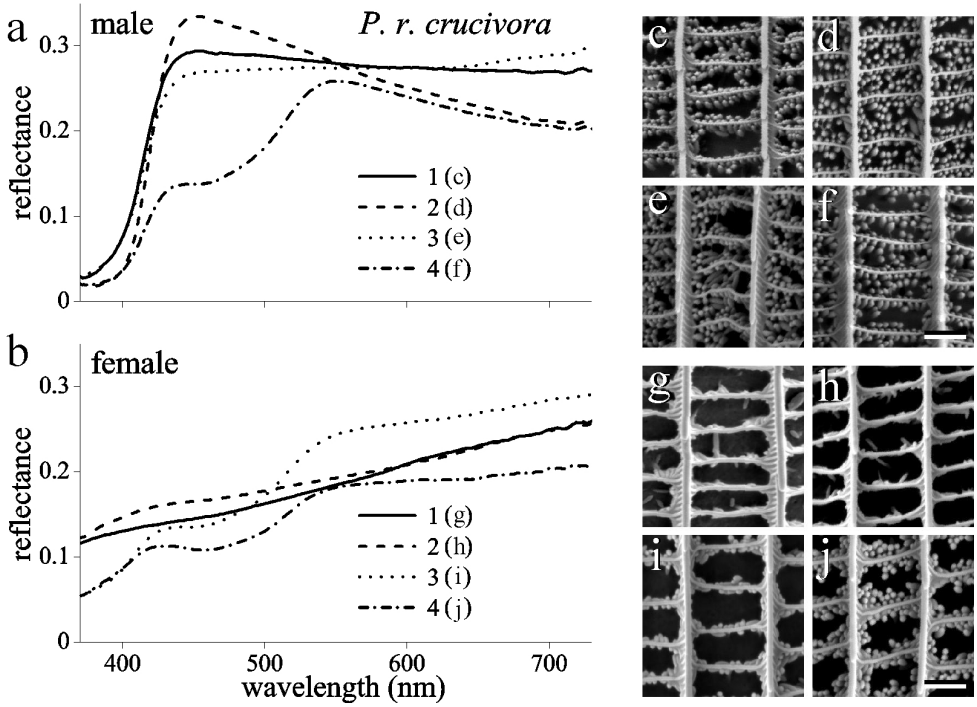

Fig. 4. Reflectance spectra of male (**a**) and female (**b**) *Pieris rapae crucivora* and scanning electron microscopy (SEM) of single scales (**c-j**). The reflectance spectra 1-4 are from single scales taken from about the same locations at the dorsal forewing (1), dorsal hindwing (2), ventral forewing (3), and ventral hindwing (4) as given in Fig. 1. They correspond to the SEM photographs [male (c-f); female (g-j)] as indicated. Bars: 1 μm.

are seen in the reflectance spectra of male and female *P. r. rapae*. [We note here that the reflectance spectra of Figs. 1 and 2 are in agreement with those reported by (Obara & Majerus 2000), their Fig. 1, except that they erroneously interchanged the reflectance spectra for the ventral and dorsal surfaces].

The colours, and accordingly the reflectance spectra, of the dorsal and ventral wings differ slightly in the studied butterflies, that is, in both sexes of the two *Pieris rapae* subspecies. In many sulphurs, the Colianidae, the difference between dorsal and ventral wings is much more extreme. The sulphurs have extensively beaded scales (Rutowski *et al.* 2005), but the pigments absorb well into the visible wavelength range, causing a yellow or orange colour (Watt 1964). The resulting colour contrast is enhanced in the dorsal wings of males of many colianid species by a brilliant iridescence, which is restricted to the UV (Ghiradella *et al.* 1972; Silberglied & Taylor 1973; Kemp *et al.* 2005). Such an additional colouration in

the UV only works in combination with a yellow, orange, or red background. In white Pieridae an additional UV reflectance would reduce colour contrast, and therefore iridescence is not found in the white wings of *Pieris* species.

In *P. rapae*, the ventral hind wings are yellowish, because the reflectance in the blue is suppressed. The ventral wing reflectance spectrum can be easily explained by assuming that scales with a blue-absorbing pterin occur simultaneously with scales containing the generally-occurring ultraviolet-absorbing pterin. However, the reflectance spectra of single scales from the ventral hind wings have the same biphasic shape as those measured from the intact ventral hind wing. The scales hence must express (at least) two types of pterins, absorbing in the UV and blue, respectively. Detailed measurements on ventral scales from the same area show that they are variable coloured; some are white, others are quite yellow or yellowish. Apparently the relative expression of the different pterins is not constant. Furthermore, the reflectance spectrum depends on the location of measurement, which correlates with the local density of beads (in preparation).

As the reflectance spectra of male and female *P. r. crucivora* only differ strongly in the ultraviolet, no striking colour difference is seen by a human observer. Nevertheless, for the Japanese Small White butterflies males and females will have distinctly different colours, because the butterflies possess a visual system with an unsurpassed rich repertoire, notably for the short wavelength range. Both male and female have three types of photoreceptors each with a short-wavelength absorbing rhodopsin, peaking in the UV, violet and blue, respectively (Arikawa et al. 2005). In males of the Japanese subspecies *P. r. crucivora*, the receptors with a violet-absorbing rhodopsin are modified into a double-peaked blue receptor by a UV-absorbing, whitish-fluorescing pigment (Arikawa et al. 2005). Presumably the short-wavelength receptors serve in the sensitive discrimination of the male and female wing colours. *P. r. crucivora* males can already discriminate females on their slightly different ventral colouration (Obara 1970). The dorsal sexual dichromatism of *P. r. crucivora* will strongly facilitate the intraspecies recognition.

Visual discrimination of females is clearly also done by male *P. r. rapae*, but this is relatively poor, and presumably therefore resting *P. r. rapae* males, when erroneously approached by other males, when searching for females, elicit a male-characteristic flutter response, which then results in no copulatory attempts. The fluttering response thus plays an important role in mate recognition, that is, it functions as a ‘mechanical isolation mechanism’ (Obara & Majerus 2000). Male *P. r. crucivora* also exhibit the flutter response, but this can be considered as rather redundant, because sexual discrimination is readily achieved visually (Obara 1970). Obara *et al.* 2000 therefore hypothesized that the flutter response of male *P. r. crucivora* is a relic of *P. r. rapae*, which is thus assumed to be evolutionary ancestral. The suppressed expression of pterin pigments and the

resulting absence of beads in the scales on the dorsal wings of female *P. r. crucivora*, which causes the strong sexual dichromatism and easy sexual discrimination of *P. r. crucivora*, hence may be subject to evolutionary forces.

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Chapter 5
Wing coloration and pigment gradients in scales of pierid butterflies

Depending on the species, the individual scales of butterfly wings have a longitudinal gradient in structure and reflectance properties, as shown by scanning electron microscopy and microspectrophotometry. White scales of the male Small White, *Pieris rapae crucivora*, show a strong gradient in both the density in pigment granules and the reflectance. After pigment extraction by aqueous ammonia, scales of male *P. r. crucivora* closely resemble the unpigmented scales of female *P. r. crucivora*. Only a minor gradient exists in the white and orange scales of the male Orange Tip, *Anthocharis cardamines*. Pigment extraction of orange scales of *A. cardamines* causes bleaching. Partial bleaching transforms the scales so that they resemble certain scales of *Phoebis philea* that have a natural extreme gradient. Reflectance measurements on an artificial stack of two overlapping scales as well as on the scale stacks existing on intact and partially denuded wings of the Large White, *Pieris brassicae*, quantitatively demonstrate the reflectance enhancement by scale stacking.

5.1 Introduction

Butterflies are among the most conspicuous animals, and their wing coloration is perhaps the most diverse in the animal kingdom. Considerable knowledge has been gained about the origin of butterfly wing patterns, which resemble pointillistic paintings where each point is formed by a coloured scale (Nijhout 1991), but only recently has research become focused on the details of how single

scales contribute to the global wing coloration (Vukusic *et al.* 1999; Yoshioka & Kinoshita 2004; Stavenga *et al.* 2006; Giraldo & Stavenga 2007a).

A butterfly wing scale generally consists of two laminae, connected by trabeculae or pillars. The lower lamina is more or less flat and unstructured, but the upper lamina consists of densely spaced ridges, which are connected by crossribs. The area framed by adjacent ridges and crossribs is called a window (Ghiradella 1998; Vukusic *et al.* 2000). Incident light is scattered by the scale structures, because the refractive index of the scale material distinctly differs from that of air (Vukusic *et al.* 1999; Stavenga *et al.* 2004). The resulting scale's colour is either determined by its structural organization or its pigmentation, or by a combination of both properties (Vukusic & Sambles 2003; Kinoshita & Yoshioka 2005; Giraldo & Stavenga 2007a).

The colour of a whole butterfly wing is an even more complex phenomenon, because the scales are usually arranged in a system of distinct, partially overlapping rows of so-called cover and ground scales (Nijhout 1991; Ghiradella 1998). The wing substrate has in general two scale layers on both the dorsal and the ventral sides, and thus light reflected and transmitted by the five elements of the wing transect (including the wing membrane itself) determines the wing colour (Stavenga *et al.* 2006).

The optical and structural properties of single scales have been specifically studied in detail for scales featuring iridescent colours. Notably the tropical brilliant blue *Morpho* butterflies occupy many of the pages of the literature written so far about wing scales (e.g., Vukusic *et al.* 1999; Yoshioka *et al.* 2004). The ridges of *Morpho* scales are elaborated into lamellae, which together form a multilayer where coherent scattering results in an intense blue reflectance. An ultraviolet version of the *Morpho* multilayer reflector is encountered in the dorsal cover scales of males of several pierid species, specifically of the subfamily Coliadinae. The coherent scattering of ultraviolet light by pierid scales is a sexually driven feature (Ghiradella *et al.* 1972; Silberglied & Taylor 1973; Kemp *et al.* 2005).

The ground scales of male Coliadinae generally scatter light incoherently, as is the case in both cover and ground scales of female Coliadinae as well as of many members of the pierid subfamily Pierinae. In the present paper we focus on an important component of this scattering, one caused by ovoid-shaped granules that partially fill the scale windows, a microscopic characteristic only of the family Pieridae. These granules, also called beads, contain pigments that belong to the class of pterins. They execute a dual function. Depending on the type of pterin, they absorb light in the short wavelength range, but outside the pigment absorption range, at the longer wavelengths, the granules strongly scatter light (Stavenga *et al.* 2004; Rutowski *et al.* 2005; Giraldo & Stavenga 2007a). The black and brown scales of pierids contain another type of pigment, melanin,

which has a broad absorption spectrum, but these scales lack the beads, and hence the melanin is located in the ridges and/or crossribs (Yagi 1954; Stavenga *et al.* 2004).

Microscopical observations of single scales reveal that the pigmentation is inhomogeneous. Here we report combined structural and optical studies on the scales of a number of pierid butterflies, and we detail how the gradient in pigmentation at the scale level will affect wing coloration.

5.2 Materials and methods

Animals

Japanese Small White butterflies, *Pieris rapae crucivora*, were obtained from Prof. K. Arikawa, University of Yokohama, Japan. The Orange Tip, *Anthocharis cardamines*, the Brimstone, *Gonepteryx rhamni*, and the Large White, *Pieris brassicae*, were collected in the Netherlands. The Orange-barred Sulphur, *Phoebis philea*, was obtained commercially.

Scale preparation and spectrophotometry

Single wing scales were isolated by gently pressing the wings to a glass plate and then were glued to the tip of a glass micropipette, which had a diameter of approximately 5 μm. Subsequently, the micropipette was mounted on a micromanipulator with one rotational and three translational degrees of freedom. For the experiments with overlapping scales, two micropipettes with scales were mounted on separate micromanipulators. Single scales were photographed with a Zeiss Axioskop microscope, applying bright-field epi-illumination or UV-induced fluorescence. Reflectance spectra were measured with a microspectrophotometer (MSP), which consisted of a xenon light source, a Leitz Ortholux microscope, and a fibre optic spectrometer (SD2000, Avantes, Eerbeek, the Netherlands). The microscope objective was an Olympus 20x, NA 0.46. The reference was a white reflectance standard (Spectralon, Labsphere, North Sutton, NH, USA). The measurements on intact and partially denuded wings were performed with an integrating sphere and the fibre optic spectrometer, as described by Stavenga *et al.* 2006. The integrating sphere integrates the reflected light over the full 2π hemispherical angle, while the MSP objective integrates light over an angle limited by its aperture. The results of both methods are nevertheless directly comparable for pierid scales, because they act as Lambertian diffusers (Giraldo *et al.* 2007).

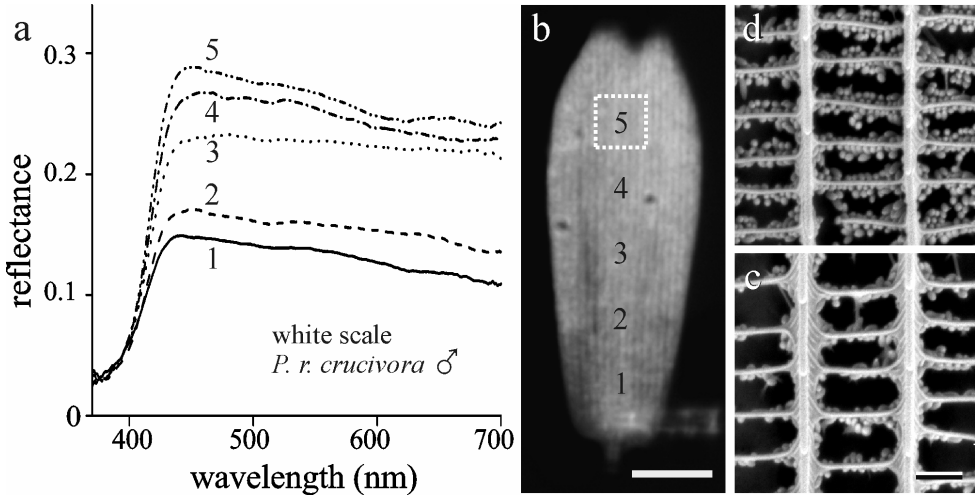

Fig. 1. a Reflectance spectra of a male *P. r. crucivora* of five different scale areas, numbered 1 to 5 from the base to the tip. **b** Reflection image of the scale photographed with an incident light microscope, with the area numbers. The dotted square represents the size of the measured area. (**c**, **d**) SEM images of the base area (1) and the tip area (5), respectively. The peak reflectance increases by almost a factor of two, when going from the base to the tip of the scale. The reflectance increase correlates well with the density of beads. Bars: 25 µm (b) and 1 µm (c, d).

Electron microscopy

After measuring reflectance spectra, the single scales were prepared for scanning electron microscopy (SEM) by sputtering the samples with palladium for 5 min at 800 V and 200 mTorr (Hummer, Technics, Alexandria, VA). The anatomy of the scales was investigated with a Philips XL-30, using a voltage of 3 kV. Small pieces of the forewing of a Brimstone, size about 1 x 4 mm^2, were cut and processed for transmission electron microscopy (TEM). Samples were immersed in agar for better handling, prefixed in 2% glutaraldehyde / 0.1 M Na-cacodylate and fixed in 1% OsO_4 / 1.5% K_4 $Fe(CN)_6$ in 0.1 M cacodylate. Subsequent washing with double distilled water, and dehydration with an alcohol series that ended with 100%, were followed by propylene oxide for 30 min and embedding in Epon. Post-microtomed samples were contrast-enhanced with uranyl acetate in methanol for 2 min, lead-water for 1 min, and then examined with a Philips 201.

Bleaching scales

Drops of 1% aqueous ammonia were put locally on the wing in order to extract pterin pigment from the granules. The drops -with the extracted pigment- were taken away with filter paper after 10 min.

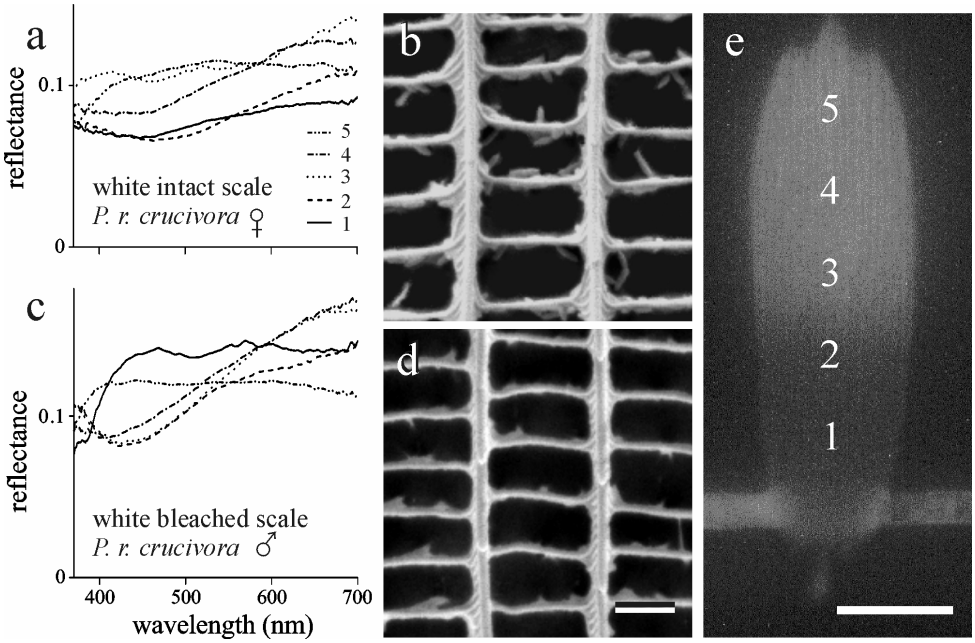

Fig. 2. Reflectance spectra and images of an intact female scale and of a bleached scale of a male *P. r. crucivora*. (a) The spectra for the five areas of a female scale from the base to the tip (1 to 5) do not reveal a clear gradient as in the case of the male, and the reflectance is much lower than that of a male scale (Fig. 1), which is due to the virtual lack of beads throughout the scale (b). (c) Reflectance spectra of a male scale where the pigmented beads were extracted with aqueous ammonia. The 10-15% peak reflectance is similar to that of the female scale. Spectrum number 1 corresponds to the base of the scale, which is not totally bleached due to the procedure used (see Section 2.4, Materials and methods). (d) The beads elsewhere vanished and the residual scale thus resembles a female scale. (e) UV-induced blue fluorescence photograph of a partly bleached scale of a male *P. r. crucivora* showing a low fluorescence in the base areas (1, 2), where pigment absorption is still present, and a high fluorescence in the tip areas (3-5), where pigment bleaching occurred. Bars: 1 µm (b, d) and 25 µm (e).

5.3 Results

5.3.1 Pigmentation of white scales of *Pieris rapae crucivora*

In order to quantitatively compare the optical and anatomical properties of single scales, we have measured for each scale the reflectance of five areas (Fig. 1a), numbered from 1 to 5 from the base to the tip (Fig. 1b). Fig. 1 presents the case of a white cover scale from the dorsal wing of a male Small White, *P. r. crucivora*. The reflectance is minor in the near UV range, virtually independent of the location, but the reflectance is high in the visible wavelength range. The peak reflectance is about 15% in area 1 and gradually increases to almost 30% in area 5 (Fig. 1a).

Scanning electron microscopy (SEM) revealed a parallel increase in the density of beads, starting from a very low density in area 1 (Fig. 1c) to a high density in area 5, where the beads occupy most of the space in the windows, between the ridges and crossribs (Fig. 1d). We did not find a clear difference in the gradient of the bead density between cover and ground scales of *P. rapae*.

A comparative study of male and female *P. r. crucivora* (Giraldo & Stavenga 2007a) revealed that the white scales on the dorsal wing of female *P. r. crucivora* have a much higher reflectance in the UV and a much lower reflectance at the longer wavelengths than do the male scales (see also Fig. 2a and Fig. 1a). Both effects are a direct consequence of the very low density of beads in the female scales (Fig. 2b). The reflectance spectra are not completely flat, presumably due to some dependency of the scattering on the size of the ridge and crossrib structures.

Whereas female *P. r. crucivora* have a reduced number of beads in a natural way, beads can also be removed artificially, namely by applying to the wings aqueous ammonia, which extracts the pterin pigments that are concentrated in the beads (Kolyer & Reimschuessel 1970; Morehouse *et al.* 2007; Wijnen *et al.* 2007). Scales of male *P. r. crucivora* wings treated with aqueous ammonia yield reflectance spectra with peak reflectances of less than 20% (Fig. 2c), very similar to those obtained from the female scales (Fig. 2a). SEM pictures indeed demonstrate that the scales of the ammonia-treated wing areas have lost the beads (Fig. 2d). The beads are not fully removed throughout the whole scale, however, as can be seen from the reflectance spectrum of area 1 (Fig. 2c), which features a low reflectance in the UV, and it is even more directly recognized from Fig. 2e, which is a photograph of the UV-induced blue fluorescence of a scale taken from an ammonia-treated wing. The cuticle of the tip area (3-5) is distinctly fluorescing, which is not seen when the strongly UV-absorbing pterin pigment leucopterin (Wijnen et al., 2007) is present.

The fluorescence is low in the base area (1, 2), because the excitation light is absorbed by the leucopterin that the ammonia has been unable to extract. This can be immediately understood, for the scales partly overlap each other, so that the base area is more or less protected.

5.3.2 Pigmentation of orange scales of *Anthocharis cardamines* and *Phoebis philea*

The effect of pigment extraction on the reflectance spectra of pierid butterfly wing scales can be favourably investigated in the orange scales of the dorsal wing tip of the male Orange Tip, *A. cardamines*. Reflectance spectra measured from the various intact scale areas are very similar, with a low reflectance at wavelengths below 500 nm, and with a high reflectance above 600 nm, with peak reflectances

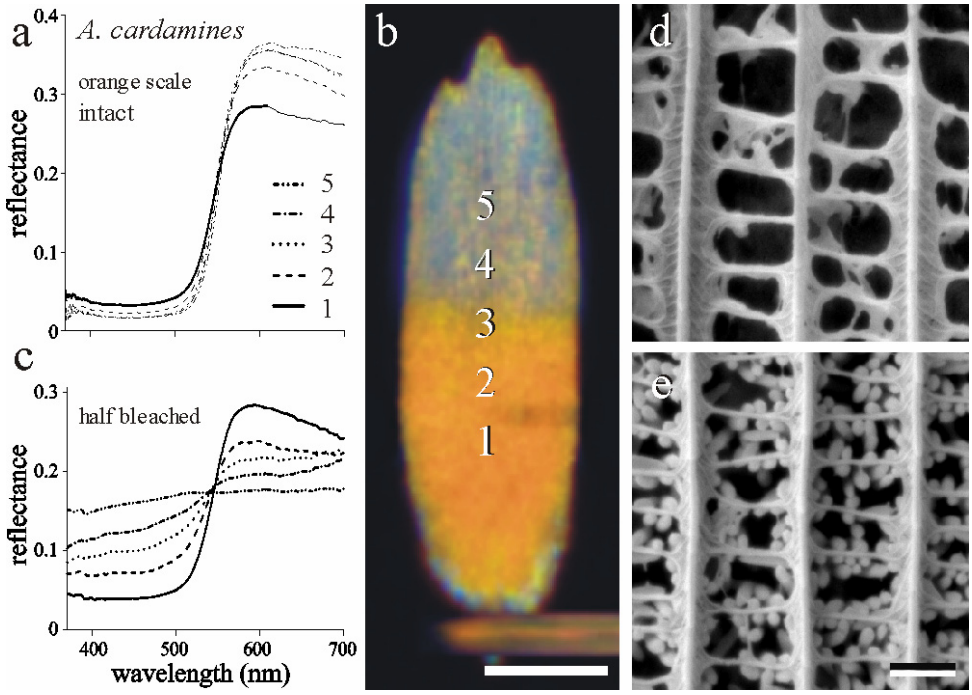

Fig. 3. Reflectance spectra of five scale areas of an intact (**a**) and half-bleached (**c**) orange scale of the male Orange Tip, *A. cardamines*. (**b**) Half-bleached scale with numbers indicating the five areas where the spectra of (c) were taken. The reflectance spectra of the intact scale reveal a gradient in long wavelengths scattering that, although important, is considerably less than that of *P. r. rapae* scales (Fig. 1a). Compared to the unbleached area 1, scattering as well as absorption is reduced in the strongly ammonia affected area 5. (**d**, **e**) SEM images of the bleached and unbleached scale areas. Bars: 25 µm (b) and 1 µm (d, e).

of about 30% (Fig. 3a). The latter value indicates a high bead density throughout the scale, an anatomical property confirmed by SEM (not shown; also the white scales of male *A. cardamines* have a fairly constant and high bead density). In agreement with the reflectance spectra and the SEM photographs, scales taken from the dorsal wing tip of a male Orange Tip normally are orange throughout the scale, but scales taken from a wing treated with ammonia very clearly show a partial bleaching, that is, a partial extraction of the short-wavelength absorbing pigment (Fig. 3b). The reflectance spectra of the partially bleached scales give a more refined picture. Reflectance spectrum 1 of Fig. 3c is almost identical to spectrum 1 of Fig. 3a, with a low reflectance in the UV and a peak reflectance approaching 30%, and hence the ammonia has hardly affected the base area. The spectra 2-5 progressively deviate from spectrum 1, however, and the reflectance at scale area 5 is flattened to a virtually constant 17% for all wavelengths. SEM pictures are in complete agreement with the spectral data. In the strongly bleached

scale tip area, beads are absent (Fig. 3d), while in the base area beads are plentiful (Fig. 3e).

The spectra of Fig. 3c clearly demonstrate the two opposite optical effects of the beads on the scale reflectance. In the absence of beads, the remaining scale elements, that is, the ridges and crossribs of the upper lamina and the lower lamina, together cause a rather wavelength-independent reflectance of about 17%. An increase in bead number means an enhanced pigment absorption, andtherefore a reduced reflectance in the short-wavelength range, which is accompanied by an enhanced scattering in the long-wavelength range.

A similar clear demonstration of the dual role of beads is encountered in a special type of scale of the female Orange-barred Sulphur, *Phoebis philea*. The ventral hindwings of the female *P. philea* are yellow with two bright white spots surrounded by orange-reddish rings. The white spot is created by unpigmented scales that strongly reflect at all wavelengths, throughout the visible as well as the ultraviolet. The orange-reddish ring is formed by scales that have an extreme colour gradient that runs from white to orange-red, similar to the gradient artificially created in the orange scales of *A. cardamines* (Figs. 3b and 4). The reflectance spectra of Fig. 4a correspond to the five scale areas indicated in Fig. 4b. Spectrum 1, from the white area, is rather flat except for a slight peak around 410 nm. Presumably this peak is due to the lower lamina, which often acts as a thin film, a phenomenon frequently observed in white and coloured scales of many butterflies. Spectrum 5, from the orange-red tip of the scale, has a peak reflectance value of only 21%, much less than the 37% peak reflectance of the male Orange Tip scale (Fig. 3a). The short-wavelength reflectance is quite low, about 5%, somewhat higher than that of the orange scale of the male *A. cardamines* (Fig. 3a), also indicating a lower bead density of the *P. philea* scale.

The structure of the *P. philea* scales indeed deviates slightly from that of the scales of *P. r. cucivora* and *A. cardamines*. The crossribs are less sharply defined, and the windows are not filled with numerous beads. The orange-red and white area 1 (Fig. 4d) has open windows. The unpigmented area 1 has a virtually constant, wavelength independent reflectance, suggesting that both the progressive decrease in reflectance at short wavelengths and the simultaneous progressive increase in reflectance at the longer wavelengths, when going from area 1 to 5, are proportional to the bead density. We have therefore further analyzed the pigmentation of the *P. philea* scale as follows. We argued that the reduction in short-wavelength reflectance with respect to the long-wavelength reflectance must be caused by an absorbing pigment that selectively absorbs light traveling through the scale and is only partly leaving the scale again as backscattered light. To derive the absorption spectrum of the pigment, we modified a classical analysis method for pigments in complex media, namely to

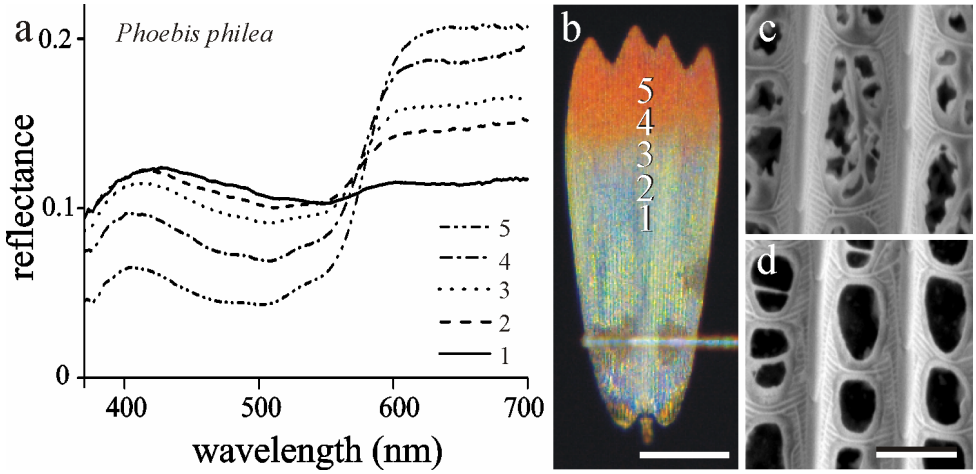

Fig. 4. A natural, partially coloured scale of a female *Phoebis philea*. **a** Reflectance spectra of five areas along the scale, which is shown in (**b**) glued to the tip of a glass micropipette; the numbers correspond to the five areas studied. SEM image of an orange-red area (**c**), and of a white area (**d**). The reflectance in the white area is rather constant, around 12%. Bars: 50 µm (b) and 1 µm (c, d).

calculate absorbance difference spectra, i.e., the spectral differences between the –log10 of the transmittance measured in states with different pigment concentrations. For each location 1-5 of Fig. 4, we have calculated a modified absorbance, by taking the –log10 of the reflectance spectrum with respect to the long wavelength reflectance (taken as the average reflectance between 620 and 700 nm). We subsequently subtracted absorbance spectrum 1 from the other absorbance spectra, assuming that pigment absorption in location 1 was negligible. We thus obtained four absorbance difference spectra (2-5), presented in Fig. 5. The spectra appeared to be perfectly proportional to each other, suggesting that the magnitudes are proportional to the density of pigmented beads. To obtain the pigment absorption spectrum, we first normalized the four absorbance difference spectra of Fig. 5, and then we calculated the average (av, Fig. 5). For comparison, we added the absorption spectrum of erythropterin, the pterin extracted from orange and red coloured wings (Fig. 5: ery; from Wijnen *et al.* 2007(Wijnen *et al.* 2007)). If indeed erythropterin is the pterin pigment of the orange-red scale of *P. philea*, then the absorption spectra of the pigment in situ (av) and in solution (ery) rather deviate, an observation also made on extractions of intact wings by Wijnen *et al.* (2007).

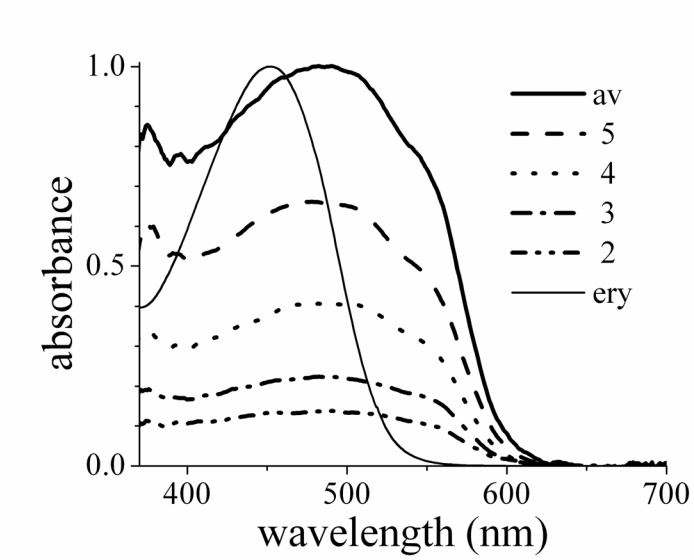

Fig. 5. Absorbance difference spectra (2-5) of the orange-red scale of *Phoebis philea* (Fig. 4b), calculated from the spectra of Fig. 4a (see text). The numbers refer to the scale locations of Fig. 4b. Normalization of spectra 2-5 with subsequent averaging yielded the pigment absorption spectrum (av). The absorption spectrum of erythropterin (ery) is given for comparison (from Wijnen *et al.* 2007).

5.3.3 Optics of pierid scales

The structural and optical observations on pierid scales presented above can be summarized with a simplified two layer model for the light flux in a scale (Fig. 6). Fig. 6a is a transmission electron microscopic section of a typical pierid wing scale (a ground scale of the dorsal wing of a male Brimstone, *Gonepteryx rhamni*). Incident light is partially reflected (back-scattered) and transmitted (forward-scattered) by the structures of the upper lamina of the scales; that is, by the ridges, crossribs and beads. The transmitted light is in turn partially reflected and transmitted at the lower lamina of the scale. The light reflected at the lower lamina is subsequently partially reflected and transmitted at the upper lamina, and so on (Fig. 6a). The resulting scale reflectance (or transmittance), which is the fraction of the incident light that is reflected (transmitted), hence is the sum total of the primary, secondary, etc., reflected (transmitted) light fractions (Fig. 6b). We have to note here, of course, that the upper lamina of a scale is not a continuous layer, especially when the scale has large open windows and when the bead density is low (Figs. 1c, 2b, 2d). A considerable fraction of the incident light then will bypass the upper lamina structures and arrive undiminished at the lower lamina. Part of the light reflected by the lower lamina can similarly pass the upper lamina through the windows. Scales with few beads, and thus little absorbing pigment, will then yield a rather flat reflectance spectrum. However, with a high

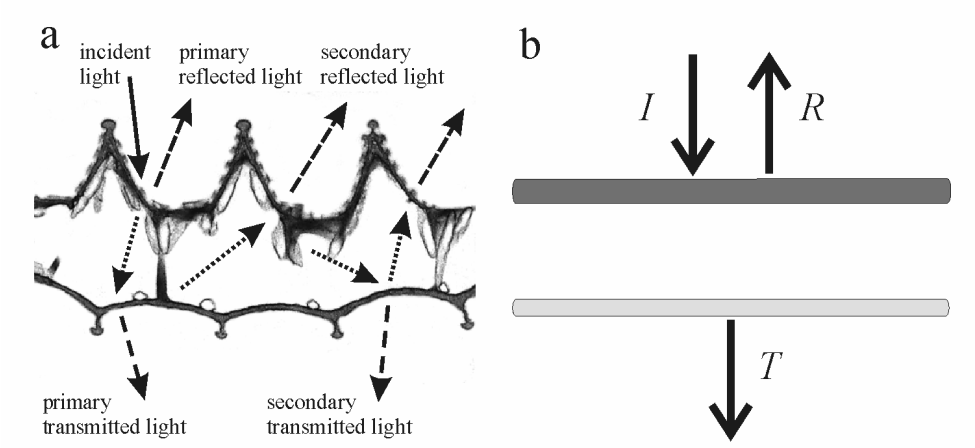

Fig. 6. a Transmission electron microscopic image of a ground scale of the Brimstone, *Gonepteryx rhamni* (Pieridae, Coliadinae). **b** Schematic indications of the light flux in the scale. The dashed arrows represent possible trajectories of the light scattered by the scale structures. Incident light is partially back-scattered by ridges, crossribs and beads located in the upper lamina. The forward-scattered light is partly reflected by the smooth lower lamina, and then scattered again by the upper lamina structures, both backward and forward. In this way, the light reflected by the lower leaf has a second chance to be absorbed by the pigmented beads, and this process is repeated numerous times. In (b), the scale reflectance, *R*, which is the fraction of the incident light flux (solid arrow) that leaves the upper lamina in the upward direction, is the sum total of the backward scattered light

bead density, the windows are largely beset by the pigmented beads, and thus with a high absorption by the beads in the short wavelength range and a strong light scattering in the long wavelength range, a low reflectance at short wavelengths and a high reflectance at long wavelengths results.

A single scale can be treated as consisting of two layers, but effectively it acts as a single layer with reflectance (*R*) and transmittance (*T*), as indicated in Fig. 6b.The intact wing similarly can be considered as a single layer, because the scales on the intact wing overlap, forming scale stacks, and thus they determine, together with the wing membrane, the overall wing reflectance and transmittance. In Section 5.3.4 we present measurements of the wing reflectance and transmittance of the Large White, *Pieris brassicae*, but first we will discuss the reflectance of the most simple scale stack, consisting of two scales (Fig. 7). Figs. 7a and 7b present the reflectance spectra measured from the usual locations (1-5, see Fig. 1b) of two white scales (A and B) isolated from the dorsal wing of a Large White. The two spectral sets are, of course, not identical, but they are very similar, with a decreasing reflectance in the UV and an increasing reflectance in the visible range, when going from the scale base to the scale tip. To study the effect of overlap, we mounted the two microelectrodes with scales A and B on two

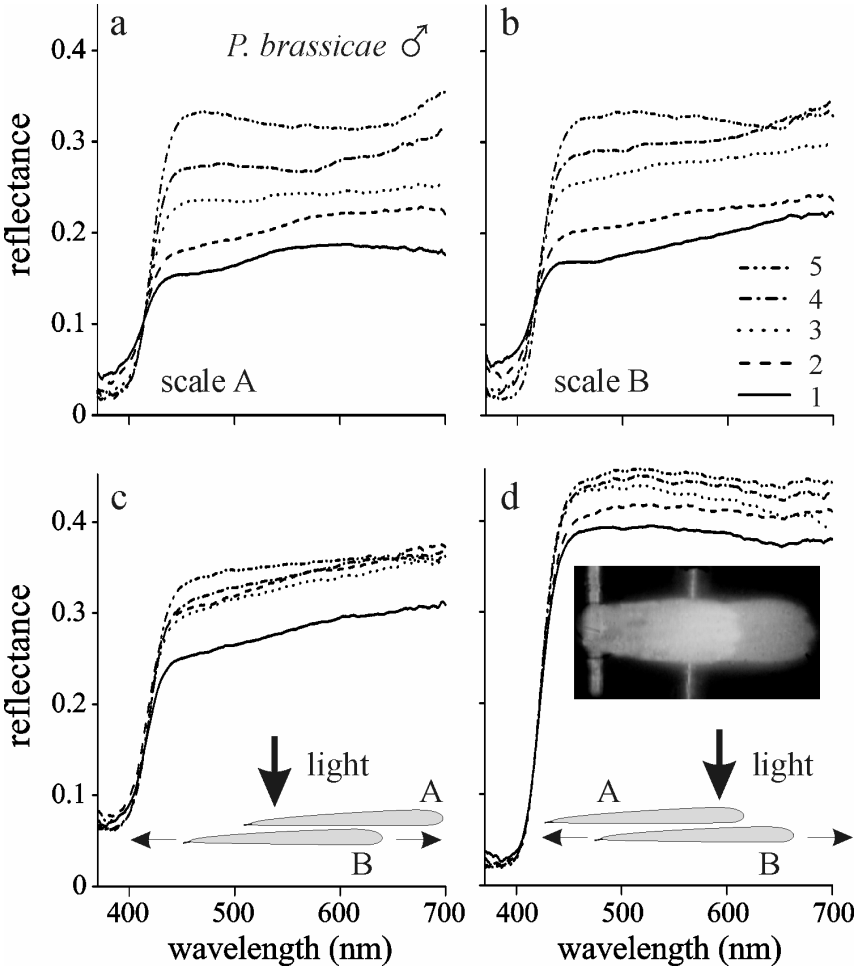

Fig. 7. Reflectance spectra of two scales of a male *P. brassicae*. Spectra of isolated scale A (**a**) and of isolated scale B (**b**). **c** Spectra of the base of scale A (location 1) over five areas of scale B (locations 1-5). **d** Spectra of the tip of scale A (location 5) over five areas of scale B (locations 1-5; see Fig. 1b). Overlapping scales have a higher reflectance than the isolated, single scales. The photograph inset shows the two overlapping scales, in a position approximating that in situ, but here glued to micropipettes. Notice that the area of the superimposed scales looks whiter. The horizontal arrows in the inset diagrams indicate that scale B was moved in steps below a stable scale A.

separate micromanipulators, which allowed precise manipulation of the scales (inset photograph, Fig. 7d). The distance of the scale planes was ca 10 µm, which is about the usual distance of scales on the wing. In the experiment of Fig. 7c, scale B was moved in small steps, so that its locations 1-5 were directly underneath location 1 of scale A, and then the reflectance was measured in each

of the five situations (see inset diagram). In the experiment of Fig. 7d, scale B was similarly moved in small steps, so that locations 1-5 were directly underneath location 5 of scale A. The vertical arrows in the inset diagrams of Figs. 7c and 7d indicate the incident light beam. The reflectance spectra show that the reflectance in the basal area is about 18% for a single scale (Figs. 7a, b), which increases to maximally about 35% for a stack of two scales (Fig. 7c). The reflectance in the tip area is about 33% for a single scale (Figs. 7a, b) and increases to about 45% for a stack of two scales (Fig. 7d). This demonstrates that the enhancement of the reflectance by scale stacking strongly depends on the characteristics of the top layer. The long wavelength-reflectance values of Fig. 7d (tip of A on top) are all larger than those of Fig. 7c (base on top). This suggests that the function of the top position of the highly reflecting scale tips is to optimize wing reflectance.

5.3.4 Optics of pierid wings

In the native, intact wing situation, scale stacks exist on both sides of the wings. We previously performed a detailed analysis of the effect of scale stacking for the Small White, *Pieris rapae* (Stavenga *et al.* 2006). Here we present the same treatment for the Large White, *P. brassicae* (Fig. 8). Briefly, we used an integrating sphere to measure the reflectance, R (Fig. 8a, b), and transmittance, T (Fig. 8c, d), of intact and denuded forewings, and we then calculated the absorptance, that is the light fraction absorbed, with $A = 1 - R - T$ (Fig. 8e, f). The measurements of Fig. 8a show that the long-wavelength reflectance of the intact wing, using incident light from the dorsal side (DWV), is higher than that when the illumination is from the ventral side (VWD), thus revealing an asymmetry in the wing optics. Fig. 8c shows a similar asymmetry for the wing transmittance. A clear asymmetry also occurs when the scales on one side of the wing are removed (for instance DW vs WD, Fig. 8a, c; or WV vs VW, Fig. 8b, d). The wing scales strongly absorb short-wavelength light, presumably due to the scale pigment leucopterin (Wijnen *et al.* 2007), and also the wing substrate appears to be slightly pigmented (Fig. 8f).

By considering the scale stack on the dorsal side (D), that on the ventral side (V), and the wing substrate (W) each as a separate layer, we can calculate the two opposite reflectances, r and s, as well as the two transmittances, t and u (Fig. 9b, inset), together with the absorptances, a, with the formalism of Stavenga *et al.* 2006. The reflectances in the visible wavelength range of the dorsal (r_D and s_D, Fig. 9a) and ventral (r_V and s_V, Fig. 9b) scale stacks were determined to be about 30-40%, similar to the measured reflectances of the artificial stacks of two scales of Fig. 7. Indeed, visual inspection shows that on the wings of *P. brassicae* in average about two scales overlap. The scale stacks on both sides of the wing, together with the wing substrate, result in a total reflectance of up to 70% (Fig. 8).

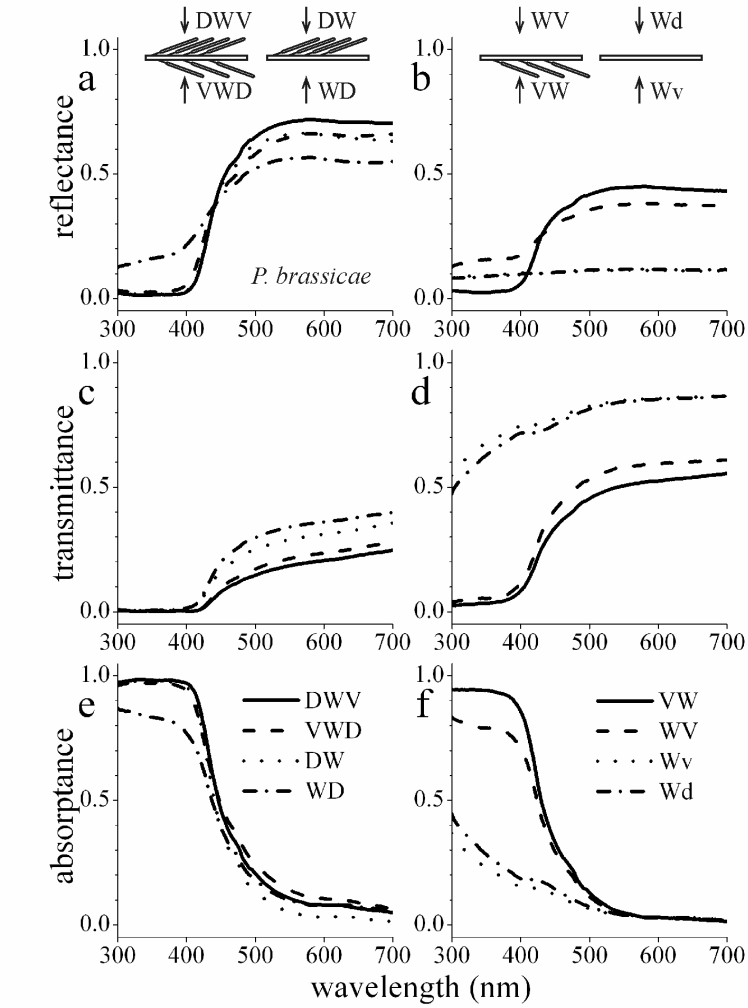

Fig. 8. Reflectance (**a**, **b**) and transmittance (**c**, **d**) spectra measured with an integrating sphere from forewings of the Large White butterfly, *Pieris brassicae*, in various conditions, together with the calculated absorptance spectra (**e**, **f**). The wing was intact for the conditions DWV and VWD, where D indicates the dorsal side of the wing, W is the wing substrate, and V is the ventral side; the order of the letters indicates the direction of the incident light. For DW and WD, the wing scales were removed from the ventral side, and for VW and WV, the wing scales were removed from the dorsal side. For Wv and Wd, both dorsal and ventral scales were removed, and the incident light came from the ventral (v) and dorsal side (d), respectively. The scales contain a strongly UV absorbing pigment, resulting in a very low transmittance and a very high absorptance in the ultraviolet. The wing scales strongly scatter in the visible wavelength range. The reflectance of the denuded wing is virtually constant throughout the whole spectral range (b), and the wing substrate contains a small amount of pigment that absorbs in the UV (f).

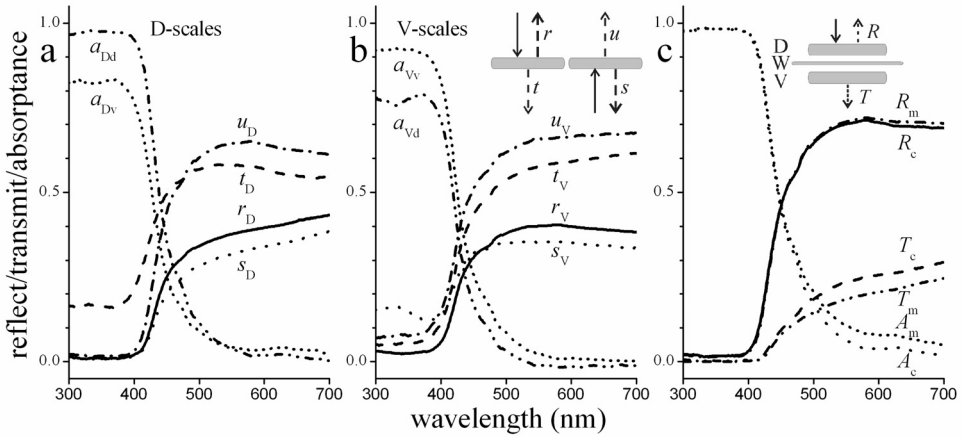

Fig. 9. Reflectances, *r* and *s*, and transmittances, *t* and *u*, of the dorsal (**a**, index D) and ventral (**b**, index V) scale layers of the dorsal forewing of *P. brassicae*, calculated with the data of Fig. 8 and the formalism of Stavenga et al. (2006). Inset in (**b**): reflectance *r* and transmittance *t* refer to incident light directed towards the wing substrate; reflectance *s* and transmittance *u* refer to incident light directed away from the wing substrate. The absorptance for the D-scales illuminated from the dorsal (d) side was calculated from $a_{Dd} = 1 - r_D - t_D$, and the absorptance with illumination from the ventral (v) side was calculated from $a_{Dv} = 1 - s_D - u_D$. **c** The reflectance and transmittance spectra measured from the intact wing with incident light from the dorsal side (DWV, see inset), R_m and T_m (continuous lines in Fig. 8a and 8c), compared with the spectra, R_c and T_c, calculated with the formalism of Stavenga et al. (2006). The calculated transmittance is slightly larger than the measured transmittance, and accordingly the calculated absorptances, calculated with $A_{m,c} = 1 - R_{m,c} - T_{m,c}$, slightly differ.

As we have argued before, this will be about optimal, as a further increase in the number of overlapping scales will enhance the reflectance only to a minor extent (Stavenga *et al.* 2006).

A basic assumption of the applied model is that light scattering in the various layers is random (Stavenga *et al.* 2006). Direct measurements of scattering by single scales of the Small White, *Pieris rapae*, indicate that this assumption approximately holds (Giraldo *et al.* 2007). A further check of the model can be performed by calculating the reflectance and the transmittance of the intact wing from the known reflectances and transmittances of the three wing layers (Stavenga *et al.* 2006). The reflectance spectrum calculated for dorsally incident light (DWV, R_c) indeed well matches the measured reflectance spectrum (R_m, Fig. 9c), but the transmittances, and accordingly the absorptances, slightly differ. Scattering in the *Pieris* scales hence is approximately, but not perfectly random (Giraldo *et al.* 2007). The absorptances indicate that ultraviolet incident light is

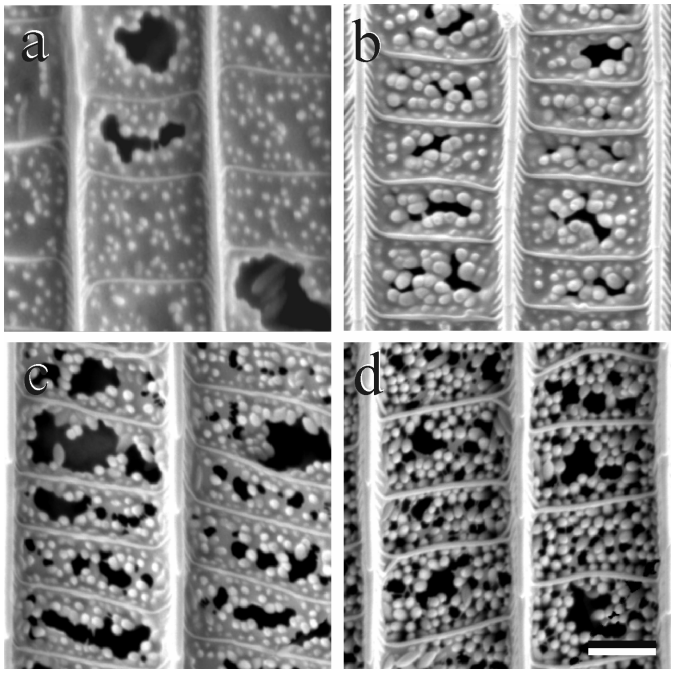

Fig. 10. Scales of *P. r. crucivora* arrested in different stages of development. **a** In the immature scales, a lamina covers the windows, and pigment granules (beads) are wrapped within the lamina material. (**b**-**d**) In more developed scales, the beads are becoming more and more separate, and connected only by thin strands of lamina material onto each other and to the crossribs. Bar: 1 µm.

virtually completely absorbed, and that the scales also absorb a substantial fraction of blue light, thus yielding the very slightly yellowish coloration of the Large White.

5.4 Discussion

The pterins of pierids are unusual among butterfly pigments, because they are localized to granular beads. The distribution of the beads in the pierid scales appears to vary strongly among species. For instance, the scales on the dorsal wings of female *P. r. crucivora* virtually lack beads, while the males have scales with a high concentration of beads, but with a distinct longitudinal gradient (Figs. 1, 2). The orange-reddish scales of *P. philea* have an extreme gradient, with only orange-red pigmentation in the scale tip (Fig. 4b).

Another variation in the scale structure is the shape of the windows, which in most pierids are wide open, well-defined by the ridges and the rather thin crossribs. In the *P. philea* scales, the windows are much less open and partly filled by a laminar membrane (Fig. 4c, d). Actually, quite frequently some membranous structure can be seen in the windows, as shown in Fig. 10, which presents scales of a male *P. r. crucivora* that are presumably arrested in various stages of development. In Fig. 10a, the upper lamina covers the windows while pigment

granules are wrapped within the lamina membrane material. The beads are separated to various degrees in other scales, where they are connected to each other and to the crossribs by thin strands of membrane material (Fig. 10b-d).

The separation in numerous distinct beads is extreme in males of the Small White and Large White, and then the beads create extreme optical effects. The beads effectively absorb short-wavelength light, but at the same time strongly scatter long-wavelength light, thus distinctly whitening the wings (Stavenga *et al.* 2004; Morehouse *et al.* 2007). When the beads were embedded in a more or less continuous membrane, as in Fig. 10a and 10b, a much lower reflectance would result. By stacking the well-scattering scales in two overlapping layers on each side of the wing, a very high wing reflectance of 60-70% is realized, a remarkable achievement given the minimal amount of material mass that is involved.

During development, butterfly wings generally have to specify several patterns at once, for instance the basic shape, venation patterning, deployment of scales, distribution of pigmentary and structural colours, the details of scale morphology. Our present findings show that additional details are the degree of pigment gradients and scale overlap.

We conclude from our combined structural and optical experiments, on single scales as well as on intact and denuded wings of pierid butterflies, that the non-iridescent coloration of pierid wings can be largely understood from the random light scattering in the overlapping layers of scales. Of course, there are several remaining questions, for instance about the development of the scales, about possible functional reasons for the variations in bead gradients among species, and about the chemical mechanisms that cause the deviant pterin absorption spectra in situ (Wijnen *et al.* 2007).

Acknowledgements

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Chapter 6
Far field scattering pattern of differently structured single butterfly scales

The angular and spectral reflectance of single scales of five different butterfly species was measured and related to the scale anatomy. The scales of the pierids *Pieris rapae* and *Delias nigrina* scatter white light randomly, in close agreement with Lambert's cosine law, which can be well understood from the randomly organized beads on the scale crossribs. The reflectance of the iridescent blue scales of *Morpho aega* is determined by multilayer structures in the scale ridges, causing diffraction in approximately a plane. The purple scales in the dorsal wing tips of the male C*olotis regina* act similarly as the *Morpho* scale in the blue, due to multilayers in the ridges, but the scattering in the red occurs as in the *Pieris* scale, because the scales contain beads with pigment that does not absorb in the red wavelength range. The green-yellow scales of *Urania fulgens* backscatter light in a narrow spatial angle, because of a multilayer structure in the scale body.

6.1 Introduction

Many butterflies have vivid, colourful wing patterns, created by rows of partly overlapping scales with intricate spatial structures that scatter incident light. The scattered light can interfere coherently or incoherently. When the scale structures have spatial periodicity, the interference of coherent light waves often results in striking iridescences. The displayed colours are then called structural or physical. Without periodicity, light scattering is incoherent or random, resulting in white scales, unless they contain pigment that selectively absorbs in a certain wavelength range. In the latter case a pigmentary or chemical colour results (Fox

& Vevers 1960; Vukusic & Sambles 2003; Kinoshita & Yoshioka 2005). Many butterfly species feature structural as well as pigmentary colours (Rutowski *et al.* 2005; Yoshioka & Kinoshita 2006b).

Each butterfly wing scale is the cuticular product of a single cell, with a rather flat, unstructured lower scale lamina and a highly structured upper lamina, typically consisting of longitudinal ridges connected by crossribs (Ghiradella 1998). The fine structure of butterfly scales is highly variable (Vukusic *et al.* 2000). For instance, the crossribs of the white scales of pierids are adorned with granules (Yagi 1954; Morehouse *et al.* 2007; Giraldo & Stavenga 2007a), the ridges of *Morpho* scales and many male Pieridae are elaborated into multilayer structures (Ghiradella *et al.* 1972; Vukusic *et al.* 1999; Kinoshita *et al.* 2002), and many papilionids and lycaenids have scales with photonic crystal properties (Vukusic *et al.* 2002; Vukusic & Sambles 2003; Kertesz *et al.* 2006).

The scales reflect only part of the incident light flux, and the remaining part is transmitted unless it is absorbed by pigment. Because the scales are arranged in layers on both sides of the wing, incident light suffers reflection and transmission in each layer of the scale stacks, and therefore the wing reflectance is not solely due to backscattering by the top layer, the cover scales, but it is the cumulative effect of the scale stacks on both wing sides. Yoshioka *et al.* 2006b investigated this phenomenon in *Morpho* wings, and to explain the reflectance spectra of intact wings from the spectra of individual scales they used a simplified scale stack model, assuming that normally incident light rays are also reflected and transmitted normally. (Stavenga *et al.* 2006) developed a more general model for the reflectance of butterfly wings to interpret reflectance measurements performed on intact as well as partly or completely denuded wings of the Small White butterfly, *Pieris rapae*. The basic assumption of the latter model, that the scales scatter randomly, was however not specifically demonstrated.

Knowledge of the spatial and spectral reflectance characteristics of single scales is essential to further develop our understanding of the coloration principles applied by butterflies. In the present study we investigate the scattering properties of wing scales of a variety of butterflies, and we correlate the scattering diagrams with the electron micrographs of the scale structure. We show that white scales of pierid butterflies are approximately random scatterers. Iridescent scales, with multilayer structures, exhibit directional reflection. Whereas some butterfly species appear to have scales with dominant iridescence, other species combine iridescence and scattering properties.

6.2 Materials and methods

Animals

We investigated the scales of a variety of butterflies. The Small White, *Pieris rapae rapae*, was obtained from a continuous culture maintained by Dr J.J. van Loon, Entomology Department, University of Wageningen (the Netherlands). The black jezebel, *Delias nigrina*, was captured near Bateman's Bay, Australia. The *Morpho aega* was purchased. The Purple Tip, *Colotis regina*, was received from the butterfly collection of the Royal Museum for Central Africa, Brussels (Belgium; curator Dr U. Dall'Asta). The moth *Urania fulgens* was a gift from Dr Marta Wolff, Entomology group, University of Antioquia (Medellín, Colombia).

Angular distribution of scattering by single scales

Single scales were isolated by gently pressing the wing of a butterfly on a glass plate. Subsequently, an isolated scale was glued to the tip of a glass micropipette (tip diameter ca 5 μm). The angular distribution setup was used (Section 2.2.3). The scale was always hanging with the longitudinal ridges of the scale oriented vertically. Light from a xenon lamp was focused on a pinhole with diameter 50 μm, which was subsequently imaged on the scale. The light beam, which had an aperture of $< 10^{o}$, passed a small hole in a screen before it reached the scale. The scale reflected (that is, back-scattered) part of the incident light. The angular spread of the reflected light was documented by photographing the light distribution at the white backside of the screen (Fig. 4a, Chapter 2). We used a Nikon Coolpix 990 (Fig. 1) and an Olympus DP70 (Fig. 5). The digital images were processed with MatLab (Fig. 5).

Spectrophotometry

The angular dependence of the light scattering by the scale was measured with a lightguide, which was mounted on a rotating motor and connected to a spectrometer (Yoshioka & Kinoshita 2006a). The scales were adjusted so that the scale plane was perpendicular to the light beam, as judged by the symmetrical reflection pattern with respect to the axial direction. The experiments were run using a Labview interface, which allowed the measurement of reflectance spectra in angular steps of 1^{o} over a 180^{o} angle. Angular reflectance curves were calculated for a series of wavelengths with 10 nm interval by sequentially averaging the reflectance spectra over 10 nm wavelength ranges. In addition, the spectral reflectances of single scales were measured with a microspectrophotometer, consisting of a xenon light source, a Leitz Ortholux microscope, and a fibre optic spectrometer. The microscope objective was an

Olympus 20x, NA 0.46. A white reflectance standard (Spectralon, Labsphere, North Sutton, NH, USA) served as the reference in all cases.

Electron microscopy

Subsequent to the microspectrophotometry, the single scales were prepared for scanning electron microscopy (SEM). Additionally, pieces of wing were cut and put on the SEM-holder in different positions to visualize the upper surface as well as cross-section of the scales. Samples were sputtered with palladium for 5 min at 800 V and 200 mTorr (Hummer, Alexandria, VA, USA). A Philips XL-30 scanning electron microscope with a voltage of 3 kV was used to investigate the scale anatomy. For transmission electron microscopy, wing pieces were prepared as usual. In brief, samples were embedded in agar for better handling, prefixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, and postfixed in 1% OsO_4 / 1.5% K_4 $Fe(CN)_6$ in 0.1 M cacodylate. Subsequent washing with double-distilled water and dehydration with an alcohol series that ended with 100%, were followed by propylene oxide during 30 min and embedding in Epon. Post-microtomed samples were contrasted with uranyl acetate in methanol for 2 min and lead in water for 1 min, and were then examined with a Philips 201 transmission electron microscope.

6.3 Results

The white scales of the Small White, *Pieris rapae*, are marked by ovoid beads that adorn the crossribs (Fig. 1a). The angular light scattering of a single scale can be readily visualized with the setup of Fig. 1a, where a beam of white light is focused on a scale via a small hole in a screen. Fig. 1b is a photograph of the screen, showing the angular distribution of the light reflected by a white scale taken from the dorsal wing of a male Small White, *Pieris rapae*. The scale scatters light approximately circular-symmetrically, suggesting that the scale acts as a diffuse scatterer.

The angular distribution of the scattering was quantitatively investigated with the setup of Fig. 1b, where the light reflected by the scale is measured as a function of angle in the horizontal plane. Fig. 1c presents the reflectance of the scale of Fig. 1b as a function of angle for a number of wavelengths, normalized to the maximal reflectance value; the angle is 0^o for the normal to the scale. The scale's reflectance spectrum for normally incident light is given in Fig. 1d. The reflectance is high in the visible wavelength range, but it is low in the ultraviolet, because of an ultraviolet-absorbing pigment, presumably leucopterin (Wijnen *et al.* 2007), which is concentrated in pigment granules, the ovoid beads (Fig. 1a).

The beads act as strong scatterers at wavelengths where the pigment absorption is negligible (Stavenga *et al.* 2004; Morehouse *et al.* 2007).

In addition to the beads, the other structures of the scale, that is the ridges and crossribs of the upper lamina of the scale as well as the lower lamina, contribute to the scattering (Fig. 1c).

Fig. 1e-h presents a similar set of data for a white scale of the dorsal forewing of a male black jezebel, *Delias nigrina*. This case is obviously very similar to that of the white *P. rapae* scale. The scale anatomy with crossribs studded with beads is very similar (Fig. 1e), the scattering is again approximately random (Fig. 1f, g), and the reflectance spectrum is also high in the visible and low in the UV (Fig. 1h).

The strikingly blue *Morpho aega* has scales where the lamellae of the ridges form multilayers (Fig. 1i). Unlike *Morpho didius*, for example, which has glass cover scales and strongly pigmented ground scales (Vukusic *et al.* 1999), cover and ground scales of *M. aega* cannot be distinguished. The scattering diagram of a scale of a *Morpho aega* is a horizontal stripe (Fig. 1j), perpendicular to the vertically oriented scale ridges. The angular dependence of the reflectance varies strongly with the wavelength (Fig. 1k), and the reflectance measured with normally incident light features a distinct peak in the blue (Fig. 1l), which is due to the multilayered structure of the scale ridges (Fig. 1i; see also (Vukusic *et al.* 1999; Kinoshita *et al.* 2002; Yoshioka & Kinoshita 2004). A reflectance peak value of more than 2 results, because the scale's scattering is highly directional and the reflectance was measured relative to the diffusely scattering white standard (Fig. 1l).

The purple scales at the dorsal wing tips of the male Purple Tip, *Colotis regina*, have ridges that are fine-structured similarly as in the case of *Morpho aega* (Fig. 1m). The purple scale features a scattering diagram with a blue stripe and a red diffuse pattern (Fig. 1n), which is a mixture of the stripe phenomenon of Fig. 1j and the diffuse patterns of Fig. 1b and 2f. The blue stripe is due to light backscattered by the fine-structured ridges, and the red diffuse pattern results from randomly scattered light, filtered by a pigment contained in granules below the multilayered ridges (Fig. 1m). The blue peaking reflectance spectrum, shown in Fig. 1p, is mainly due to reflection by the ridges, and the red band, above 600 nm, is mainly caused by the light scattering pigment granules (Fig. 1p).

A green-yellow reflecting scale of the moth *Urania fulgens* has between the upper and lower scale laminae (Fig. 1q) an elaborate multilayer system, which yields a spatially restricted, directional scattering diagram (Fig. 1r, s). The high amplitude of 3 of its reflectance spectrum is again due to the directionality of the scale reflectance (Fig. 1t). The reflectance spectrum features a distinct band, peaking at

590 nm, with halfwidth about 120 nm, indicating that an interference reflector is involved.

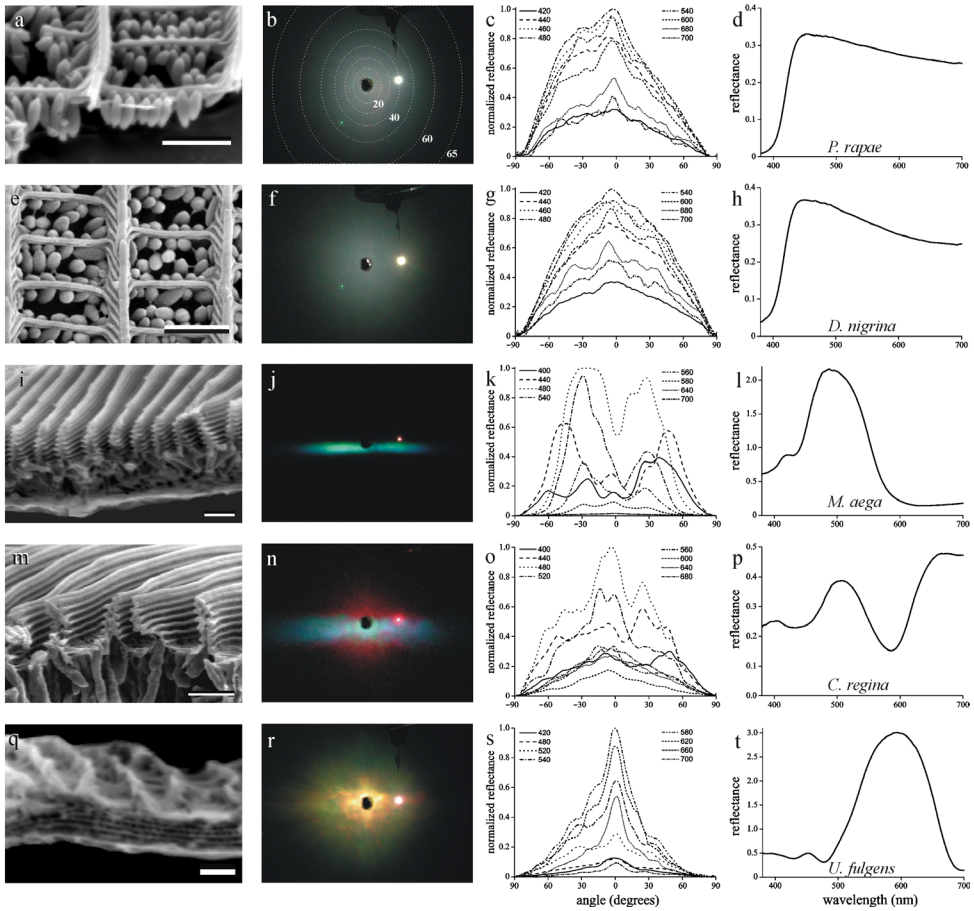

Fig. 1. Angular and spectral reflectance of isolated butterfly scales. **a-d** White scale of the dorsal forewing of a male *Pieris rapae*. **e-h** White scale from the dorsal forewing of a male black jezebel, *Delias nigrina*. **i-l** Blue scale of a *Morpho aega*. **m-p** Purple scale from the tip of the dorsal forewing of a male Purple Tip, *Colotis regina*. **q-t** Orange-white scale of *Urania fulgens*. First column: scanning electron microscopy of scale cross-sections made by cutting pieces of wing with a razor blade.. Second column: photographs of the angular distribution of light reflected by a scale and subsequently reflected by a white screen, where the scale was illuminated with a narrow beam of white light (see Fig. 1a). The scales were glued at the tip of a glass micropipette, which was suspended from above on a micromanipulator; see the black shadow in the upper part of (b) and (f). The bright spot at the right of the central black hole is due to light forward scattered by the scale. The ellipses in b (due to the slightly oblique position of the camera) represent directional angles in steps of 10^o, from 10^o to 60^o, as well as that for 65^o. The hole in the screen is ca 8^o. Third column: angular dependence of the reflectance in the horizontal plane, measured with the setup of Fig. 1b. Fourth column: reflectance spectra of single scales measured with a microspectrophotometer. Bars: 1 µm

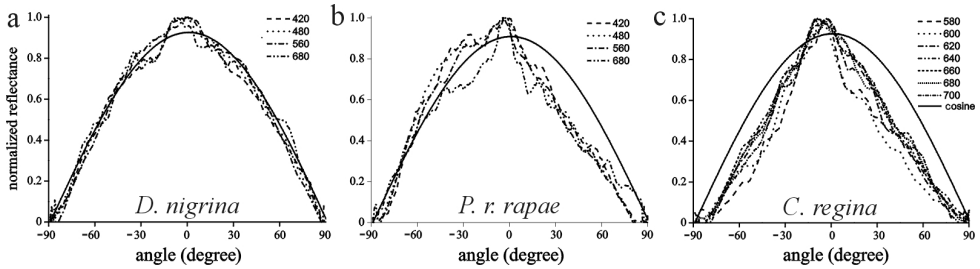

Fig. 2 Normalized angular reflectances measured from the scales of the three pierid butterflies, *P. rapae*, *D. nigrina*, and *C. regina*, compared to a Lambertian reflector (bold line). Angular reflectance curves are shown in the wavelength range where the reflectance is high, that is, where pigment absorption is low. Four wavelengths covering the visible range are shown for *P. rapae* (**a**) and *D. nigrina* (**b**). The scattering diagrams of *D. nigrina* well approximate a Lambertian reflector, but the angular dependence of the scattering by *C. regina* (**c**) shows regular peaks, which indicates that the light scattered by the pigment granules is affected by the multilayered ridges.

To evaluate whether the beaded scales of *Pieris rapae, Delias nigrina* and *Colotis regina* act as diffuse scatterers, the angular reflectances of these species were normalized and compared with a Lambertian reflector (Fig. 2). The wavelengths selected are outside the absorption bands of the pigments, which means that the wavelengths chosen for *P. rapae* and *D. nigrina* are in the visible spectrum, but for *C. regina* only in the red wavelength range. The white scale of *D. nigrina* (Fig. 2b) well approximates a Lambertian reflector. The angular distribution patterns of the white scale of *P. rapae* slightly deviates from the ideal curve (Fig. 2a), but notably the purple scale of *C. regina* is not a perfectly diffuse red scatterer. Presumably this is due to the highly structured, multilayered ridges.

6.4 Discussion

The relationship between the optical properties of butterfly scales and their structure has been the topic of several studies (e.g. Kinoshita *et al.* 2002; Vukusic *et al.* 1999; Vukusic *et al.* 2002). Most of the previous investigations have focused on iridescent scales. Here, we have compared the reflection pattern of five differently structured single butterfly scales that scatter light coherently or incoherently, or both.

We started with a simple white scale common to many species of the pierid subfamily Pierinae. Due to the characteristic beaded structure light scattering is strong, thus causing the intense white colour. We find that the white pierid scales approximate the properties of a Lambertian diffuser, at least in the wavelength range where pigment absorption is negligible (Fig. 2).

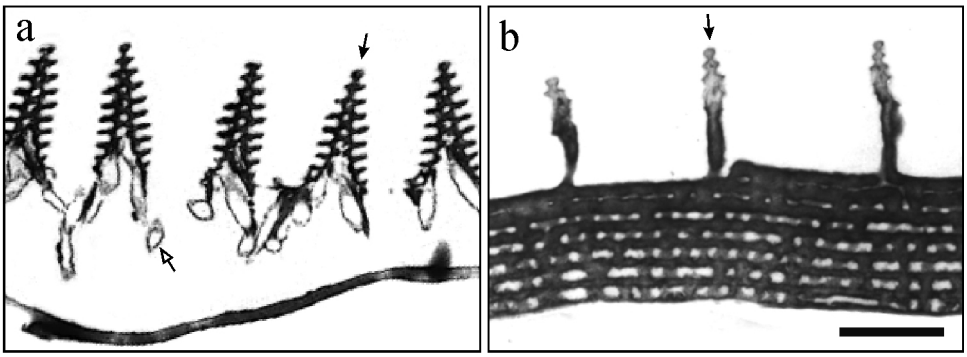

Fig. 3 Transmission electron microscopy images of a cover scale of the dorsal wing of a male brimstone, *Gonopteryx rhamni* (**a**), and of a scale of *Urania fulgens* (**b**). Ridges are indicated by closed arrows. The brimstone scale has multilayers restricted to the ridges. The space between ridges and crossribs is studded with pigment granules (open arrow). *U. fulgens* has continuous multilayers connected by numerous pillars that result in spreading of the scattering. Bar: 1 μm.

The ridges of the scales of *Morpho* butterflies cause a blue colour. Melanin pigment below the multilayered ridges absorbs stray light over the whole visible wavelength range, including the ultraviolet, thus supporting the strikingly blue wing colour (Yoshioka and Kinoshita, 2006a). The pigment of the scales in the dorsal wing tips of *C. regina* also absorbs stray light, but not in the long-wavelength range. The remaining red light together with the blue iridescence causes the purple colour.

A similar, however short-wavelength-shifted case is formed by the yellow scales of the dorsal wings of many male butterflies of the pierid subfamily Coliadinae, where a UV and blue absorbing pigment is combined with UV iridescence (Ghiradella *et al.* 1972; Rutowski *et al.* 2005). Fig. 3a is a transmission electron micrograph of a yellow cover scale from the dorsal wing of a male brimstone, *Gonepteryx rhamni*. The UV iridescent, yellow scales of the male brimstone have numerous beads, which contain the yellow pigment xanthopterin (Wijnen *et al.* 2007). The beads are seen in Fig. 3a as empty ovoids, but this is presumably due to the procedures for transmission electron microscopy (Morehouse *et al.* 2007; Giraldo & Stavenga 2007a). The scattering diagram of *Urania fulgens* is not perfectly directional (Figs. 2r, s), which should have been the case when the scales consisted of an ideal multilayer. Transmission electron microscopy shows that between the multilayers exist pillars (Fig. 3b), which presumably cause the spread in the scattering diagram. Of course, the scales -and therefore the multilayers- are also not perfectly flat. The ridges (see Figs. 2q and 4b) will further contribute to some diffuse scattering.

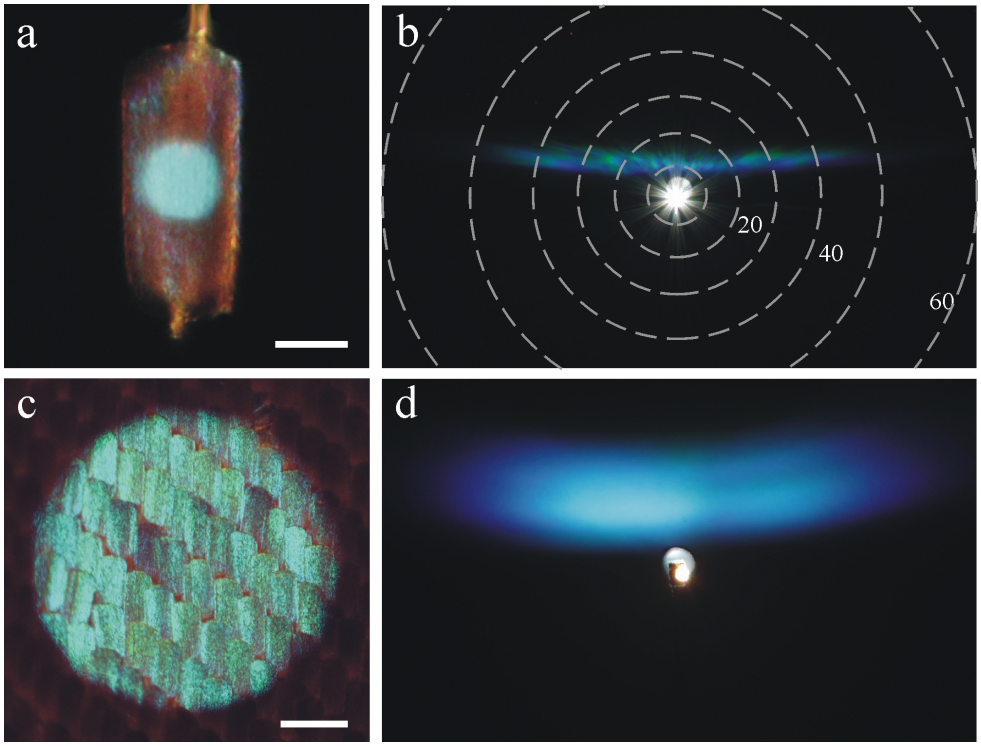

Fig. 4 Scattering by scales of *Morpho aega*. **a** Single scale about normally illuminated by a beam with about 50 µm diameter; the outline of the scale is visible because of an additional, obliquely illuminating beam. **b** Far field scattering pattern of the single scale photographed at a white screen (see Fig. 2j). The bright white spot is due to light scattered at the hole in the white screen and direct scattering by the scale. The interrupted circles indicate the angular directions of the scattering in steps of 10^{o}. **c** A wing piece about normally illuminated by a beam with about 900 µm diameter; cover and ground scales cannot be distinguished. **d** Far field scattering pattern of the wing piece. The white spot is due to light scattered at the hole in the white screen and to direct scattering by the scale. Bars: 50 µm (a) and 200 µm (c).

Knowing how light is scattered by individual scales is necessary for a proper understanding of butterfly wing coloration. As has been previously demonstrated, wing reflectance is a cumulative effect due to multiple reflection and transmission by the layers of scales on the butterfly wing (Yoshioka & Kinoshita 2006b). The scales are often arranged in quite regular rows and have approximately the same orientation with respect to the wing surface, but of course there are deviations from perfect order. This will be rather irrelevant in the case of the Small White, *Pieris rapae*, where scattering is approximately diffuse, but in cases where scattering is highly directional, as in *Morpho* butterflies, disordered scale

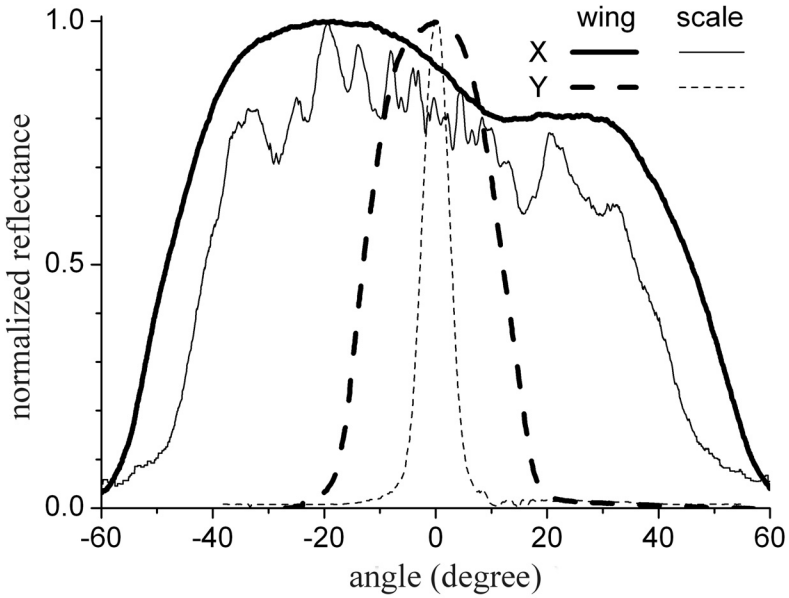

Fig. 5 Spatial profiles of the scattering diagrams of Figs. 5b and 5d. The extent of the scattering in the horizontal plane (X) is much broader than the scattering in the vertical plane (Y) for both the single scale as for the intact wing piece. The angular width in the vertical plane of the scattering by the single scale is < 10^o, but the width in the horizontal plane is about 100^o. The vertical angular extent of the scattering by the wing piece is about 30^o, meaning a broadening of about 20^o with respect to the single scale, and the extent in the horizontal plane is similarly broadened.

arrangement will result in broadening of the scattering diagrams. This is illustrated in Fig. 4, where scattering by a single scale of *Morpho aega* (Fig. 4a) is compared with scattering by an array of scales on the wing (Fig. 4c). The far-field scattering diagram of a single scale is a narrow stripe (Fig. 4b; see also Fig. 1j), but that of a set of scales is a distinctly broadened stripe (Fig. 4d). The angular extent of the scattering by the single scale in the vertical plane is <10^o, but that of the scale set is about 30^o (Y, Fig. 5). The broadening of about 20^o will be due to a rotational variation of the scale plane around the long axis of the scales. The tilt angle, that is the angle of the scale plane with the wing, will have a similar variation, as the scattering diagram of the scale set is about 20^o wider than that of the single scale (X, Fig. 5). Further broadening of the scattering diagram will occur when reflection by the complete wings of a *Morpho* butterfly is considered. The dynamic changes of light scattering by a *Morpho* flying in a natural environment, and how the butterflies will be perceived by conspecifics and predators will be interesting themes for future research.

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Chapter 7
Optical and structural properties of the silver scales of the Silverspot Butterfly, *Dione juno*

Spectrophotometry as well as structural analyses were performed on the scales that form the silver spots in the ventral wings of fritillary butterflies, specifically *Dione juno*. Electron microscopy shows that the scales have a very thin body and a rather smooth upper surface, in large contrast with most butterfly wing scales, which have a highly structured upper lamina. The silver scales have clear ridges, and the spaces in between the ridges form longitudinal, semi-cylindrical channels. Transmitted light through a single scale forms a linear diffraction pattern perpendicular to the direction of the ridges. The diffraction pattern agrees with calculations using the anatomical ridge distance. The far-field scattering profile of light reflected by a single silver scale is a non-uniform, coloured stripe, also perpendicular to the direction of the ridges. This pattern resembles the scattering profile of *Morpho* butterflies, but the physical basis is very different, because the *Morpho* scales have multilayered ridges. The scattering pattern of *D. juno* scales is primarily determined by reflection at the semi-cylindrical channels. The far-field distribution of light reflected by silver spot areas on the wing is a broadened white stripe, which results from the summed scattering by multiple single scales.

7.1 Introduction

Butterfly wings have perhaps the most diverse colorations and patterning in biological nature. Different purposes have presumably driven the formation of such patterns, including sexual selection, camouflage, conspecific recognition and mimicry. A butterfly wing is formed by a so-called wing substrate, which is a two-cell layer of transparent cuticular material, and rows of scales stacked at both

sides of the wing substrate. Different wing areas have distinct colours, depending on the structure and/or pigmentation of the scales. To understand the coloration of the wing as a whole, it is therefore necessary to understand first the mechanisms of coloration of single scales. In general, two mechanisms are distinguished. One is due to pigments that absorb light in a restricted wavelength range and therefore is called chemical or pigmentary coloration. The other is due to regular structures that cause coherent scattering of certain wavelengths. This is called physical or structural coloration. The scale structures are usually not developed uniformly throughout the scale. In Pierids, the bead density increases from the base to the tip. This is a morphological characteristic that has functional consequences (Giraldo & Stavenga 2007b).

In a study of single butterfly scales of different species, Giraldo *et al.* (2007) showed that the spatial profile of the light reflected by the scales is highly dependent on the scale structure. In pierids, the intricate upper lamina of the scales is usually beset with pigment granules, which scatter light incoherently, similar to scattering by a Lambertian reflector. In *Morpho* butterflies, the scales scatter light coherently, due to the multilayered ridges of their upper lamina. Some scales, as in the purple tip of the dorsal forewings of *C. regina* combine blue coherent scattering with pigment absorption and incoherent scattering of the pigment granules (Giraldo *et al.* 2007).

In the present study we describe the anatomical and optical properties of the silver scales of fritillary butterflies, specifically the Silverspot butterfly, *Dione juno*. We analysed the optical properties of single scales as well as that of large wing areas so to increase our understanding of wing coloration.

7.2 Materials and methods

Animals

We studied the silver spots of various fritillary butterflies belonging to the nymphalid tribe Heliconiini (Simonsen (2006, 2007). *Dione juno* (Silverspot butterfly) was a donation of Dr. Marta Wolf from the Entomology Group of the University of Antioquia, Medellín, Colombia, *Dione moneta* (Mexican Silverspot) was a gift from Andrés Vélez from the Ecological Park of Piedras Blancas, Santa Elena, Colombia, and *Agraulis vanillae* (Gulf Fritillary) was donated by Mr Ric Wehling, Eglin, Florida.

Spectrophotometry

Single scale reflectance was studied with the microspectrophotometer described in Section 2.2.1. The microscope objective used was an Olympus 20x, NA 0.46.

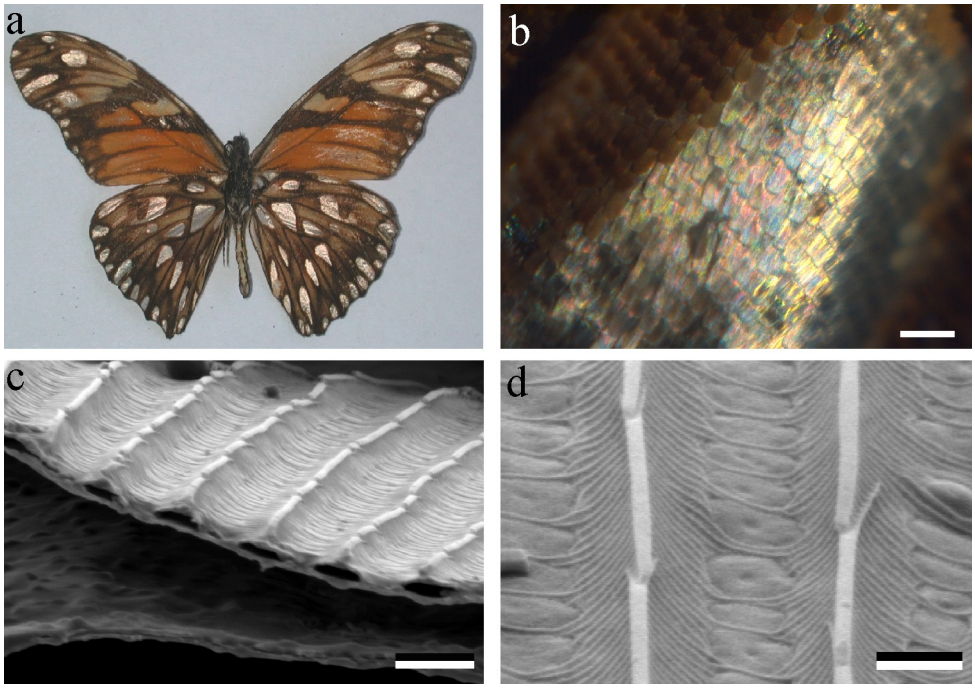

Fig 1. Silver spots and scales of *Dione juno*. **a** Photograph of the ventral wings. **b** Magnification of a silver spot. **c** SEM image of the cross section of a cut silver scale and wing substrate, showing that the scale body is thin and formed by two laminae. **d** SEM image of the upper lamina of a scale, showing that the windows are closed. The smoothness of the upper lamina contrasts largely with most butterfly wing scales. Bars: 200 µm (b), 2 µm (c), 1 µm (d).

The angular distribution of the reflected light was studied with the optical system described in Section 2.2.3. In brief, a light beam passed through a pinhole and was imaged on a scale. The reflected light was collected with a fiber optic and relayed to a spectrometer (SD2000, Avantes, Eerbeek, the Netherlands). The scattering pattern appearing on a white screen, either put between the imaging lens and the scale (reflected light) or behind the scale (transmitted light) was photographed. The reflectance and transmittance spectra of semi-denuded silver wing areas were measured with an integrating sphere (AvaSphere-50-Refl; Section 2.2.2). The dorsal orange and brown scales, opposite to the silver spots, were therefore fully removed by using adhesive tape. A white reflectance standard (Spectralon, Labsphere, North Sutton, NH, USA) served as a reference.

7.3 Results

The Silverspot butterfly, *Dione juno*, derives it name from the metallic shining spots on the ventral wings (Fig. 1a). The scales at the opposite, dorsal side of the

wings of this species are rather inconspicuously orange-brown coloured. The specular properties of the silver spot areas are prominently clear when observed with a light microscope (Fig. 1b). To understand the silver scale optics we performed scanning electron microscopy. Fig. 1c shows a cross section of a silver scale and the wing substrate. The thickness of the scale is approximately the same as that of the wing substrate (~0.5 μm), which is about half the thickness of pierid scales (for example, Giraldo and Stavenga 2007c). The scale is composed of two closely spaced laminae. The windows, which are the areas between ridges and crossribs, are closed (Fig. 1d), in contrast with the orange and brown scales of the same wing of *D. juno* as well as the scales of most butterflies, which have open windows.

For comparison, we also performed scanning electron microscopy on two other frittilaries, *Dione moneta* and *Agraulis vanillae*. The scales of silverspot areas of *D. moneta* were indistinguishable from those of *D. juno*, as also their windows were fully closed. The silver scales of *A. vanillae* were slightly different in that a small percentage of the window panes appeared to have small pores. Similar observations were very recently reported by Simonsen (2007).

The specular characteristics of the silver scales are presumably derived from the smoothness of the upper scale lamina. In order to verify this hypothesis, we have studied single scales as well as scales on the wing under transmitted and reflected light conditions. The reflectance spectrum of Fig. 2a was measured with a microspectrophotometer from a single silver scale, The spectrum increases monotonically, is high throughout the visible wavelength range, and suggests the presence of an ultraviolet-blue absorbing pigment.

In addition to the single scale microspectrophotometry, we performed experiments on semi-denuded wings with an integrating sphere (see Section 2.2.2). We removed the orange and brown scales from the dorsal wing to evaluate the optical properties of the silver spots without the contaminating effect of the scales on the opposite side of the wing. Fig. 2b shows the reflectance and transmittance measured from a silver spot illuminated with a white light beam of about 2 mm diameter. The absorptance was calculated by subtracting the summed reflectance and transmittance from 1. The absorptance spectrum shows that the cuticular material possesses a UV-blue absorbing pigment.

Because the scales on the dorsal wing were removed, the experimental spectra correspond to a stack of approximately two overlapping scales plus the wing substrate. The reflectance of the semi-denuded wing reaches a maximum of 0.45 at long wavelengths, considerably lower than the single scale reflectance measured with the microspectrophotometer. This difference must be due to the different apertures of the experimental arrangements. The measurements were in

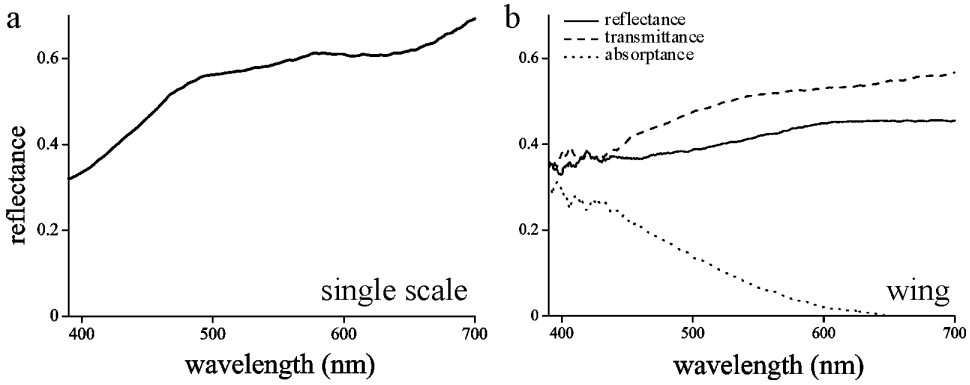

Fig. 2. Reflectance spectra of silver scales. **a** Reflectance spectrum of a single scale measured microspectrophotometrically. **b** Reflectance and transmittance measured with an integrating sphere, together with the calculated absorptance of a semi-denuded silver spot. The absorptance curve shows that the cuticular material contains a UV-blue absorbing pigment.

both cases done with a white diffuser as a reference. The integrating sphere collects all the back-scattered light, from all angles, but the microspectrophotometer objective has a limited numerical aperture and therefore collects preferentially specularly reflected light.

Knowledge of the spatial profile of the reflected light hence is clearly of crucial importance to understand the optical properties of single silver scales, and therefore we have studied the angular distribution of the reflected and transmitted light into further detail. For measurements of the reflected light profile, the setup described in Section 2.2.3 was used. A single scale, with the ridges positioned vertically, was illuminated with a narrow light beam (Fig. 3a). The far-field spatial profile of the reflected light then is a stripe perpendicular to the ridges (Fig. 3b). The stripe is not uniform, but rather consists of coloured spots with the highest intensity for longer wavelengths. The reflectance as a function of angle of a single scale was measured for seven different wavelengths, chosen throughout the visible spectrum. The curves, which have been normalized to the maximum reflectance (reached at 680 nm), slightly vary for the different wavelengths (Fig. 3c).

The reflectance spectra like that of Fig. 3c showed some fine structure, indicating interference effects, and therefore we performed measurements of light transmitted by single scales with the angular distribution setup of Fig. 4a, which is a slight modification of the optical system used to study the angular reflection. The transmitted light appeared to display a diffraction pattern perpendicular to thedirection of the ridges (Fig. 4b), similarly as the linear profile of reflected light

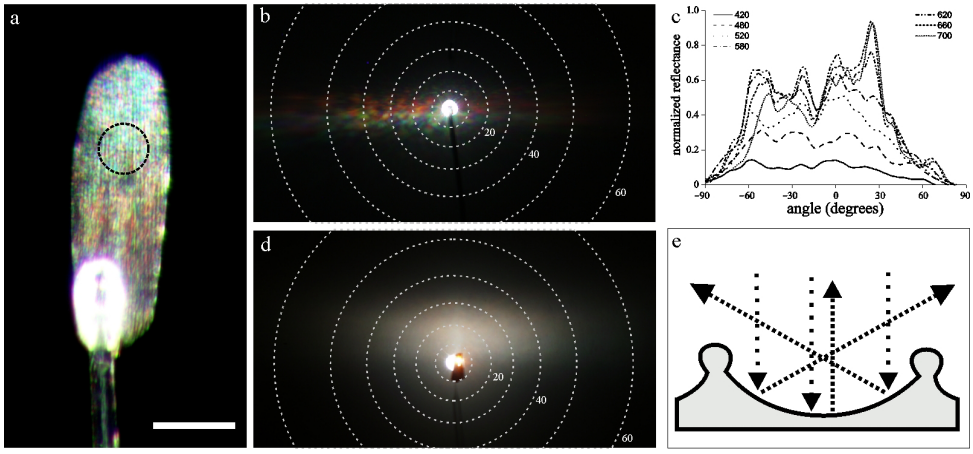

Fig. 3. a Microphotograph of a single silver scale attached to a glass micropipette. The dashed circle depicts the illumination area and the approximated size of the light spot (~30 µm); bar 50 µm. **b** Spatial reflection pattern of a single scale of *D. juno*. The dashed circles represent angles in degrees. The dark spot in the centre is the hole in the screen through which the light beam passed. The vertical dark line below the hole is the glass pipette holder. **c** Angular reflectance for seven wavelengths along the visible spectrum. **d** Spatial reflection pattern of an intact silver spot area illuminated with a light beam of approximately 400 µm diameter. **e** Diagram showing the mechanism that produces a linear pattern of the light reflected by a Silverspot Butterfly scale. Incident light is reflected into a plane due to the cylindrical shape of the area between the ridges.

(Fig. 3b). The angular distribution of the maxima was studied for five different wavelengths throughout the visible range. The grating constant was calculated for each wavelength and yielded a value 2.1 ± 0.2 µm, similar as the anatomical distance between the scale ridges.

Since the actual observed coloration of a silver spot depends upon the stack of scales, we have also photographed the scattering pattern of an intact silver spot area. The diameter of the illumination beam was about 400 µm. The resulting scattering profile retained roughly its linear shape, although it was considerably broadened (Fig. 3d), as expected from the cumulative effect of a large number of scales that are not perfectly aligned. The latter consideration also explains why the resulting stripe is no longer spotted, but uniform and white.

7.4 Discussion

A single scale from a silver spot of a fritillary butterfly scatters incident light in approximately a plane, perpendicular to the scale ridges (Fig. 3b). The assembly of scales on the intact wing scatters light in a broader angle (Fig. 3d), resulting in a white, silvery colour. The physical mechanism of the silver spots displayed by

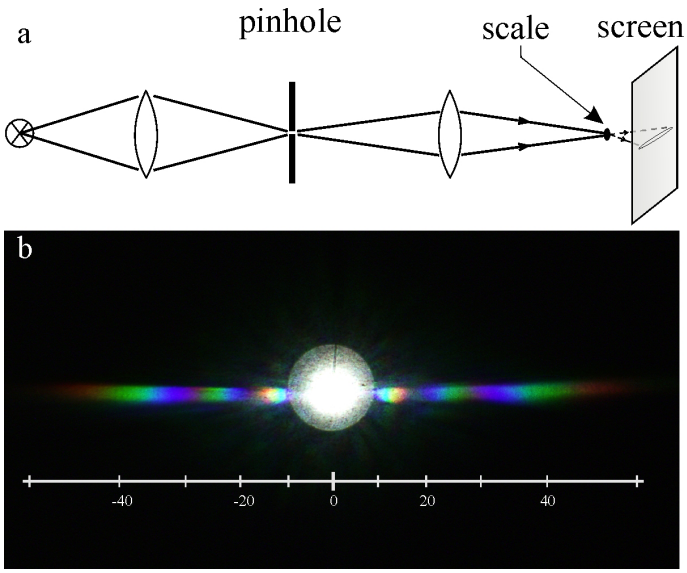

Fig. 4. a Diagram of the optical system used to study the transmission of a scale. The setup described in Section 2.2.3 was modified. A semitransparent screen was put behind the illuminated scale and then photographed. **b** Photograph of the semitransparent screen showing the diffraction pattern produced when light is transmitted through the scale.

several frittilary species of the nymphalid tribes Argynnini and Heliconiini (Simonsen 2007) has so far not been elucidated. Mason (1926) suggested, based on immersion experiments, that the pearl white of the silver scales is produced by a multiple-film system. Similarly, Simonsen (2007), who performed an extensive comparative survey of silverspot scales using scanning electron microscopy, also stated that the specular properties might be caused by a fine structured scale lumen. He regarded this nevertheless unlikely, because his anatomy favoured as the most parsimonious explanation that the only difference between normal and silver scales exists in the windows, which are usually open but are closed in the silver scales. Our scanning electron microscopical images agrees with that interpretation and, furthermore, do not provide any evidence for multilayers. The silver spot scales indeed appear to be similar to the classical bilayer scales with a smooth lower lamina and an elaborated upper lamina, with ridges like in the orange and brown coloured scales of *D. juno*. However, the closed windows create between the ridges a smooth, semicircular cylindrical surface, which acts as a cylindrical mirror. A cylindrical mirror reflects a small-aperture incident light beam into a stripe pattern in the far-field. Because the upper lamina of a silver scale forms a series of parallel cylindrical mirrors, the reflection pattern is a combined result from the specular and diffraction properties of the scale, resulting in a somewhat irregular stripe pattern.

The reflectance properties of single scales have previously been studied for a number of butterfly species (Vukusic *et al.* 1999; Yoshioka & Kinoshita 2004; Giraldo *et al.* 2007) Especially scales of *Morpho* butterflies have received most of

the attention, presumably due to their eye catching characteristics and special anatomy. The multilayered ridges that characterize their structure have also been found in other species and thus are called *Morpho* type scales. The light reflected by the *Morpho* scale type forms a linear pattern, similar to the one reported here for *D. juno*, but the reflectance is restricted to ultraviolet or blue wavelengths (Vukusic *et al.* 2000; Yoshioka & Kinoshita 2004; Giraldo *et al.* 2007). A light beam hitting the narrow multilayers in the ridges of a *Morpho* scale is diffracted in a plane determined by the mirror law. As we argued above, although the far field reflection pattern of the *Morpho* scale type resembles that of *D. juno* silver scales, the optical mechanism of the silver scales is probably that of a cylindrical mirror with diffraction at the ridge lattice, rather than that of an assembly of slender multilayers, which is the case in a *Morpho* scale. The narrow coloured stripe seen in the far-field reflection pattern of a single silver scale of *D. juno* contrasts with the broader white stripe created by an intact silver spot (Figs. 3b and d). A similar broadening of the reflection pattern was also reported by Giraldo *et al.* (2007) for *Morpho aega*.

The reflectance of a single scale was determined to be of the order of 0.5-0.6 (Fig. 2a), which is extremely difficult to reconcile with a simple bilayered scale. Measurements with a microscope objective that samples the light reflected by a single scale inevitably overestimates the reflectance when using a random diffuser as the reference, because of the directionality of the light back-scattered by a single scale. The reflectance of a semi-denuded wing, being the wing substrate with only on one side rows of two overlapping silver scales, was determined to be of the order of 0.4 (Fig. 2b). Assuming that the reflectance of the wing substrate is about 0.1, as in pierids (Stavenga *et al.* 2006; Giraldo & Stavenga 2007b), the reflectance of a single scale still has to be higher than (0.4-0.1)/2 = 0.15 (see Stavenga *et al.* 2005). This is about what might be expected for two layers of cuticular material with refractive index ~1.6 (Vukusic *et al.* 1999). However, a proper optical analysis of the reflectance of silver scales will require a more detailed analysis, as the two layers of a scale have thin film properties, which may have distinct wavelength effects. Furthermore, SEM micrographs indicate that the upper and lower lamina approach each other in the midplane between two adjacent ridges. In fact, there the two layers could form a multilayer, where wave optical interference effects may occur. Transmission electron microscopy is hence necessary to acquire precise geometrical data about scale layer thicknesses and distances.

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Chapter 6 was based on

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Chapter 7 was based on

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Summary and general discussion

The use of colour is ubiquitous in nature. Evolution has produced complex visual organs, as well as systems that cause a variety of colourful optical effects. Vision is considered a fundamental sense in a vast majority of animals. The struggle for survival has induced the creation of a large diversity of systems to manipulate environmental light (pigments, nanostructures). Butterfly wings have perhaps the most diverse coloration in the animal kingdom. As described in Chapter 1, the colour of an intact butterfly wing is however a complex optical phenomenon.

In many butterfly species, dorsal and ventral sides of the wing can have very different patterns. When observed with a microscope, wings resemble pointillistic paintings where each point is formed by a coloured scale. How to study the fine structure and optical properties of single scales is treated in Chapter 2.

Scales are on both sides of the wing substrate arranged in partially overlapping rows of cover and ground scales. Incident light thus is reflected and transmitted by five layers. Chapter 3 shows how the stack of five layers determines wing coloration. We have investigated a number of accessible species of the butterfly family Pieridae. A rather high wing reflectance, up to 70%, is due to the superposition of effectively reflecting single scales. But, why are there two overlapping scales and not more? We have investigated intact, denuded and semi-denuded wings of the Small White, *Pieris rapae rapae*. Experimentally measured reflectance and transmittance spectra were interpreted with a theoretical model for a pile of reflecting plates. We concluded that the actual system of two superimposed scales on both wing sides is close to optimal. By adding more layers (scales), the reflectance does not increase substantially; but the weight does. However, some of the shiniest butterflies like *Morpho aega* or *Morpho cypris* have on their dorsal wings only one layer of blue scales, which scatter light coherently, causing iridescence. The reflectance is very high, and two overlapping scales can produce other effects, to the detriment of the iridescence (e.g. in *Morpho peleides* and *Morpho deidamia*).

Although the architecture of a scale depends upon the species, general characteristics can be recognized. A scale generally consists of two laminae, the one facing the viewer (abwing) and the one facing the wing proper (adwing), which are connected by trabeculae, also called pillars. The lower lamina is rather flat and unstructured, but the upper lamina is typically formed by densely spaced

ridges, connected by crossribs. The scales of the family Pieridae have an unusual feature. The ridges and crossribs are adorned with beads. When incident light is incoherently scattered by the scale structures a white colour results, unless there is pigment that absorbs in a given wavelength range.

After having explored the macroscopical effect of the scale distribution on the wing, we have studied the anatomical and optical properties of single scales. Chapter 4 presents measurements on the wings of two subspecies of *P. rapae*. Spectrophotometric analyses of the wings of the European subspecies, *P. r. rapae*, show virtually no dependence on sex; the wings of both sexes strongly absorb ultraviolet light. However, the Japanese subspecies, *P. r. crucivora*, displays a definite sexual dichromatism. As in the European case, dorsal wings of males absorb UV light, but those of females do not. Examination of the microscopical structure of single scales, from different areas in dorsal and ventral wings, reveals anatomical differences in male and female scales. Beads appear to be absent in female dorsal scales. Thus, by comparing the structure and optical properties of scales, we concluded that the scale pigment is concentrated in the beads. Furthermore, they appeared to have a dual role in butterfly coloration. They store the pigment and also enhance the scattering of light outside the pigment absorption range. This spectral range is rather wide for the scales of *Pieris rapae*, as its pigment, presumably leucopterin, absorbs only ultraviolet light. For other coloured species of Pieridae, the spectral range narrows, since their beads contain pigments that also absorb in the blue, green or yellow wavelengths, causing yellow, orange or red scales.

Scales of pierids have another peculiarity; the distribution of beads, and consequently of the pigment, is not uniform throughout the scale. Chapter 5 demonstrates that instead, the granule density increases gradually from the base of the scale to the tip. Spectrophotometric analyses show also a gradient of the scale reflectance, proportional to the bead density. Extreme gradients can be artificially produced in scales by extraction of pigment with aqueous ammonia. Reflectance spectra of different areas of those scales permitted to quantify the relative contribution of the two scale laminae to the scale reflectance. Experiments with manipulated overlapping scales were performed, which allowed to elucidate a functional reason for the scale gradient. By having a non-uniform distribution of beads, with beads being high in the area not covered by neighbouring scales (the base), incident light is effectively reflected.

Pigmentary coloration, also called chemical coloration, usually produces (in butterflies) yellow to red colours, that is, the material reflects is in the long-wavelength part of the visible spectrum. Besides pigmentary coloration, another way of generating colour is to produce ordered nanostructures, which because of their regularity create coherent light scattering. This physical or structural coloration usually produces ultraviolet, blue or green colours. As illustrated in

Chapter 6, the scale colour is either determined by its pigmentation or its structural organization, or by both. We have compared the spatial reflectance profiles of various species. We found that scales of white pierids (*Delias nigrina* and *Pieris rapae*) scatter light randomly, in close agreement with Lambert's cosine law, which can be well understood from the randomly distributed beads. Since pierid scales with different colours do not differ in bead distribution but only differ in the type of pigment stored in the granules, white, yellow, orange or red scales will have a similar scattering profile. The *Morpho* butterfly scales, on the other hand, have elaborated ridges that form a system of multilayers. The system causes constructive interference of blue wavelengths (normal illumination), and so blue light is specularly reflected. The organization of the multilayers in the spaced ridges causes also diffraction. When the multilayers are not localized in the ridges but in the body of the scale, between the upper and lower laminae, the reflected light is not diffracted. Instead, light is backscattered in a narrow spatial angle. That is the case for the green-yellow scales of the moth, *Urania fulgens*. The purple scales in the dorsal wing tips of the pierid male *Colotis regina* act similarly as the *Morpho* scale in the blue, due to multilayers in the ridges. The scattering in the red occurs as in the *Pieris* case, because the scales have ridges with multilayers and contain beads with pigment that does not absorb in the red wavelength range.

This thesis has elucidated the optical and structural mechanisms that determine the wing coloration of notably the pierid butterflies, but also the optical characteristics of some other species with differently structured wing scales have been clarified. The macroscopic features of butterfly wings are now beginning to be understood from the microscopic level. Many butterfly species have spectral properties that require further studies, for instance the pigmentation of nymphalids and the colours produced by the photonic crystals occurring in the wing scales of lycaenids and papilionids.

Samenvatting en discussie

In de natuur speelt kleur een prominente rol. De evolutie heeft geresulteerd in complexe visuele organen, en ook systemen die een grote verscheidenheid in kleurrijke effecten veroorzaken. Het zien van deze kleuren is een algemene eigenschap van dieren. De natuurlijke selectie heeft een grote diversiteit van systemen opgeleverd die het licht manipuleren (pigmenten, nanostructuren). De vleugels van vlinders hebben wellicht de meest diverse kleuren in het dierenrijk. Hoofdstuk 1 beschrijft de complexe optische effecten, die bij de vlinderkleuren optreden.

In veel vlindersoorten hebben de dorsale en ventrale vleugels (de boven- resp. onderzijden) zeer verschillende patronen. Bekeken met een microscoop lijkt een vlindervleugel op een pointilistisch schilderij, waar ieder punt gevormd wordt door een gekleurde schub. De methoden waarmee de fijnstructuur en de optische eigenschappen van individuele vlinderschubben bestudeerd zijn worden beschreven in hoofdstuk 2.

Schubben zijn aan beide zijden van het vleugelsubstraat gerangschikt in gedeeltelijk overlappende rijen van dek- en grondschubben, en dus wordt de reflectie en transmissie van opvallend licht bepaald door in totaal vijf lagen. Hoofdstuk 3 laat zien op welke manier die vijf lagen de kleur van een vlindervleugel bepalen. We hebben daarvoor een aantal toegankelijke vlindersoorten van de familie Pieridae bestudeerd. Deze hebben vleugels met een tamelijk hoge reflectantie, tot 70%, dankzij de stapeling van sterk reflecterende schubben. De vraag dringt zich dan op waarom er maar twee overlappende schubben aan iedere vleugelzijde zijn en niet meer. We hebben daarom reflectie- en transmissiemetingen verricht aan zowel volledig intacte vleugels als aan vleugels waarvan de schubben deels verwijderd waren, en wel van het kleine koolwitje, *Pieris rapae rapae*. De metingen zijn geïnterpreteerd met een theoretisch model voor een stapel reflecterende lagen. De conclusie was dat het gerealiseerde stelsel van twee overlappende schubben aan beide kanten van de vleugel ongeveer optimaal is, omdat met meer lagen (schubben) de reflectantie slechts in geringe mate hoger wordt, terwijl het gewicht van de vleugel toeneemt evenredig met het aantal lagen. Een geheel andere situatie bestaat bij de prachtige, iridescente *Morpho aega* en *Morpho cypris*. Die hebben aan de dorsale vleugelzijde slechts één laag van blauw-reflecterende schubben, waarin opvallend

licht coherent verstrooid wordt. De hoge reflectantie en de iridescentie wordt gemakkelijk verstoord bij overlappende schubben (zoals gebeurt bij *Morpho peleides* en *Morpho deidamia*).

Alhoewel de ruimtelijke structuur van een schub afhangt van de vlindersoort zijn er een aantal algemene karateristieken. Een schub bestaat uit twee lagen (laminae), verbonden door pilaartjes (trabeculae). De onderlaag is meestal glad, maar de bovenlaag wordt gevormd door evenwijdige richels, verbonden door dwarsribben. Alleen de schubben van Pieridae hebben korrels, welke als het ware hangen aan de dwarsribben. Opvallend licht wordt door die structuren verstrooid, hetgeen een witte kleur oplevert, tenzij de schub absorberende pigmenten bevat.

In aanvulling op het onderzoek aan het macroscopisch effect van de stapeling van schubben op de vleugels hebben we de anatomische en optische eigenschappen van geïsoleerde schubben bestudeerd. Hoofdstuk 4 beschrijft metingen aan twee ondersoorten van het kleine koolwitje. Spectrofotometrie aan de vleugels van de Europese ondersoort *P. r. rapae* leert dat beide seksen vrijwel dezelfde refectiespectra hebben. Dit in tegenstelling tot de Japanse ondersoort *P. r. crucivora*, waarbij de dorsale vleugelzijde van het mannetje sterk ultraviolet absorbeert, net zo als bij de Europese mannetjes, echter de UV-absorptie is afwezig bij de Japanse vrouwtjes. De anatomie van de schubben van de Japanse mannetjes en vrouwtjes verschilt evenzeer, want de korrels aan de dwarsribben ontbreken bij de vrouwtjes. Door vergelijken van structuur en optische eigenschappen konden we concluderen dat het pigment van de koolwitjes geconcentreerd is in de korrels aan de dwarsribben. De korrels blijken een dubbele functie te hebben. Ze bevatten pigment en absorberen daardoor licht, maar ze verstrooien daarnaast in aanzienlijke mate licht in het golflengtegebied buiten het absorptiespectrum van het pigment. In het geval van de schubben van *Pieris rapae* beslaat dit golflengtegebied het gehele zichtbare spectrum, omdat het pigment (leucopterin) slechts in het ultraviolet absorbeert. Andere Pieridae hebben een beperkter golflengtegebied waar het licht verstrooid wordt, omdat de pigmenten niet alleen UV, maar ook blauw, groen of geel licht absorberen, hetgeen resulteert in gele, oranje of rode schubben.

De schubben van Pieridae vertonen nog een bijkomende speciale eigenschap, namelijk dat de korrels niet homogeen over de schub verdeeld zijn. Hoofdstuk 5 laat zien dat de korreldichtheid geleidelijk toeneemt van de basis naar de top. De lokaal gemeten reflectantie blijkt evenredig te zijn met de korreldichtheid. Extreme verschillen in korreldichtheid is te realiseren door pigmentextractie met behulp van ammonia, waardoor daarmee evenredige grote verschillen in reflectantie worden gecreëerd. De functionele gevolgen van de gradienten in korreldichtheid zijn experimenteel onderzocht door de reflectantie te meten van gestapelde schubben, waarbij de mate van overlap gevarieerd werd. Bij een niet-uniforme verdeling van de korrels, waarbij de korreldichtheid hoog is in het

gebied dat niet door een andere schub wordt overlapt, wordt invallend licht effectief gereflecteerd.

Pigmentkleuren, ook wel chemische kleuren genoemd, zijn bij vlinders meestal geel, oranje of rood, dus in het lange golflengtegebied. Behalve door pigmentatie wordt kleur ook veroorzaakt door geordende nanostructuren, waarin coherente lichtverstrooiing optreedt. Deze fysische of structurele kleuren beperken zich meestal tot het ultraviolet, blauw of groen. Hoofdstuk 6 beschrijft hoe de kleur van vlinderschubben bepaald wordt door de pigmentatie, of door de schubstructuur, of door beide. We hebben verder de ruimtelijke reflectieprofielen van een aantal vlindersoorten bestudeerd. De schubben van (kool)witjes (*Delias nigrina* en *Pieris rapae*) verstrooien licht volledig diffuus, in overeenstemming met de cosinuswet van Lambert, en zoals verwacht voor onregelmatig verdeelde korrels. Omdat gekleurde schubben van Pieridae niet verschillen in de verdeling van de korrels, maar alleen verschillen in het type pigment hebben ze alle eenzelfde strooiingsprofiel. De schubben van *Morpho* vlinders hebben sterk gestructureerde richels, in de vorm van multilagen. Dit stelsel veroorzaakt constructieve interferentie van blauwe golflengten en dus spiegeling van blauw licht. De smalle richels veroorzaken echter ook diffractie, waardoor het gereflecteerde licht uitwaaiert. Wanneer de multilagen niet beperkt zijn tot de richels maar uitgestrekt zijn binnenin de schub, tussen boven- en onderlaag, zoals bij de mot *Urania fulgens*, dan wordt invallend licht gereflecteerd binnen een nauwe ruimtehoek. De purpergekleurde schubben in de dorsal vleugeltip van het mannetje C*olotis regina* functioneren in het blauw net als de schubben van de *Morpho* vlinders, maar rood licht wordt verstrooid zoals dat gebeurt bij de witte Pieridae, omdat de schubben zowel richels met multilagen hebben als pigmentkorrels die niet in het rood absorberen.

Dit proefschrift behandelt de optische en structurele mechanismen die de kleur van vlindervleugels bepalen, in het bijzonder van de Pieridae. Daarnaast worden de optische karakteristieken van de schubben van enkele andere vlindersoorten verhelderd. Hoe de macroscopische fenomenen van vlindervleugels veroorzaakt worden door de microscopische eigenschappen wordt langzamerhand duidelijk. De spectrale eigenschappen van veel vlindersoorten verdienen echter nog verdere bestudering, bijvoorbeeld de pigmentatie van de Nymphalidae en de kleuren die bij Lycaenidae en Papilionidae veroorzaakt worden in schubben met fotonische kristaleigenschappen.

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About the author

Marco Antonio Giraldo was born in the city of the eternal spring (Medellín, Colombia) the 28th June of 1978. The son of don Juan Antonio Giraldo and doña Stella Cadavid, shoes merchants, showed since the beginning an appetite for knowledge in general, although rather inclined to the natural sciences. After sporadic unsuccessful incursions in football, tae kwon do, and a list of other sports, he understood that the best was to focus on science and decided to study Physics in the University of Antioquia. During his carrier, he became interested in astrophysics and biophysics; and it was in medical biophysics where he started as instructor in the University of Antioquia and San Martin teaching introductory courses to students of Medicine and veterinary. In 2003 he received his degree and travelled to Groningen to start his PhD research project in the Department of Neurobiophysics, with Prof. Doekele Stavenga.

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