Chapter 7

Spatial reflection patterns of iridescent pierid butterfly wings and the dependence of visibility on scale curvature

Abstract

The males of many pierid butterflies have iridescent wings, which presumably function in intraspecific communication. The iridescence is due to nanostructured ridges of the cover scales. We have studied the iridescence in a few Coliadinae, Gonepteryx aspasia, G. cleopatra, G. rhamni, and Colias croceus, and in two members of the Colotis group, Hebomoia glaucippe and Colotis regina. Imaging scatterometry demonstrated that the structural colouration is highly directional, whereas the pigmentary colouration is diffuse. Angle-dependent reflectance measurements demonstrated that the directional iridescence distinctly varies among closely related species. The species-dependent scale curvature determines the spatial properties of the wing iridescence. Narrow beam illumination of flat scales results in a narrow far-field iridescence pattern, but curved scales produce broadened patterns. The restricted spatial visibility of iridescence presumably plays a role in intraspecific signalling.

Introduction

Butterfly wings are patterned by differently coloured wing scales. The colour is due to the combined reflections of the cover scales and the underlying ground scales (Nijhout 1991; Stavenga et al. 2006). The scale colours are due to structural elements and/or due to pigments. A butterfly wing scale consists of a flat lower lamina and a structured upper lamina. The upper lamina of scales is often marked by longitudinal ridges, connected by cross-ribs. The components of the butterfly wing scales are commonly not very regularly arranged, and then incident light is scattered more or less diffusely. When the nanostructures in the scales are regularly arranged, light interference results in structural (or physical) colours, as is the case in the famous iridescent Morpho butterflies (Vukusic and Sambles 2003; Kinoshita et al. 2008; Kinoshita 2008).

Melanin, a pigment that absorbs throughout the visible wavelength range, is frequently employed to enhance the structural colours (Mason 1926; Mason 1927; Yoshioka and Kinoshita 2006). When the wing scales contain pigments that absorb in only a restricted wavelength range, a pigmentary (or chemical) colour results. Pierid butterflies package their wing pigments, the pterins, into small granules which increase the scattering efficiency in the wavelength range where pterin absorption is low. Thus, the granules enhance the pigmentary colouration (Stavenga et al. 2004; Rutowski et al. 2005). Many pierid butterflies additionally have structurally coloured wings. Usually only males exhibit iridescence, presumably for impressing females, but in some cases both sexes are iridescent (Kemp 2006; Kemp 2007; Kemp and Rutowski 2007). The structural colouration is combined with
yellow, orange or red pigments, depending on the species. In several species of the subfamily Coliadinae both the forewing and the hindwing are iridescent, e.g. *Colias eurytheme*, *Colias electo*, *Eurema hecabe*, *Phoebis argante* (Scott 1986; Rutowski et al. 2007a; Rutowski et al. 2007b; Wijnen et al. 2007), but in most members of the *Colotis* group, belonging to the subfamily Pierinae (Braby et al. 2006), iridescence is restricted to the tips of the forewings (Stavenga et al. 2006; Wijnen et al. 2007). The iridescence originates in the cover scales, specifically in the ridges, which consist of a stack of elaborate lamellae, thus forming a multilayer reflector. Electron microscopic analysis demonstrated that the multilayered structures in pierids are very similar to those found in *Morpho* butterflies: the cross-section of the longitudinal ridges resembles the shape of a Christmas-tree (Ghiradella et al. 1972; Ghiradella 1989; Vukusic et al. 1999; Giraldo et al. 2008; Wilts et al. 2011).

While the absorption spectra of the pterin pigments as well as the reflectance spectra and patterns of the iridescent wing areas of pierid butterflies have been described in considerable detail (Rutowski et al. 2005; Stavenga et al. 2006; Rutowski et al. 2007a; Wijnen et al. 2007; Wilts et al. 2011), only a few pioneering studies on the spatial distribution of the reflected light have been made so far (Rutowski et al. 2007b; Giraldo et al. 2008). In this study, we study six pierid species, i.e. the members of the Coliadinae *Gonepteryx aspasia*, *G. cleopatra*, *G. rhamni*, and *Colias croceus*, and the Colotis group members *Hebomoia glaucippe* and *Colotis regina*. We focus on the spatial radiation patterns of the structural and pigmented wing colouration, using imaging scatterometry (ISM) and angle-dependent reflectance measurements (ARM). We have found that the iridescence spectra and far-field radiation patterns distinctly depend on the species. The scale curvature plays a crucial role in the angular distribution of iridescence, which is presumably important for intraspecific signalling, both in a static display and during flight.

### Materials and Methods

#### Animals

Specimens of the Clouded Yellow, *Colias croceus*, were caught in the littoral part of Slovenia; the Cleopatra Brimstone, *Gonepteryx cleopatra*, near Argelliers (Hérault, France); the Common Brimstone, *Gonepteryx rhamni*, in Groningen (the Netherlands) and in Ljubljana (Slovenia); the Great Orange Tip, *Hebomoia glaucippe*, in Taiwan; the Queen Purple Tip, *Colotis regina*, was obtained from Dr. U. Dall’Asta (Royal Museum of Central Africa, Tervuren, Belgium); and the Lesser Brimstone, *Gonepteryx aspasia*, from prof. K. Arikawa (Sokendai, Hayama, Japan).

#### Photography

The upper sides of the wings of the butterfly specimens were photographed with a Nikon D70 Mk I camera equipped with a Nikkor 70 mm f1.4 macro objective. The red channel of the CCD chip of this camera has sufficient UV-sensitivity to allow UV photographs when using a blacklight lamp and visible-wavelengths-blocking filters (Schott glasses UG3 and BG17). The peak sensitivity in the UV was at about 370 nm.

#### Imaging scatterometry

The spatial distribution (far-field) of the light scattered from single scales was visualized with an imaging scatterometer, built around an ellipsoidal mirror collecting light from a full hemisphere around the first focal point containing the sample (Stavenga et al. 2009; Wilts et al. 2009). Small pieces were cut from the wings using a razor blade and fine scissors. The pieces were glued to the tip of a glass micro-pipette. A small piece of magnesium oxide served as a white diffuse reference
object. RGB images were taken with an Olympus DP-70 camera. For measuring the effect of scale curvature, the spot size was reduced by inserting a small pinhole into the primary beam of the scatterometer, and the scale was moved so that the spot illuminated different parts of it. The vertical intensity profiles of the far-field scattering patterns were obtained from the blue channel of the images by averaging the pixel values in each row of vertically oriented rectangular regions.

Angle-dependent reflectance measurement
The angular distribution of the light scattered by the intact wings was measured with a set-up consisting of two optical fibres, one for illumination and the other for light detection, attached to two goniometers with the same rotation axis. The wings were placed on a black cardboard and positioned at the rotation axis of the goniometers (Fig. 1). We measured the reflectance spectra along the central meridian in the plane defined by the wing base and apex and the normal to the wing plane. The angles of illumination ($\alpha$) and the sensor ($\beta$) along the meridian could be adjusted. The coordinate system was defined with the illumination and measurement meridians running from the wing base ($-90°$) to the wing apex ($+90°$). The illumination and the sensor arm were tilted to about $10°$ to the sides of the central meridian, thus avoiding occultation but still detecting the reflections from the central parts of the iridescent lines.

Reflectance spectra from iridescent wing areas were recorded with a diode-array microspectrometer (UV-VIS range 180-1100 nm, AvaSpec 2048-2; Avantes, Eerbeek, the Netherlands), coupled to a 600 $\mu$m quartz fibre (FC-UV-600, Avantes) equipped with quartz collimators (Col-UV/Vis, Avantes). The same quartz optics was used for the illumination arm. A diffusely scattering reference tile (Avantes WS-2) served as the white standard. The light source was an ozone-free Xenon lamp (XBO).

The diameter of the illuminated area was about $3\text{ mm}$ and that of the measurement circle about $9\text{ mm}$, respectively, in the normal (vertical) position ($a = \beta = 0°$; Fig. 1). The sensor field of view was bigger than the illuminated spot at all combinations of illumination and detection angles. The sampling angle (full width at half maximum, FWHM) of the setup was $3.9°$ at 600 nm and $4.6°$ at 300 nm (see also Stavenga et al. 2011). Consequently, at a fixed detection angle (e.g. $\beta = 0°$), a diffuse scatterer yielded the same reflectance value at all illumination angles $a$. In order to simulate a distant point source illuminating a unit area (e.g. the sun), the reflectance measurements were transformed to the reflectance per unit area (projected reflectance) by a cosine projection, i.e. $R^* = R \cos(\alpha)$. We measured reflectance spectra at the illumination angles $a = [0°, ±30°, ±45°, ±60°]$ by varying the measurement angle $\beta$ between $-75°$ and $+75°$ in $5°$ or $10°$ steps (for an example of the measurement grid, see Suppl. Fig. 1). Additionally, we followed the line of optimal iridescence by stepping the illumination angle, $a$, from $-75°$ to $+75°$, always adjusting the measurement angle $\beta$ until the peak reflectance in the iridescence band was maximal.

Band reflectances were measured in the ranges $340±10\text{ nm}$, $420±10\text{ nm}$, and $700±10\text{ nm}$. The overall iridescence was measured as the average reflectance in the wavelength range starting at $300\text{ nm}$ and ending at $430\text{ nm}$ (G. rhamni forewing), $450\text{ nm}$ (G. cleopatra hindwing and G. aspasia forewing), $460\text{ nm}$ (H. glaucippe tip), $470\text{ nm}$ (G. cleopatra forewing), $480\text{ nm}$ (C. croceus) or $560\text{ nm}$ (C. regina), respectively.

The data was analysed with Matlab (Mathworks, USA). Average band reflectance at each angle pair ($a,\beta$) was interpolated into a mesh with $5°$ angular resolution using the routine griddata (Matlab) or gridfit (by John D’Erico). The boundary values ($a,\beta = ±90°$) were set to zero before interpolation. The shape of the $R^*(a,\beta)$ distribution for an ideal diffuser is a two-dimensional cosine function. The interpolation errors at this level of analysis were found to be negligible (data not shown).
Figure 1
Diagram of the angle-dependent reflectance measurement (ARM) setup. The butterfly wing is positioned in the origin of a coordinate system determined by the two fibres, which rotate in the same vertical plane. The plane of the wing determines the horizontal plane of the coordinate system. The angles between the wing normal and the illumination and detection fibres are $\alpha$ and $\beta$, respectively. The wing is positioned so that its base corresponds to $\alpha = \beta = -90^\circ$ and its apex to $\alpha = \beta = +90^\circ$.

Figure 3
Shape and scatterograms of single cover scales from the forewing tips of *H. glaucippe* (a-c) and *C. regina* (d-f). a,d Top-view of the scales. A distinct iridescence line is seen on top of the orange and red coloured scale, respectively. b,e Side-view of the scales; upper side to the left (see inset of e, lower side to the right). The cover scale of *H. glaucippe* (b) is flat, except at the tip, and the cover scale of *C. regina* (e) is strongly curved. Bar (a,b,d,e): 50 μm. c,f. Full hemisphere scatterograms from single scales, illuminated with white light. The white circles in the polar plots indicate angles of 5°, 30°, 60° and 90° with respect to the normal to the wing plane. The orange and red reflections are diffuse and fill the whole hemisphere. The short-wavelength iridescent reflections are spatially limited, for *H. glaucippe* (c) to a narrow angular space and to a wider space for *C. regina* (f).
Figure 2
The Lesser Brimstone, Gonepteryx aspasia. a RGB photograph (bar: 1 cm). b UV photograph with wide aperture illumination. c UV photograph with illumination from the left side. Only the right wing reflects towards the camera. d Reflectance spectra with variable illumination angle $\alpha$, observed normal to the wing ($\beta = 0^\circ$). The reflectance in the UV is highly directional.

Figure 4
Local illumination for the differently curved scales of H. glaucippe and C. regina yielding different scattering patterns. a,b Scatterogram of a H. glaucippe scale illuminated near the root (a) and at the middle (b) of the scale. c Angular profiles of the scatterograms from a and b. d,e Scatterogram of a C. regina scale illuminated near the root (d) and at the middle (e) of the scale. The blue and red curves (c,f) represent the angular profiles vertically across the scatterograms (yellow rectangle in d). The black curves in c and f, representing the sum of the red and blue curves, show the limited spatial scattering by the scale of H. glaucippe and the wider spatial scattering of the C. regina scale.
Results

The upper sides of the wings of the investigated male pierids have different reflection characteristics in the short wavelength range compared to those in the long wavelength range (Ghiradella et al. 1972; Ghiradella 1989; Rutowski et al. 2007a; Rutowski et al. 2007b). The wing reflection in the long wavelength range is diffuse and the reflection in the short-wavelength range is directional, as is demonstrated on the Lesser Brimstone, Gonepteryx aspasia (Fig. 2). The RGB photograph (Fig. 2a) shows that the forewing upper side is distinctly yellow; the hindwing upper side is somewhat paler, presumably due to a lower density of pterin pigment, most likely xanthopterin (Wijnen et al. 2007). Both the forewing and hindwing have orange spots, probably due to the presence of erythropterin. The UV photograph (Fig. 2b), made with a wide aperture blacklight source, shows that the forewings (except their edges) and the orange spots on the hindwings, reflect UV light. When the specimen is illuminated from the left side, the UV reflection of only the right wing is seen (Fig. 2c).

We measured the directionality of the UV reflection on the forewings with the ARM setup. The measurement fibre was set at the wing normal (β = 0°), and the illumination direction was changed in steps of 5°. The UV reflectance was maximal with the illumination direction from the basal side (α = −45°, R=0.8, Fig. 2d). The diffuse reflectance in the long-wavelength range remained almost constant, confirming that the field of view of the detector captured the full illumination spot, independent of the illumination angle (see Methods).

The directional reflection at short wavelengths is due to the multilayered ridges of the cover scales (Wilts et al. 2011). Preliminary investigations on a variety of pierid butterflies showed that the directionality strongly depended on the species. To gain a quantitative understanding of the directional reflections, we first investigated the shape of isolated short-wavelength-reflecting cover scales using light microscopy. The cover scales in the wing tips of the Great Orange Tip, Hebomoia glaucippe, and the Queen Purple Tip, Colotis regina, appeared to have very different shapes. The scales of H. glaucippe are orange and strongly reflect UV-violet light (Wilts et al. 2011); the latter can be seen in RGB photographs as a faint violet reflection (Fig. 3a). The scales are virtually flat and only near the tip slightly curved (Fig. 3b). The scales of C. regina are red and additionally reflect blue light (Fig. 3d). When observing a scale of C. regina with an epi-illumination light microscope, the blue reflection changes in position when the scale is rotated, indicating that the scale has a curvy shape (Fig. 3e).

We studied the far-field scattering patterns of the cover scales of H. glaucippe and C. regina with an imaging scatterometer, applying a narrow aperture white illumination focused at a small area of the scale. The spatial scattering properties in the long and the short wavelength range clearly differ (Fig. 3c,f). The scales of both species reflected a diffuse orange (Fig. 3c) or red (Fig. 3f) scattering pattern, respectively, when illuminated in a small, central area of the scale. The scattering pattern was filling virtually the full hemisphere. In the scatterogram of H. glaucippe a narrow violet line emerged (Fig. 3c), while in the scatterogram of C. regina a broad, blue band overlapped the diffuse pattern (Fig. 3f). The narrow violet line of the H. glaucippe scale is reminiscent of a similar narrow blue line observed in scatterograms of Morpho scales (Stavenga et al. 2009), which was explained by diffraction of light by the slender, multilayered ridges (Vukusic et al. 1999; Kinoshita et al. 2002). Indeed, the structures in iridescent pierid butterflies are similar (Rutowski et al. 2007b; Giraldo et al. 2008; Wilts et al. 2011). The difference in colour is due to the difference in spacing of the ridge lamellae (Wilts et al. 2011).

The results of Fig. 3 suggest that the strongly curved scales of C. regina cause the broadened scattering of blue light. This assumption was tested in the experiment of Fig. 4, where the scales of H. glaucippe and C. regina were illuminated at two different locations (insets Fig. 4c,f). In the case of the H. glaucippe, the blue channel of the scatterograms showed almost identical strip-like spatial scattering patterns (Fig. 4a,b). The scale of C. regina produced a broad scattering pattern, the extent of which depended on the location of illumination (Fig. 4d,e). We have quantified the angular spread
of the scattering by measuring a vertical profile (see rectangle in Fig. 4d) in the polar plots of Fig. 4a,b and 4d,e. The far-field scattering profiles are shown in Fig. 4c,f. The cover scales in the wing tips of H. glaucippe (Fig. 4c) reflect short-wavelength light into a narrow spatial angle, with a sharp, almost symmetric bell-shaped profile, while the scales of C. regina (Fig. 4f) reflect light into a broader angle, with an additional asymmetric tail. The FWHM is about 16° for H. glaucippe and about 40° for C. regina.

In our previous study on the morphology and spectral behaviour of pierid wing scales (Wilts et al. 2011), we noticed that the angle between the wing plane and the multilayer reflectors in the scale ridges varied among the investigated species. Here, we have therefore studied the reflection and scattering patterns of a number of pierids in more detail with the ARM setup: C. croceus, G. rhamni, G. aspasia, G. cleopatra (both forewing and hindwing), H. glaucippe, and C. regina (Fig. 5, right column). We have measured reflectance spectra with the angle of illumination and detection adjusted so that the amplitude of the short-wavelength reflectance peak was maximal. All studied species had short-wavelength peaks in the UV, except for C. regina, where the peak was in the blue wavelength range (Fig. 5a). We note here that the UV reflectance peak of H. glaucippe extends into the visible violet. This allowed studying the multilayer reflections with the scatterometer (Fig. 3c, 4a,b), despite its limited wavelength range (above 380 nm). Further, due to a predictable shape of the short-wavelength scattering pattern along the line (Fig. 4c), a representative measurement can be taken at the centre of the line, thus simplifying the ARM measurements to a two parameter set of (α, β) (see Methods).

The spectral and spatial reflection characteristics of the short-wavelength iridescence depend on the angle of illumination and detection. To visualise the spatial dependence of the iridescence peak, we changed the illumination angle α in steps of 5° and then determined the detection angle β where the amplitude of the short-wavelength band was maximal. In other words, we measured the lines of optimal iridescence β_max(α) for each species (Fig. 5b). If the iridescence had been due to a continuous flat multilayer, a line given by β = −α (the mirror line) would have been obtained. For all investigated species the relationship was approximately linear, but it deviated from the mirror line, indicating that the reflectors were tilted with respect to the wing plane. The total tilt angle, γ, follows from β = −α + 2γ, or γ = (α + β)/2. The tilt angle γ can thus be read as half the vertical distance between the optimal line and the mirror line (Fig. 5b). The tilt angle is the largest in C. regina (γ ≈ −33°) and almost as large in G. cleopatra forewing (γ ≈ −30°) and G. rhamni (γ ≈ −28°) forewing, but smaller in G. aspasia forewing, C. croceus hindwing (both γ ≈ −22°) and G. cleopatra hindwing (γ ≈ −19°). The tilt is the smallest in H. glaucippe (γ ≈ −9°). The tilt angle derived for H. glaucippe should correspond to the angle between the wing and the scale, because in this species the ridge lamellae are parallel to the scale plane (Wilts et al. 2011). The situation is more complicated in C. regina, due to the pronounced scale curvature. In the other species, the lamellar multilayers of the scale ridges are tilted with respect to the scale surface, and the measured tilt angle should be a combination of the lamellar tilt angle (4°~6°) and the scale tilt angle with respect to the wing base.

In our measurements of the angle dependence of the direction of optimal reflectance (Fig. 5b) it appeared that the peak wavelength of the short-wavelength reflectance changed with the angle of illumination. We have further analysed these spectral changes in Fig. 6. The waterfall plots (Fig. 6, left column) show the reflectance spectra measured at the lines of optimal reflectance, β_max(α); that is, for each angle of illumination, α, the cosine-projected reflectance spectrum at the optimal angle β is shown (see Fig. 5b). For all species, the short-wavelength peak wavelength was maximal approximately when α = β = γ. The peak wavelength shifted hypsochromically (towards shorter wavelengths) when going away from the optimal point, as expected for a multilayer reflector (Wilts et al. 2011). The short-wavelength reflections thus exhibit iridescence. The long-wavelength part of the reflectance spectra changed only in amplitude but not in shape, as expected for non-iridescent, diffusive materials.
Figure 5
Reflectance spectra and angular dependence of the optimal iridescence line of the investigated pierid butterflies. a Reflectance spectra with the maximal short-wavelength reflectance measured with the ARM setup (at $\alpha \approx \beta$; for exact illumination and detection angles, see points in b). The line colours of the spectra are the same as those at the left of the photographs of the butterflies (right column; bars: 1 cm), from top to bottom: C. croceus, G. rhamni, G. aspasia, G. cleopatra, H. glaucippe, C. regina. b Angular position of the detector ($\beta$) where the structural colouration is maximal as a function of the angular position of the illumination ($\alpha$). The inset shows the arrangement of the scales on the wing, indicating the angles $\alpha$, $\beta$ and the multilayer tilt angle $\gamma$. The optimal line of an ideal mirror is shown as a dash-dotted line. The expected positions of maximal iridescence for an ideal tilted multilayer are shown with a dotted line. Reading of twice the tilt angle ($\gamma$) is indicated for H. glaucippe.

Figure 7
Spatial reflectance profiles for normally incident illumination ($\alpha = 0^\circ$) for three different wavelengths. a 340 nm; b 420 nm; c 700 nm.

Supplementary Figure S1
Spatial map of G. rhamni indicating the measurement positions with black dots.
Figure 6
Reflectance spectra measured with the ARM setup. a C. croceus; b G. rhamni; c G. aspasia; d G. cleopatra HW; e G. cleopatra FW; f H. glaucippe; g C. regina. The left column shows the spectra for the optimal iridescence line (angles $\beta$ can be read from Fig. 5). The right column shows the spectra measured at the fixed angle of incidence $\alpha = 0^\circ$. 
In the measurements shown in Fig. 6, left column, the directions of both the illumination and the detection changed simultaneously. A simpler, yet realistic, situation occurs with a moving observer, static illumination and a static wing. We therefore measured the reflectance spectra at various detection angles while keeping the illumination angle fixed, at $a = 0^\circ$ (normal illumination; Fig. 6, right column). The short-wavelength reflectance bands then were limited to a small angular range in three cases, around $\beta \sim 2\gamma$ (Fig. 6a,d,f, right column), but in the other cases the angular range is more spread out (Fig. 6b,c,e,g, right column). This can be directly understood from the curvature of the cover scales. The cover scales of *C. croceus* (Fig. 6a), *G. cleopatra* hindwing (Fig. 6d) and *H. glaucippe* (Fig. 6f) are flat, while those of *G. rhamni*, *G. aspasia*, *G. cleopatra* forewing, and *C. regina* are curved. The differences in scale curvature are shown for *H. glaucippe* and *C. regina* in Fig. 4.

Fig. 7 gives an alternative representation of the far-field scattering profiles of the different pierid scales. The reflectances measured at various detection angles $\beta$ with normal illumination, $a = 0^\circ$ (see Fig. 6, right column), were averaged over three wavelength ranges, $340 \pm 10$ nm, $420 \pm 10$ nm, and $700 \pm 10$ nm. The graphs show narrow angular reflectance distributions at 340 nm (Fig. 7a) and 420 nm (Fig. 7b), and a much broader distribution at 700 nm (Fig. 7c). The angular spread of the reflectance depends on the species. The two brightest and sharpest reflectance profiles in the UV are from the flat scales of *H. glaucippe* (FWHM $\approx 19^\circ$) and *G. cleopatra* hindwing (FWHM $\approx 35^\circ$; Fig. 7a). The curved scales of the forewings of the studied *Gonepteryx* species have a double-peaked angular reflectance profile. The broadest profile, covering almost the whole hemisphere, was measured at 420 nm from the scales of *C. regina* (Fig. 7b). The asymmetric tail of *C. regina* is also visible in the scatterometer measurement (Fig. 4f). The short-wavelength angular reflectance profiles of *H. glaucippe* and *C. regina* measured from the wing patches with the goniometer (Fig. 7a,b) are, however, broader than the corresponding profiles obtained from single scales with the scatterometer (Fig. 4c,f). The additional spread is most likely due to varying orientations of the multilayered ridges and the scales on the wing.

The long-wavelength reflectance is for all species similar and has a cosine-like angular relationship (Fig. 7c). The departures from the symmetric, central position and the cosine shape towards negative values of the detection angle, $\beta$, i.e. towards the wing base, are presumably caused by differences in the tilt of the scales with respect to the wings and also by the scale curvature. Furthermore, shading due to scale stacking could occur. The reflectance maxima of the diffuse reflections, at long wavelengths, differ between the species, ranging between 0.4 in *C. croceus* and 0.7 in *G. cleopatra* forewing, roughly in accordance with the measurements performed with the integrating sphere (Wilts et al. 2011). The amplitude differences presumably result from different concentrations of the pigments and differences in scale stacking.

The angular spread of the iridescent wing coloration should have important consequences for the visibility of the wing pattern. We therefore investigated this aspect in more detail. To provide a general and comparative visualisation of the far-field iridescence patterns, we have averaged the reflectance over the iridescence bands (for wavelength ranges, see Methods), at all measurement combinations of $(a, \beta)$. The measurement grid was interpolated, resulting in a spatial map of short-wavelength reflectance (Fig. 8). The resulting spatial maps show that the angular width of the iridescence signal strongly varies among the different pierid species. While some species, like *H. glaucippe* and *C. croceus*, reflect incident light into a limited spatial angle, others, like *G. rhamni* and *C. regina*, produce a considerably broadened far-field scattering pattern. A comparison of the scattering patterns with the scale shapes for all species clearly shows that the curvy scales produce scattering patterns with an extended angular spread (Fig. 8a,c,e,g); the flatter scales produce a narrow scattering pattern (Fig. 8b,d,f). Fig. 8h presents the scattering pattern in the long-wavelength range, at 700 nm, for *C. regina*, where the pigmentary colouration causes diffuse scattering. The pattern closely approximates that of a Lambertian diffuser. The waterfalls for spectral bands at 340, 380, 420 and 460 nm are shown in Suppl. Fig. 2.
Discussion

The colouration of the investigated male pierids is quite diverse and characteristic for each species. The diversity is threefold: first, the wing colouration patterns vary (Wijnen et al. 2007, Wilts et al. 2011); second, the reflectance spectra are different in terms of iridescence peak amplitude and position and the long wavelength cut-off (Fig. 5a); third, the width of the far-field pattern of iridescence varies (Figs. 7,8). Some general principles can be nevertheless recognized. The pigmented colours, created by granular pigment masses inside the scale lumen, are restricted to a few classes, that is, white, yellow, orange and red, depending on the expressed pterins. The concentration of pigment presumably varies among the studied species, as indicated by the differences in the diffuse reflectance in the long wavelength range (Fig. 7c).

The UV- or blue-iridescence reflectance peak of the studied pierids is separated by a through (i.e., a minimum) from the long-wavelength, pigmented colouration. The structural and pigmented coloration together create a highly chromatic signal. With a narrow aperture illumination, the iridescence pattern remains restricted to a limited spatial angle, meaning that the directionality of the structural colouration becomes important in direct sunlight. An iridescent butterfly in flight under the sun will radiate flashes of short-wavelength light while the diffuse long-wavelength light will be less modulated. Flapping *H. glaucippe* wings, with flat scales, will radiate brief UV flashes, whilst the wings of *C. regina*, with curvy scales, will show longer blue flashes, assuming the same flapping frequency (Figs. 7 & 8, Suppl. Fig. 2). We note that under natural conditions, the flashes would always be superimposed over a persistent background signal, especially in the UV wavelength range due to Rayleigh scattering.

In the studied species, the optimal iridescence line is directed away from the mirror line (Fig. 5b). Some angular shift is a necessary consequence of the scale stacking (e.g. \( \gamma \approx -9^\circ \) in *H. glaucippe*). The additional angular shift in the case of for instance *G. cleopatra* forewing (\( \gamma \approx -30^\circ \)) must be due to the combined effect of the lamellar multilayer tilt with respect to the scale plane and the scale curvature. Possibly, the tilt serves a purpose: it is there to optimize the signal’s visibility under a certain positioning of the sun, the wing and the observer.

The wing reflectance spectra are probably tuned to the spectral sensitivities of the photoreceptors in the compound eyes (Stavenga and Ariikawa 2006). The set of photoreceptors are known in considerable detail for two pierids, namely the Small White, *Pieris rapae* (Qiu et al. 2002; Stavenga and Ariikawa 2006), and of the Eastern Pale Clouded Yellow, *Colias erate* (Pirih et al. 2010). The two species both employ UV receptors (360 nm), violet/blue receptors (420-480 nm), green receptors (560 nm) and red receptors (620-660 nm). Very likely other pierids have photoreceptors with very similar spectral sensitivities: we may assume that a generic pierid eye contains one UV receptor class, at least one blue/violet class, a green class and at least one red class. Male pierids with UV reflecting wings will be well detected by the UV receptors. The short-wavelength flashes of flying males will be detected as a distinct, short-lived signal on top of the longer-lasting signal created by the non-iridescent wing reflections, for instance via a two or three receptor opponency mechanism (e.g. UV+/violet−/green+). The blue/red (purple) combination requires photoreceptor types in the blue and red range, a condition met by both *Colias* and *Pieris*. Nevertheless, in the majority of iridescent pierids the signalling of the iridescent flashes will be detected by the UV receptors.

We conclude that there are several realisations of combined structural and pigmentary colouration, having different spectral and spatial signatures, which are determined by the pigment, the multilayer nanostructure and the shape of the scale. It will be interesting to study how the colouration is detected through the eyes of the con specifics and the predators, both in terms of the wing pattern (near-field) and in terms of the far-field signalling during flight. An especially intriguing question is the relative importance of the two signals in terms of mating choice.
Spatial maps for the integrated short-wavelength reflectance of the iridescent wing parts of the investigated pierid butterflies. a G. rhamni; b C. croceus; c G. cleopatra FW; d G. cleopatra HW; e G. aspasia; f H. glaucippe; g C. regina. The scales of the species in the left column are curved, while the scales of the species in the right column are flat, resulting in differences of the angular width of the iridescence. h Integrated diffusive (long-wavelength) part the spectrum of C. regina.
Supplementary figure S2
Spatial maps of a C. croceus; b G. rhamni; c G. aspasia; d G. cleopatra HW; e G. cleopatra FW; f H. glaucippe; and g C. regina integrated over wavelength bands of 340 ± 10 nm, 380 ± 10 nm, 420 ± 10 nm, 460 ± 10 nm and 700 ± 10 nm. The colour coding is mapped to the absolute reflectance range $R^* = [0 .. 1]$. 
References


The classification of five external senses of humans, attributed to Aristotle, is: vision, hearing, smell, taste, touch. Vision and hearing convey spatial information from the remote environment and are therefore the remote imaging senses. Aquatic vertebrates are using two more senses to image the environment: electrorception and the lateral line, implementing remote touch. The human sense of touch may be regarded as an aberrant imaging sense since it does not operate remotely and since it is most often used in the scanning mode (e.g. reading Braille). Chemical senses do not have continuous modalities (e.g. frequency, colour) but stimulus classes (e.g. sweet, sour). Chemical senses are also without the spatial modality; in order to get the spatial picture, an animal needs to scan the environment, e.g. by sniffing. Many animals rely heavily on smelling pheromone plumes to track their conspecifics and their physiological state. There is evidence that the humans are also not stripped of this sense, nor able to ignore it completely. In the case of humans, and many other animals living in illuminated environments, vision is the important sense. What makes vision special?

Vision extracts the information from light, a medium that conveys signals over distances, for all practical earthly living purposes instantly, preserving the directionality of the signal; in other words, light is a medium conveying information about the environment. Moreover, light, due to its wave nature, has two submodalities - wavelength and polarisation – which both can convey information. Vision can remain a passive sense in all environments where there is ambient illumination: practically all materials reflect at least a few percent of the incident light, and the energy that fuels transfer of information is ultimately provided by the Sun (or the stars). The detection is fully passive and static in the sense that it works without either the receiver or sender of the information actively emitting light. The workable range of vision as a passive sense spans from broad sunlight to a starry night, or in water, down to depths of several hundred metres. Further towards the bottom, vision cannot remain passive. Deep water fish often have special light organs.

In some other murky aquatic environments, such as turbid terrestrial waters and caves, active light organs would be either bringing more harm than good, or would simply be too energetically demanding. In these environments, vision is often excelled or completely replaced by one of the two other senses that retain directionality: electrorception and remote touch, as implemented by the fish lateral line (also known as svenning, after its discoverer Sven Dijkgraaf). These two senses have a limited range, due to the fact that they are near field detectors. Their range is directly proportional to the physical size of the sensor array. Further, their acuity is directly proportional to the spacing
between the elements of the array, due to the fact that they are not using focusing optics. These two senses are hardly simultaneously static and passive, because the flow of energy is less constant and predictable in the case of mechanical waves and electrical waves than in the case of light. As a consequence, either stationary objects cannot be readily detected or the receiver must reveal itself by emitting energy that would then be reflected from stationary objects (e.g., production of the electric field in weakly electric fish, or any kind of self-movement for svenning).

While terrestrial arthropods do use mechanoreceptors to detect air flow, a full analogue of the lateral line has not yet been found. The physical constraints of a low-density medium and a relatively small body size prohibit this sense from having imaging capacity that would extend significantly into the environment. Terrestrial vertebrates have lost the ability of either remote touch and electroreception. In most terrestrial animals groups, hearing became an important accessory, or sometimes the main sense. Hearing (i.e., detection of far-field mechanical waves) is a sense developed in both terrestrial and aquatic forms of most animal groups. Binaural hearing however cannot provide a full image of the environment, because it relies on comparison between only two sensory units; for a more precise determination of the sensory image, binaural hearing is often used in conjunction with scanning (e.g., turning the ears or the body). Sometimes, especially in dark environments, binaural scanning combined with emission of energy, prevails over vision. The terrestrial case for this is the sonar of bats; the wet case are dolphins and whales.

Going beyond the intuitive understanding of information extraction, the concept of information capacity of any sense, but especially of vision, may be quantified with a rather computerised unit of bits per second. The information capacity is related to the signal to noise ratio and vision is often optimised for that. The flow of visual information can be overwhelmingly large; however, not all information may be relevant: the signals from static background structures may not always be informative. We can therefore use the concept of relevant information capacity and the signal-to-background ratio. Vision is optimised to extract the relevant signals from the environment and filter out the irrelevant ones. The neural mechanisms downstream further reduce the information and abstract the relevant signal to the point where the nervous system will make a behavioural decision that will be executed by the motor circuitry: attack or retreat, turn left or right. An important concept here is also, that extraction of information costs energy: more bits need more ATP.

The information yielded from the environment is often enhanced by combining the input from several senses or several modalities of the same sense. We may combine sound frequency and direction while we are subconsciously separating the basso continuo from the flamboyant voix singing Cadró, ma quai si mira. We may combine smell and vision to locate the defunct power transistor on the electronic board. We may make combined use of smell, vision and hearing in order to make an educated (?) guess about the genetic compatibility with the person who just entered the party. Humans and birds wear shiny feathers to be statically noticed. When they sing and dance, the display becomes even more persuasive. In the case of butterflies, the pheromones may be used to attract the
conspecifics from a distance. From close, the adornment on the wings seems to be the medium they use to present their genetic quality. The display, be it static (e.g. the pattern of spots) or dynamic (e.g. wing flapping), must be tuned to the vision of the species. As these traits are under sexual selection, we may expect that the tuning will be mercilessly efficient. To complicate things further, a butterfly does not live in a benevolent environment: predators, too, can also use the colouration to detect the butterfly. The colouration is tuned both to the environment and to its vision, often walking on the thin line between being conspicuous and concealed. A similar triangle exist also between hearing, sound production and environment. However, when comparing colouration with sound production, the principle of passivity is again there: colourful adornment is static and will be seen when the two interacting conspecifics are in line of sight, while sound production requires energy and will potentially be detectable in a larger space.

Several aspects of a particular sense must be considered in the studies: the physical modalities, temporal precision, spatial acuity, reach (contact, near field, far field), imaging and scanning capability, the source of energy (the sensor itself, the detected object, extrinsic source), the relevant information, the background, the intrinsic noise. When adding to the picture the animal’s interest in its conspecifics, the aspects of visibility and conspicuousness come into play. The lack of our own experience of a modality sometimes shows up in the lack of research and even the lack of the word for the sense. Outside mammals, UV and polarisation vision reveals a world which looks different than ours. The lateral line sense of amphibians and fish has been named with a synthetic expression (remote touch) and, less well known, with a novel word *svenning*. Although the major interest in studying this sense is due to the fact that it shares the sensory cell type (hair cells) with human hearing, a paradigm-shifting research of the field is more likely to be about how the image of the world is *svenned* by the lateral line.

Even more radically, although vertebrates sense pheromones with a special organ (the vomero-nasal organ of Jacobsen) with has a special neural connection with the brain (terminal cranial nerve, nerve zero), there is no word that would describe the sense, though the sensing action itself is named from German as *flehm* response (the lip-rolling behaviour seen in horses; an overdone human pendant was simulated by Anthony Hopkins in the film *Silence of the Lambs*). The English verb could be *to flehm* (-ed, -ing), the adjective *flemy* or *flehmish*. An alternative English word to describe the sense could be a chimera made from pheromone smell; thus, *phell* as the noun, *to phell* (-ed, -ed, -ing) as the verb, and *phelly* as the adjective. The naming for the sense seems to be due also because it seems more and more plausible that humans, too, sense pheromones.

A student of sensory neuroscience should not be ignorant due to its own disability to detect a certain modality and should care not to get biased by the limitations of its own senses, when the modality is detected by the humans. This way, sensory neuroscience will be able to advance in the directions where biodiversity will provide for new concepts and maybe an extension of the list of external senses. These new paradigms may eventually be put into human use via biomimetics.
Chapter 9

General discussion

In this thesis, we have studied insect vision and butterfly colouration. In Chapter 2, we have dealt with the primary processes of phototransduction in the fruit-fly *Drosophila melanogaster*. In Chapters 3&4, we have studied the eye regionalisation and photoreceptor types in the Eastern Clouded Yellow Butterfly *Colias erate*. In Chapter 6, we have described an instrument, tailored for studies of spatial and spectral patterns of colouration. In Chapters 5&7, we have dealt with the morphology, reflectance spectra and spatial scattering patterns from the wings of iridescent pierid butterflies from the Colias group (*Colias croceus*, *Gonepteryx rhamni*, *G. cleopatra*, *G. aspasia*) and the Colotis group (*Hebomoia glaucippe*, *Colotis regina*).

**The role of arrestin in photoreceptors**

The insect phototransduction cascade is triggered by the conversion of rhodopsin into the active metarhodopsin state, which is subsequently deactivated by binding of arrestin. When arrestin becomes depleted, the photoreceptor has a sluggish repolarisation and may eventually end up in the state of prolonged/persistent depolarising afterpotential (PDA), even after there is no more light to excite the photoreceptor (Dolph et al. 1993).

In the UV and blue receptors and in the main blue-green sensitive photoreceptors of higher flies, the rhodopsins have bathochromic (long wavelength-shifted) metarhodopsins. The spectral distribution of natural light is such that the metarhodopsin fraction remains low and a limited arrestin fraction suffices to prevent the afterpotentials (Stavenga and Hardie 2011). Under experimental conditions, however, it is possible to achieve that metarhodopsins outnumber arrestin. In Chapter 2, we used short-wavelength light stimuli to elicit persistent afterpotentials in the fruitfly main photoreceptors (R1-6). In the hypomorphic arrestin mutant, the afterpotential was elicited at lower metarhodopsin fractions (3%) as in the wild type (30%). We note that in the fruitfly, the afterpotential can be elicited with prolonged monochromatic illumination of extremely low intensities (Belušič and Pirih, unpublished), showing that removal of metarhodopsin and renewal of rhodopsin is negligible. A persistent afterpotential also can be experimentally elicited in the UV receptors of the owlfly (Pirih, unpublished).

The afterpotential is prolonged but not persistent in the blowfly *Calliphora erythrocephala* (Minke and Kirschfeld 1984; Hamdorf and Ramzjoo 1978), presumably because it has a higher arrestin fraction which eventually deactivates the metarhodopsin. A higher arrestin fraction in the blowfly presumably speeds up the information transfer rate of the phototransduction system (Juusola and Hardie 2001). A higher rate comes at the cost of a higher energy consumption, but it seems that the small fruitfly cannot or does not need to afford this luxury (Niven et al. 2007).

On the other hand, the green rhodopsin of the main receptors of Hymenoptera, Orthoptera, lower Diptera, as well as the R8 cells of higher Diptera (blowflies and fruitflies) and the receptors R3-8 of Lepidoptera, has a hypsochromic (short-wavelength shifted) metarhodopsin. A non-persistent afterpotential, which echoed out into individual bumps, has been measured in the green
photoreceptors of the grasshopper (Horridge and Tsukahara 1978). A non-persistent afterpotential has been hypothesised on the basis of optical measurements of the pupil in butterflies (Stavenga 1979). It has been shown that in the green photoreceptors of butterflies, the metarhodopsins are degraded and restored as rhodopsins by the visual pigment turnover cycle (Bernard 1983; Vanhoutte and Stavenga 2005). Natural illumination creates a high (>60%) metarhodopsin fraction in green photoreceptors (Stavenga and Hardie 2011). Although not proven, it is very likely that the green receptors have a large arrestin fraction in order to avoid afterpotentials under natural conditions. We may conclude that the turnover of visual pigment and arrestin control cooperate in forming a subtle adaptation system in green photoreceptors to allow them to function over a broad range of light intensities.

Multispectral imaging in butterfly eyes

Vision is most often the most important sense of diurnal butterflies. It may be used to find food, escape predators, find host plants and find mates. Information from the environment can be contained in the modalities of intensity, spectral content or polarisation. Several types of adaptations of the eye design presumably optimize the spectral information throughput. Duplication of opsin genes and addition of red and yellow filtering pigments lead to tuning of spectral sensitivities and to diversification of ommatidial types which contain subsets of photoreceptor classes. In several butterfly groups, the basic triad of UV, blue and green visual pigment genes has been expanded to four or five. Gene duplications have been shown to occur in all three opsin clades (green, blue/violet, UV; Briscoe 2008). Differential distribution of ommatidial types and formation of acute zones results in regionalisation of the eye. The distribution of photoreceptor classes throughout the butterfly eye often follows two concepts: local (quasi) random mosaic patterning and regionalisation along the dorso-frontal axis of the eye. The dorsal part of the eye is very often without any screening pigments (Arikawa and Stavenga 1997; Stavenga et al. 2001; Stavenga 2002).

We described the anatomical and optical regionalisation of the retina of the Eastern Pale Clouded Yellow Butterfly (Colias erate) in Chapter 3, and the functional consequences—a set of photoreceptor expanded to nine classes, in Chapter 4. The Eastern Pale Clouded Yellow (Colias erate) has a duplicated blue/violet rhodopsin (Awata et al. 2009). One rhodopsin peaks in the violet at 420 nm, and the other most likely in the blue range at about 460 nm (Chapter 4). The dorsal ommatidia are devoid of red screening pigments and consequently, the spectral sensitivities of the photoreceptors in the dorsal eye are probably ranging only from UV to green.

There is a single red screening pigment in the three ventral ommatidial types. The efficiency of screening is different in different ommatidial types; it is increased through constrictions of the rhabdomal waveguides, causing a red sensitivity shift in the proximal, green rhodopsin photoreceptors (Chapter 3). We have found two red photoreceptor classes, peaking at about 630 nm and 650 nm, respectively. The far red class is so far the farthest red-peak receptors found in insects: in Pieris rapae crucivora (Qiu and Arikawa 2003) the two red receptor classes peak at 620 and 640 nm, respectively. In Colias, similarly as in the males of P. rapae crucivora, one ventral ommatidial type additionally contains a distal fluorescing pigment (Chapter 3) which adds to the diversification of violet-blue sensitivity classes (Chapter 4).

Several general trends may be elucidated from photoreceptor and ommatidial specialisation in butterflies. The set of spectral sensitivity classes expands towards red, becomes dense in the blue region, the sensitivity curves are becoming narrower, and all this is done at the expense of absolute sensitivity. The goal of this diversification is presumably to enhance the colour vision range and accuracy. There are several types of colour vision (Chapter 1; Kelber and Osorio 2010).
Experimentally, the most important difference is whether the behaviour resulting from colour vision is innate (colour-specific) or plastic (and thus possibly trainable; true colour vision). For any behaviour that involves colour discrimination, the following questions arise: which part of the eye (region, ommatidial types, photoreceptor classes) are involved in colour discrimination, what are the opponency mechanisms and which level of colour vision is involved. The swallowtail butterfly, for instance, has eight receptor classes of which four (UV, blue, green and red) are used in foraging (Koshitaka et al. 2008). Innate colour preference has been shown for drumming (a prequel to egg-laying) in female cabbage white Pieris brassicae (Kolb 1982). In Papilio aegus, a dichromatic mechanism involving three receptors is used for finding the host plant (Kelber 1999).

It could well be that in Colias, subsets of photoreceptors are used for different behaviours: (1) finding food, (2) finding hosts, (3) finding mates. It is reasonable to assume that Pieris and Colias also use tri- or tetrachromatic colour vision for foraging. Given the abundance of receptor classes, it is even possible that a difference mechanism similar to the one in Papilio aegus (Kelber 1999) exists in one of the main colour channels, enhancing wavelength discrimination in a certain range, while the overall system functions as tri- or tetrachromatic colour vision. Further, it may be possible that the red receptors are used for finding and quality assessment of host plants. For instance, a red–green opponency mechanism could be used for finding the host plant; a two-channel difference mechanism, based on the two sharply tuned red receptor classes, could possibly be used for assessing the level of chlorophyll or water in the host plant (Chapter 4). The involvement of colour vision in the task of finding mates, i.e. the connection between vision and colouration, is discussed in the following section.

The local mosaic structure and the overall regionalisation are presumably related to the existence of several parallel neural pathways, starting with different subsets of photoreceptor classes, which feed the information to the brain, where there may be different circuits designated for different pattern and colour-processing tasks. As a contrast, humans strive to use the photopic and scotopic visual subsystems to perform the same tasks, and often fail miserably in that.

**Colour on the wing and in the eye**

Butterflies are indisputably among the most colourful animal groups on this planet. The patterns on the wings may go from almost uniform to extremely complex and colourful, and may serve different functions: camouflage, predator deterrence, mimicry, intraspecific discrimination (see Chapter 1). Here, we focus on intraspecific recognition of potential mates and competitors and territorial signalling.

The basic unit of the wing pattern is a scale. There are two colouration mechanisms – chemical (pigmentary) and physical (structural). Pierid butterflies employ both mechanisms. Their chemical colouration is based on black/brown melanin and yellow/orange/red pterin pigments. Their physical colouration is due to multilayers and results in iridescence, i.e. in reflectance, the hue of which is angle dependent. In Chapters 5&7, we have studied the colouration of the iridescent males of several pierid species from the Coliadinae subfamily (sulphurs) and the Colotis group (orange and purple tips). The combination of structural and pigmentary colouration in all cases results in spectra with two reflectance bands, the iridescent band being in the short wavelength region and the pigmentary band being in the long wavelength region, with a trough in between. The iridescence band peaks either in the UV (360 nm) or is shifted to peak in the green-blue, at around 480 nm. In the former case, it is accompanied with either yellow or orange pigmentary colouration, and in the latter case with red pigmentary colouration. This two-peaked, “UV-purple” or “blue-purple” colouration is rather uncommon in the usual environmental background: the single-peaked green of
the vegetation, or the UV-rich, red-poor skylight. The reflectance minimum between the two bands is either in the violet (420 nm), blue-green (450-500 nm) or in the orange (580 nm).

It is quite likely that the spectral sensitivities of some of the photoreceptor classes in the butterfly eye are tuned to the wing colouration (Stavenga and Arikawa 2006). Both the amplitude of the iridescent peak (Kemp et al. 2006; Kemp and Rutowski 2007) and the hue of pigmentary colouration may be informative in choosing the mate.

In this case, a mechanism involving three receptor types, coinciding with the two peaks and the trough, could be a good way of detecting the conspecific males. We may suppose that the photoreceptor set of all studied butterflies is very similar to the one found in *C. erate* (Chapter 4). In this case, the UV-purple colouration would be well detected with the UV, nB, and bG classes for the yellow wings of *G. rhamni* and *G. cleopatra*, and by UV, nB and nR classes for the orange wings of *C. croceus* and *G. cleopatra*. Further, the nB/bG difference mechanism, which is probably most sensitive for hue changes in the range 440-480 (Chapter 4), could be used to detect subtle hue shifts of yellow colouration (the steepest slope at about 480 nm) which is related to the pterin pigment concentration. Incidentally, all these receptor classes reside in the fluorescent type I ommatidia in *C. erate*.

In the case of *Colotis regina*, the purple colouration would be well detected with a bB-like receptor and two green rhodopsin based receptors, either an unscreened bG-like receptor and the fR receptor, or two red receptors peaking at about 580 and 650 nm. It is even possible that in the case of *C. regina*, the fR-like receptor would be pushed even further into the far red than in *C. erate*, in order to optimize the contrast.

It seems that in pierid butterflies, the UV-purple colouration is more common whereas the blue-purple colouration is limited to a few species of the genus *Colotis*. It is possible that the ecological pressure from predators pushes the purple colouration towards the shorter wavelengths; after all, not all predators see in the UV and yellow colour is far more common, and hence less conspicuous in the environment, than the blue-purple. It would be interesting to see whether the purple colouration tuning is related to the lifestyle of the species – for instance, *C. croceus*, living in the open fields, has a less pronounced UV iridescence peak (Chapter 5) than the sympatric *G. rhamni* and *G. cleopatra*, which seem to prefer less open habitats. In the case of the genus *Gonepteryx*, it is also an intriguing question, what is the predation cost of the more conspicuous orange wing of *G. cleopatra*?

Pierid butterflies seem not to employ the combination of UV-iridescence with white, UV-absorbing leucopterins (e.g. peak at 320 nm, trough at 380 nm, pass-band from 420 nm on). This configuration would require two receptor classes with two different UV rhodopsins and a bG-like receptor class. Possibly, this combination does not exist because the UV rhodopsins also serve for providing the spectral resolving power, or that the intensity of the environmental illumination below 330 nm is not sufficient for signalling.

Wing colouration may serve for detection when in close contact, but it can also be used for distance signalling. In the latter case, the spatial distribution of the colouration signal is important. Using the setup described in Chapter 6, we have found that within the iridescent pierids, the curvature of the iridescent scales greatly influences the far-field scattering pattern in the short wavelength band: flat scales produce a sharp line pattern whereas curved scales cause a dispersion of the reflectance into a much broader spatial angle. The far-field scattering pattern in the long-wavelength band is in all cases very close to an ideal diffuser (Chapter 7).

The sharpness of the short-wavelength spatial pattern may be important when the male is flying: possibly the Great Orange Tip butterfly, *H. glaucippe*, with flat iridescent scales, employs the strategy of lighthouse signalling: during each flap of the wings, a UV beacon line is swept through the
environmental space. This may be detected as a conspicuous short flash in the UV, a signal that may well be understood by the conspecifics, but not by the predators. A similar strategy is possibly employed by the blue-flashing butterflies from the genus *Morpho*. There, the erratic flashing may be additionally used as a confusing signal to avian predators (Young 1971). Another possible aspect of double colouration of pierids is that the information for the conspecifics is conveyed in the difference between the spatial scattering pattern of the short- and long-wavelength bands (Chapter 7). All these aspects influence visibility, a loose concept dealing with how far away can the colouration signal be detected by the interested conspecifics or the predators.

In order to gain further insight into the ways how the butterflies communicate visually, (1) spatial colouration patterns should be linked to the relevant colour vision model and to environmental illumination; (2) relevant environmental backgrounds should be included; (3) a behavioural paradigm for testing visibility should be developed; (4) the neural mechanisms related to proximity and distance signalling in the higher centres of the butterfly brain should be mapped and physiologically assessed.

References

Young AM (1971) Wing coloration and reflectance in Morpho butterflies as related to reproductive behavior and escape from avian predators. Oecologia 7:209-222
Summary of the PhD thesis

“Vision, Pigments and Structural Colouration of Butterflies”

The theme of this thesis is the interplay of seeing and being seen. Butterflies are wonderful study objects for this question.

In Chapter 1, a general introduction is given to the topics of physiology of insect vision, physiological optics of butterfly eyes, and the optics of butterfly wing colouration.

In Chapter 2, we studied the primary processes of phototransduction (light sensing) in the visual sense (photoreceptor) cells of the fruitfly Drosophila melanogaster. We studied the interplay between the visual pigment, rhodopsin and its controlling protein, arrestin, under stimulation with light of different intensities and wavelengths. The role of arrestin is to inactivate the active form of visual pigment, metarhodopsin. If all available arrestin is used up, the photoreceptors remain spuriously activated even in the absence of light. We compared the electrical responses to light in the white-eyed wild-type fruitfly and in a hypomorphic mutant containing low levels of arrestin2 at a range of stimulus wavelengths and intensities. The reduced level of arrestin2 in the mutant modestly increased the light sensitivity, decreased the photoreceptor dynamic range and made the termination of the electrical response to light slow; the transition between full repolarization and spurious afterpotential occurred at a lower metarhodopsin fraction than in the wild type. We hypothesise that fruitflies, in comparison to bigger flies, economise on safety mechanisms: the only price paid under natural circumstances seems to be a reduced speed of light sensing.

The next two chapters are dealing with the physiological optics of butterflies. Their compound eye consists of ommatidia. Their central parts, the rhabdoms, are fused and form a wave guide. The ommatidia of pierid butterflies have three tiers: the distal tier has four photoreceptors. Two of them express UV or violet/blue rhodopsins. The other seven photoreceptors express a green rhodopsin.

In Chapter 3, we studied the eye regionalisation and the ultrastructure of the eye of the pierid butterfly, the Eastern Pale Clouded Yellow, Colias erate (family Pieridae). Its eye has distinct dorsal and ventral parts. In the ventral eye part, there are three types of ommatidia, which all use a red screening pigment to modify the spectral sensitivities of the photoreceptors. The UV cells always contribute less microvilli to the rhabdom than the violet/blue cells. All three ommatidial types have a constriction of the waveguide between the two tiers, which adds to the efficiency of the screening of the red pigment. The eye shine, resulting from tapetal reflections, peaks in the red (at 660 nm) or in the far red (730 nm), indicating that the sensitivity of proximal photoreceptors is shifted from green to red. The red ommatidia fluoresced under violet excitation, implying the presence of a violet-absorbing pigment that acts as a short-wavelength filter. The ommatidia in the dorsal part of the eye are devoid of screening pigments. The dorsal part of the eye has a brighter red eyeshine and is presumably only sensitive in the UV-green spectral range.

In Chapter 4, we studied the photoreceptors in the eye of Colias. We stimulated the eye with pulses of monochromatic light and used intracellular electrodes to measure the spectral and polarisation sensitivities of individual photoreceptors. We have identified one UV, four violet-blue, two green and two red photoreceptor classes, presumably based on rhodopsins peaking in the UV, violet, blue and green (360, 420, 460 and 560 nm, respectively). The four violet-blue photoreceptor classes are presumably based on a mixture of the two violet/blue rhodopsins, screened by a violet-absorbing distal pigment. The green classes have reduced sensitivity in the ultraviolet range. The two red...
classes have primary peaks at about 650 and 665 nm. This peak shift, achieved by tuning the effective thickness of the red perirhabdomal screening pigment, is so far the largest among insects.

The next three chapters are dealing with the colouration of butterflies. Their wings are covered with two layers of scales, which form pixelated patterns. We have studied the optics of colouration of the iridescent males from a few pierid butterflies from the Coliadinae subfamily (Colias erate, Gonepteryx rhamni, G. cleopatra, G. aspasia) and the Colotis group (Hebomoia glaucippe, Colotis regina).

In Chapter 5, we have dealt with the morphology of single scales and reflectance spectra from the wings of iridescent pierid males. We used scanning electron microscopy (SEM) to describe the ultrastructure of their scales. The pigmentary colouration is based on pterin pigments which are located in small beads in the scales. The ridges on the upper surface of the scales are elaborated into multilayers, which result in iridescent structural colouration. The integrated reflection efficiency is correlated with the number of multilayers. We have measured the dependence of the peak reflectance wavelength on the illumination angle and found that it is in agreement with classical multilayer theory. The iridescence in pierid butterflies is in most cases in the ultraviolet wavelength range, but some species have a blue-peaking iridescence. We hypothesise that the spectral properties of the pigmentary and structural colouration are tuned to the spectral sensitivities of the butterflies’ photoreceptors.

In Chapter 6, we have described an instrument, tailored for studies of spatial and spectral patterns of colouration. The instrument, an imaging scatterometer (ISM) is built around an ellipsoidal mirror and essentially compresses a full hemisphere of reflections into a smaller angle which is then imaged with a commercial CCD camera. The instrument’s performance is illustrated by measurements of the scattering profiles of the blue-iridescent dorsal wing scales of the nymphalid Morpho aega and the matte-green ventral wing scales of the lycaenid Callophrys rubi.

In Chapter 7, we have studied spatial reflection patterns of the wings of iridescent pierid males. Imaging scatterometry demonstrated that the pigmentary colouration is diffuse whereas the structural colouration is more or less directional. The scattering pattern of structural colouration is elongated into a line. The directionality of structural correlation depends strongly on the scale curvature. In the case of the males of the Cleopatra brimstone Gonepteryx cleopatra, the fore- and hindwings have scales with different pigments and different scale curvatures. We hypothesise that the curvature modifies the spatial visibility of iridescence and may play a role in intraspecific signalling.

In Chapter 8, in an essay, we compared different senses, with an emphasis on imaging senses. The thesis is concluded by a short discussion in Chapter 9.
Samenvatting van het proefschrift

“Zien, pigmenten en structurele kleuren van vlinders”

Het centrale thema van dit proefschrift is de wisselwerking van zien en gezien worden. Vlinders zijn hiervoor uitmuntende onderzoeksobjecten.

Hoofdstuk 1 is een algemene inleiding in de fysiologie van het zien van insecten, de fysiologische optica van vlinderogen en de optica van de kleuren van vlindervleugels.

Hoofdstuk 2 beschrijft de primaire processen van de fototransductie (lichtdetectie) in de visuele zintuigcellen (de fotoreceptoren) van de fruitvlieg *Drosophila melanogaster*. We bestudeerden de interactie van het visuele pigment, *rhodopsine*, met het regeleiwit, *arrestine*, de lichtintensiteit en de golf lengte van het licht. Arrestine inactieveert de actieve vorm van het visuele pigment, *metarhodopsine*. Bij uitputting van het arrestine blijft de zintuigcel na belichting geactiveerd. We vergeleken de elektrische responsie als functie van stimulusgolf lengte en lichtintensiteit van het witogige wildtype met die van een mutant met weinig *arrestin2*. Het gereduceerde *arrestin2*-niveau van de mutant verhoogde de lichtgevoeligheid enigszins, verminderde het dynamische bereik van de fotoreceptor en vertraagde de afval van de responsie aan het einde van de belichting; de overgang van de volledige repolarisatie in een blijvende napotentiaal vond plaats bij een lagere *metarhodopsinearctie* dan die in het wildtype. We veronderstellen dat fruitvliegen, vergeleken met grotere vliegen, bezuinigen op veiligheids systemen: de enige prijs die onder natuurlijke omstandigheden betaald wordt is een tragere lichtdetectiesnelheid.

De volgende twee hoofdstukken behandelen de fysiologische optica van de ogen van dagvlinders. Het samengestelde oog van vlinders bestaat uit ommatidia. Centraal staan de gefuseerde rhabdomen, die werken als een lichtgeleider. De ommatidia van de witjes (Pieridae) hebben drie lagen: de distale laag bevat vier fotoreceptoren. Twee ervan hebben UV- of violet/blauw-gevoelige *rhodopsines*. De andere zeven fotoreceptoren maken gebruik van een groen-gevoelige *rhodopsine*.

Hoofdstuk 3 gaat over de regionalisatie en ultrastructuur van het oog van een witje, de luzernevlinder *Colias erate*. Het oog bestaat uit een dorsaal en een ventraal deel. De drie types van ommatidia in het ventrale deel bevatten allen een rood afschermend pigment, dat de spectrale gevoeligheid van de fotoreceptoren wijzigt. De bijdrage van de microvilli van de UV cellen aan het rhabdoom is minder dan die van de violet/blauw-gevoelige cellen. De rhabdomen hebben een insoering, waardoor het spectrale effect van het rode pigment versterkt wordt. De vlinderogen vertonen een spiegelend roodgevoelige reflectie van reflecterende tapeta, afhankelijk van het type ommatidium. Uit de reflectiespectra, die een piek hebben in het rood (660 nm) of in het ver-rood (730 nm), kan worden afgeleid dat de spectrale gevoeligheid van de proximale fotoreceptoren verschoven moet zijn van het groen naar het rood. Het type ommatidia met piek in het rood fluoresceert bij excitatie met violet licht, hetgeen aangeeft dat er een violet-absorberend pigment is dat functioneert als een optisch filter. In het dorsale deel van het oog hebben de ommatidia geen afschermende pigmenten, en er is daar een sterkere rode oogspiegelging. Vermoedelijk is dit deel van het oog alleen gevoelig in het ultraviolette tot groene deel van het spectrum.

In hoofdstuk 4 worden de fotoreceptoren van *Colias* beschreven. Het oog werd gestimuleerd met pulsen monochroomatisch licht, en we gebruikten intracellulaire electrodos om de spectrale en polarisatiegevoeligheid van individuele fotoreceptoren te meten. We identificeerden een UV, vier violet-blauwe, twee groene en twee rode klassen van fotoreceptoren, welke vermoedelijk gebaseerd
zijn op rhodopsines met piekgevoeligheid in het UV, violet, blauw en groen (resp. 360, 420, 460 en 560 nm). De vier violet-blauwe fotoreceptorklassen hebben waarschijnlijk een mengsel van twee violet-blauwe rhodopsines, die gefilterd worden door een violet-absorberend pigment. De groene klassen hebben een verminderde ultraviolet-gevoeligheid. De twee rode klassen zijn maximaal gevoelig bij 650 en 665 nm. De hiervoor noodzakelijke piekverschuiving, gerealiseerd door het rode afschermende pigmentfilter, is voor zover bekend de grootste onder de insecten.

De volgende drie hoofdstukken betreffen de kleuren van vlinders. Hun vleugels zijn bedekt met twee lagen schubjes, die een patroon van pixels vormen. We bestudeerden de optica van de kleuren van de iridescente mannetjes van enkele witjes: luzernevlinders (Colias croesus, Gonepteryx rhamni, G. cleopatra, G. aspasia) en vlinders behorend tot de Colotis groep (Hebomoia glaucippe, Colotis regina).

**Hoofdstuk 5** beschrijft de morfologie van de schubjes en de reflectiespectra van de vlindervleugels. We gebruikten scanning-electronen-microscopie (SEM) om de ultrasculptuur van de schubjes te bestuderen. De pigmentkleuren van de schubjes zijn het gevolg van pterines, pigmenten die geconcentreerd zijn in kleine kraaltjes. De richels aan de bovenkant van de schubjes zijn geplooid en vormen multilagen, die de iridescente kleuren veroorzaken. De totale reflectie blijkt evenredig te zijn met het aantal lagen. De golflengte van maximale reflectie als functie van de hoek van lichtinval is in overeenstemming met klassieke multilaagtheorie. De iridescentie van de mannelijke witjes is voornamelijk gelocaliseerd in het ultraviolet, maar sommige soorten iridesceren in het blauw. We veronderstellen dat de spectrale eigenschappen van zowel de pigmentkleuren als van de structurele kleuren afgestemd zijn op de spectrale gevoeligheden van de fotoreceptoren van de vlinders.

**Hoofdstuk 6** beschrijft een instrument toegespitst op het meten van ruimtelijke en spectrale kleurpatronen. Deze beeldvormende (imaging) strooimeter (ISM) is gebouwd rond een ellipsoidale spiegel, die de ruimtelijke reflecties in het bovenste halfrond van een voorwerp compimeert in een kleine hoek, zodat vastleggen met een digitale camera mogelijk wordt. Metingen aan de blauw-iridescerende vleugels van *Morpho aega* en de matgroene vleugels van *Callophrys rubi* illustreren de kwaliteiten van het instrument.

**Hoofdstuk 7** bestudeert de ruimtelijke reflectiepatronen van de iridescente vleugels van mannetjeswitjes. De strooimeter toonde aan dat de pigmentkleuren diffuus zijn, terwijl de structurele kleuren min of meer gericht zijn. Het strooingspatroon van de structurele kleuren is langgerekt tot lijnvormig, en de gerichtheid hangt sterk af van de kromming van de schubjes. In het geval van de mannetjes van het Cleopatra citroentje, *Gonepteryx cleopatra*, hebben de voor- en achtervleugels schubjes met verschillende pigmenten en kromming. We veronderstellen dat de kromming de ruimtelijke zichtbaarheid van de iridescentie beïnvloedt, wat een rol kan spelen in het geven van signalen naar individuen van de eigen soort.

In **hoofdstuk 8**, een *essay*, worden verschillende zintuigen vergeleken, met de nadruk op de afbeeldende zintuigen. Het proefschrift wordt afgesloten met een korte discussie in **hoofdstuk 9**.
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Dear friends,

I decided not to fall for emotional cheapness, I chose not to walk the edge of (un)intentionally forgetting you. I succumbed to the vanity of writing a few words into your copy instead. That is, if you wished so ...

... the flowerpot will feel it ...

Primzi
Chinese Peacock *Papilio bianor*. Bar 1 cm.