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Reflections on colourful ommatidia of butterfly eyes

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Summary

The eye shine of butterflies from a large number of ommatidia was observed with a modified epi-illumination apparatus equipped with an objective lens of large numerical aperture. A few representative cases are presented: the satyrine *Bicyclus anynana*, the heliconian *Heliconius melpomene*, the small white *Pieris rapae* and the small copper *Lycaena phlaeas*. The colour of the eye shine is determined mainly by the reflectance spectrum of the tapetal mirror and the transmittance spectrum of the photoreceptor screening pigments, if present near the light-guiding rhabdom. Reflectance spectra measured from individual ommatidia show that tapetum and screening pigments are co-expressed in fixed combinations, thus determining different ommatidial classes. The classes are distributed in an irregular pattern that can be rapidly assessed with the novel epi-illumination apparatus. Many butterfly species appear to have red-reflecting ommatidia, which is interpreted to indicate the presence of red-sensitive photoreceptors.

Key words: butterfly, eye shine, colour, vision, photoreceptor, red sensitivity, regionalization, heterogeneity, tapetum, screening pigment.

Introduction

Many insect species have a well-developed visual system with the capacity to see colour, i.e. objects in their environment are discriminated by their spectral content. Butterflies are considered to be highly visual animals and are generally believed to possess colour vision. Nevertheless, definitive evidence for colour vision was only recently obtained for two papilionid species, the Japanese yellow swallowtail *Papilio xuthus* (Kinoshita et al., 1999) and the Australian orchard butterfly *Papilio aegeus* (Kelber and Pfaff, 1999). In the classical example of insect colour vision, the honeybee *Apis mellifera*, three photoreceptors form the standard set of photoreceptors underlying colour vision, with spectral sensitivities in the ultraviolet, blue and green, respectively (Menzel and Backhaus, 1989), corresponding well with the absorption spectra of three identified rhodopsins (Townson et al., 1998). These rhodopsins are assumed to be expressed in anatomically well-defined photoreceptors (Menzel and Backhaus, 1989).

The organization of the spectral types of photoreceptor appears to be much more complex for butterflies. At least six rhodopsins are present in papilionids (Briscoe, 1998), five of which have been shown to be expressed in the retina (Kitamoto et al., 1998, 2000). Parts of the sensitivity spectra of the (at least) five photoreceptor types of *Papilio xuthus*, with peak sensitivities in the ultraviolet, violet, blue, green and red (Arikawa et al., 1987), deviate strongly from known rhodopsin absorption spectra. Some of the deviations are due to filtering by red and yellow screening pigments, present in two distinct groups of ommatidia (Arikawa and Stavenga, 1997). The pigments are concentrated in four clusters, more-or-less symmetrically grouped around the rhabdom, i.e. the cylindrical structure that contains the visual pigments and acts as an optical waveguide. The screening pigments selectively absorb short-wavelength light and, thus, fine-tune the sensitivity spectrum of long-wavelength receptors (Arikawa et al., 1999b). Moreover, a proportion of the ommatidia that contain red screening pigment also harbour an ultraviolet-absorbing pigment, which sharpens a photoreceptor class with an ultraviolet rhodopsin into a narrow-band violet receptor (Arikawa et al., 1999a). The five rhodopsins and three photostable pigments are expressed in the retina in unique combinations, determining three classes of ommatidia (Kitamoto et al., 2000). The three classes are randomly distributed.

Ommatidial heterogeneity appears to be a widespread characteristic of compound eyes, as follows from anatomical and spectrophotometrical evidence; e.g. flies (Franceschini et al., 1981; Hardie, 1986; Salcedo et al., 1999), sphecid wasps (Ribi, 1978a), moths (Meinecke and Langer, 1984), backswimmers (Schwind et al., 1984) and butterflies (Arikawa and Stavenga, 1997; Stavenga et al., 2001). The heterogeneity in the eyes of butterflies can be most exquisitely observed by epi-illumination microscopy (Bernard and Miller, 1970). Diurnal butterflies, but not Papilionidae (Miller, 1979), exhibit a colourful eye shine due to a reflecting tapetum, present in each ommatidium proximal to the rhabdom (Miller and

Bernard, 1968). The tapetum is formed by a tracheole folded into a stack of layers, alternately consisting of air and cytoplasm, thus creating an interference reflection filter. Incident light that has travelled through the rhabdom without being absorbed is mirrored by the tapetum. The eye shine is the fraction of light also escaping absorption on its way back.

In an early study, Ribi (1979a) compared the colour of the tapetal reflection as seen in eye slices, from which the photoreceptor layer had been removed, with the colour of the eye shine, i.e. with the tapetum in the intact eye. He found that tapetal reflection and eye shine colours were virtually identical in Nymphalidae, Satyridae and Lycaenidae, but not in Pieridae. In pierids, a major part of the eye exhibited a prominent red eye shine, whereas the colour of the tapetum with the retina removed was green-yellow. The anatomy of the eye of the small white *Pieris rapae* showed that the red eye shine is the result of the presence of a red screening pigment, which exists in four clusters near the rhabdom, where it selectively absorbs short-wavelength light propagating along the rhabdom (Ribi, 1979b). Evidently, the function of the pigment clusters in *Pieris rapae* is identical to that of the corresponding pigment clusters in *Papilio xuthus*, namely to suppress short-wavelength light, thereby shifting the sensitivity spectrum of the long-wavelength receptors into the red to produce a spectrum corresponding to sensitivity spectra measured electrophysiologically (Shimohigashi and Tominaga, 1991).

Recent anatomical work has revealed that the red pigment of *Pieris rapae* eyes consists of two types of photoreceptor screening pigment, coloured red and deep-red; i.e. the four proximal photoreceptors of an ommatidium are either red- or deep-red-pigmented. The two different types of ommatidium are arranged in a random, heterogeneous pattern in the retina, which can be observed *in vivo via* the eye shine (Qiu et al., 2002).

In an extensive comparative study of butterfly eye shine, we found that the colour of the light reflected from individual, neighbouring ommatidia often varies substantially in many species, testifying to the strong heterogeneity of butterfly eyes (Stavenga et al., 2001). Moreover, a substantial proportion of the ommatidia appeared to reflect in the red. As in the established case for the pierids, this red reflection is presumably the result of red pigment filtering the light flux in the rhabdoms, suggesting that the red-reflecting ommatidia contain photoreceptors with peak sensitivity in the red.

Inspection of the eye shine is a very attractive method of rapidly surveying the distribution of red-reflecting ommatidia within the heterogeneous ommatidial lattice. However, classical epi-illumination microscopy has serious shortcomings because only low-power objectives with small apertures can be successfully applied. This paper demonstrates that these limitations can be largely overcome with a special apparatus that exploits the optical properties of the butterfly compound eye. The apparatus allows a large-aperture objective to be used so that the tapetal reflections of numerous ommatidia can be observed simultaneously. This approach will facilitate the charting of butterfly eyes and thus stimulate further understanding of eye regionalization and heterogeneity (Stavenga, 1992; Stavenga et al., 2001). In addition to presenting a few exemplary cases of butterfly eye shine, it is argued that the physiological functions of the tapetal mirrors and the screening pigments can be inferred from reflectance spectra measured from individual ommatidia.

Materials and methods

Animals

The eye shine of a number of butterfly species was investigated with the apparatus depicted in Fig. 1. The satyrine *Bicyclus anynana* and the heliconian *Heliconius melpomene* (see Fig. 2) were obtained from cultures maintained by the Institute of Evolutionary Sciences, University of Leiden, the Netherlands. The small white *Pieris rapae* (see Fig. 2) and the small copper *Lycaena phlaeas* (see Fig. 3) were captured locally. Specimens were immobilized with wax and mounted on the platform of a goniometer.

Apparatus

The optical apparatus (Fig. 1) is, in principle, a modified epi-illumination microscope. The rationale of the instrument is that incident light applied to a butterfly eye is channelled by the facet lens and crystalline cone into the light-guiding rhabdom. Light reaching the ommatidial tapetum is reflected and guided back through the rhabdom (see Fig. 1 inset); when not absorbed there, it leaves the eye again and is then observable as the eye shine. Because butterfly eyes, like those of most insects, are locally more-or-less spherical, the visual axes of the ommatidia intersect at the eye's centre of curvature. Hence, the optimal way to fill ommatidia with light is to position the eye's centre at the focal point of an objective lens (L1 in Fig. 1) so that a point source at infinity is focused on the eye's centre.

The point source, which is in reality a slightly extended light source, is created by a white light source focused by lens L2 onto diaphragm D1, which is placed in the focal plane of lens L3. The parallel beam leaving L3 is mirrored by a semi-reflecting mirror, angled at 45 ° with respect to the optical axes of L1 and L3. L1 and L3 form a telescopic lens pair because they are confocal.

The reflected light beams leaving the individual ommatidia diverge slightly, depending on the extent of the visual fields of the ommatidia. The beams intersect each other in the eye's centre; the image created there is called the deep pseudopupil (DPP) (Franceschini and Kirschfeld, 1971). In the case of the butterfly, it is also called the luminous DPP (Stavenga, 1979). When the DPP is adjusted to the focal point of L1 and this point coincides with the centre of the goniometer, the eye shine in various areas of the eye can be rapidly scanned.

Lens L4 is placed confocal with L1 and, hence, the telescopic lens pair L1 and L4 images the DPP in the back focal plane of L4, where diaphragm D2 is positioned. The eye shine at the level of the corneal facet lenses is finally imaged by lens L5, placed confocal with L4. The projected image is then photographed with a photomicroscope. To obtain an optimal picture, the areas

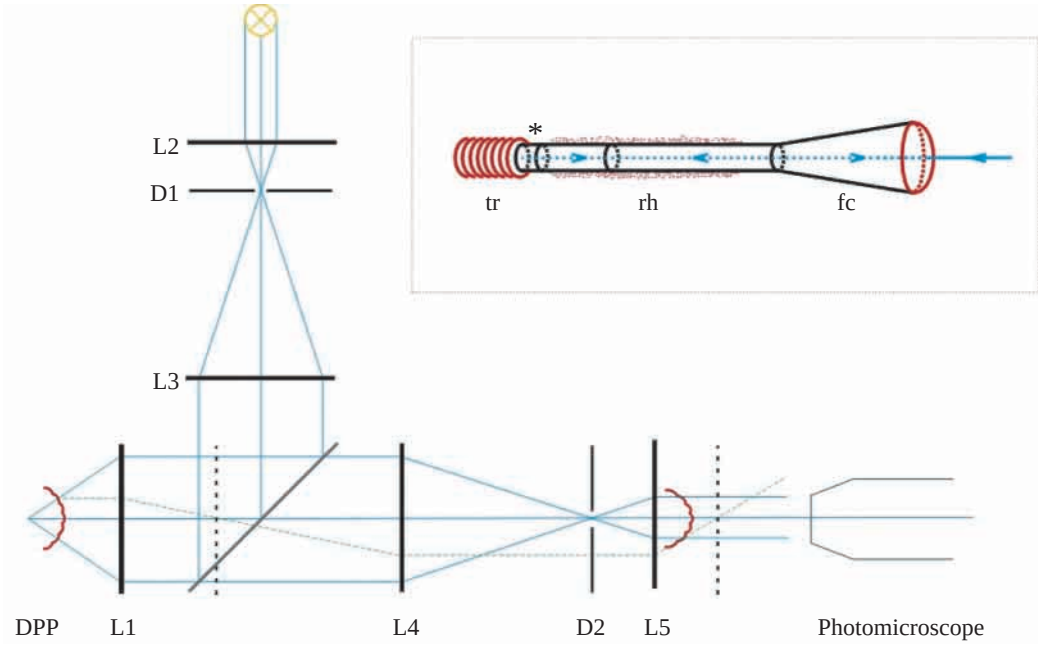

Fig. 1. The optical apparatus used to photograph the eye shine of butterfly eyes. The objective lens L1 has a large aperture. A light source is focused by lens L2 in its back focal plane, where the field diaphragm D1 is positioned. D1 is in the focal plane of lens L3, which is confocal with L1 because of a half-mirror placed at 45° with respect to the optical axes of L1 and L3. A more-or-less parallel beam, depending on the size of D1, enters L1 and is focused on the deep pseudopupil (DPP) in the centre of the butterfly's eye. The telescope lens pair L1 and L4 images the DPP in the back focal plane of L4, where diaphragm D2 is positioned. The image of the corneal eye shine, projected by lens L5, confocal with L4, is photographed by a photomicroscope. The dotted lines are the back-focal planes of L1 and L5. Inset: incident light entering a butterfly ommatidium is focused by the facet lens and crystalline cone (fc) into the rhabdom (rh) and then propagates to the tapetal reflector (tr), where it is mirrored back into the rhabdom and out of the eye again, unless it is absorbed by visual pigments in the rhabdom or by screening pigments in the medium surrounding the rhabdom. The rhabdom organization of a pierid butterfly is indicated schematically: the distal part of the rhabdom consists of the rhabdomeres of photoreceptors R1–R4, the proximal part consists of the rhabdomeres of photoreceptors R5–R8 and the most basal part consists only of rhabdomere R9, which is indicated by an asterisk (see Qiu et al., 2002). The rhabdom is surrounded by photoreceptor screening pigment that absorbs light from the propagating light wave.

of diaphragms D1 and D2 must be adjusted so that they are slightly wider than the image of the DPP. The number of ommatidia contributing to the eye shine depends directly on the aperture of objective L1. A large number can be captured with a Leitz LM32 0.60 objective, which combines a high numerical aperture with a long working distance. L2–L5 are 80, 100, 80 and 15 mm Spindler and Hoyer (Goettingen, Germany) lenses, respectively. The photomicroscope, with a Zeiss 3.2 0.07 objective, is equipped with a Kodak DC120 digital camera.

The actual experimental apparatus used in the present study has two epi-illumination beams supplied by a 50 W halogen lamp and a 100 W mercury lamp. The halogen lamp provided the white light source in Figs 2 and 3, and the mercury lamp was used in Fig. 3 for applying monochromatic light at 670 or 550 nm (*via* Schott DAL interference filters, half-width approximately 15 nm). Although stray light and unwanted reflections are largely eliminated, some reflection on the lens surfaces of the microscope objective remains, and this is visible as a central 'hot spot'. Its prominence can be diminished by reducing the bandwidth of the illumination beam, as was done in Fig. 2B. A long-pass filter, >550 nm, was used in that case since the eye shine had no components in the shorter wavelength range.

The eye shine photographs were made from dark-adapted eyes. Exposures were shorter than 1 s so that contamination by the pupil mechanism (Stavenga et al., 1977) was circumvented. The exposures lasted a few seconds with the 670 nm illumination (Fig. 3), but this long-wavelength light did not activate the pupil.

The apparatus resembles the ophthalmoscopes developed for the analysis of the visual fields of fly eyes by Franceschini (1975) and van Hateren (1984); the main difference is the added epi-illumination arm. Land and Osorio (1990) used an ophthalmoscope with a slightly different design to investigate the spatial properties of butterfly eyes (Land, 1984).

Reflectance spectra

Reflectance spectra (see Fig. 4) were measured with a conventional epi-illumination microscope (Leitz Ortholux) equipped with a Leitz NPL10, 0.22 objective. The goniometer with butterfly was positioned on the stage of the microscope. The eye shine due to illumination with a broadband, white (150 W Xe) light source was measured from a single facet by adjusting a diaphragm in front of an Oriel diode array spectrophotometer attached to the microscope.

The measured reflectance spectra can be formally interpreted by realizing that light emerging from an ommatidium has travelled twice through the length of the rhabdom while having been reflected at the tapetum. Or, the reflectance spectrum, $R_r(\lambda)$, is given by:

$$R_r(\lambda) = M(\lambda)T_r^2(\lambda)\,,$$

where λ is the wavelength of the light, $M(\lambda)$ is the reflectance spectrum of the tapetal mirror and $T_r(\lambda)$ is the (single pass) transmittance spectrum of the rhabdom (Stavenga et al., 1977). The transmittance of the rhabdom is affected by two components: transmitted light is absorbed both by the visual pigments in the rhabdom interior and by the screening pigments in the exterior medium surrounding the rhabdom. The measurements of Fig. 4 were performed after prolonged pre-illumination that bleached the long-wavelength (green) visual pigment. The absorption of the screening pigments then determines the rhabdom transmittance in the long-wavelength range, above 550 nm, because absorption by the visual pigment has become negligible there.

Results

Eye shine

Fig. 2 presents the eye shine of two tropical butterflies, the satyrine *Bicyclus anynana* and the heliconian *Heliconius melpomene*, as well as that of the small white *Pieris rapae*, photographed with the apparatus depicted in Fig. 1. The frontal, i.e. forward-looking, areas were selected because the interommatidial angle in this area is smallest and, hence, the number of shining ommatidia captured by the objective aperture is largest (see Stavenga et al., 2001). In all three cases in Fig. 2, ommatidial heterogeneity is strikingly apparent, with the ommatidia reflecting predominantly yellow or red. The heterogeneity in the eye shine pattern in *Bicyclus anynana* is restricted to the ventral eye area (Fig. 2A); dorsally, only one type of reflection, that of the yellow-reflecting ommatidia, occurs. A survey of the eye of *Heliconius melpomene* (Fig. 2B) shows that two ommatidial types co-exist throughout the eye. The ommatidia in the majority of the eye of *Pieris rapae* are red or deep-red, the latter ommatidia appearing rather dark in Fig. 2C; the dorsal ommatidia reflect a mixture of yellows, and the ommatidia in a transition zone of approximately 15 rows between the dorsal and ventral areas reflect rather uniformly red (Fig. 2C).

Another example of a butterfly with a distinct dorsal area is the small copper *Lycaena phlaeas* (Fig. 3). This eye was photographed from four directions, differing by 30 ° from each other, in a vertical plane. In the main fronto-ventral area, two classes of ommatidium can be distinguished, reflecting predominantly in the green or red. The two classes can be easily discriminated by using suitable monochromatic light, i.e. with wavelengths of 670 or 550 nm. In the dorsal area, a mixed population of bluish-green-reflecting ommatidia

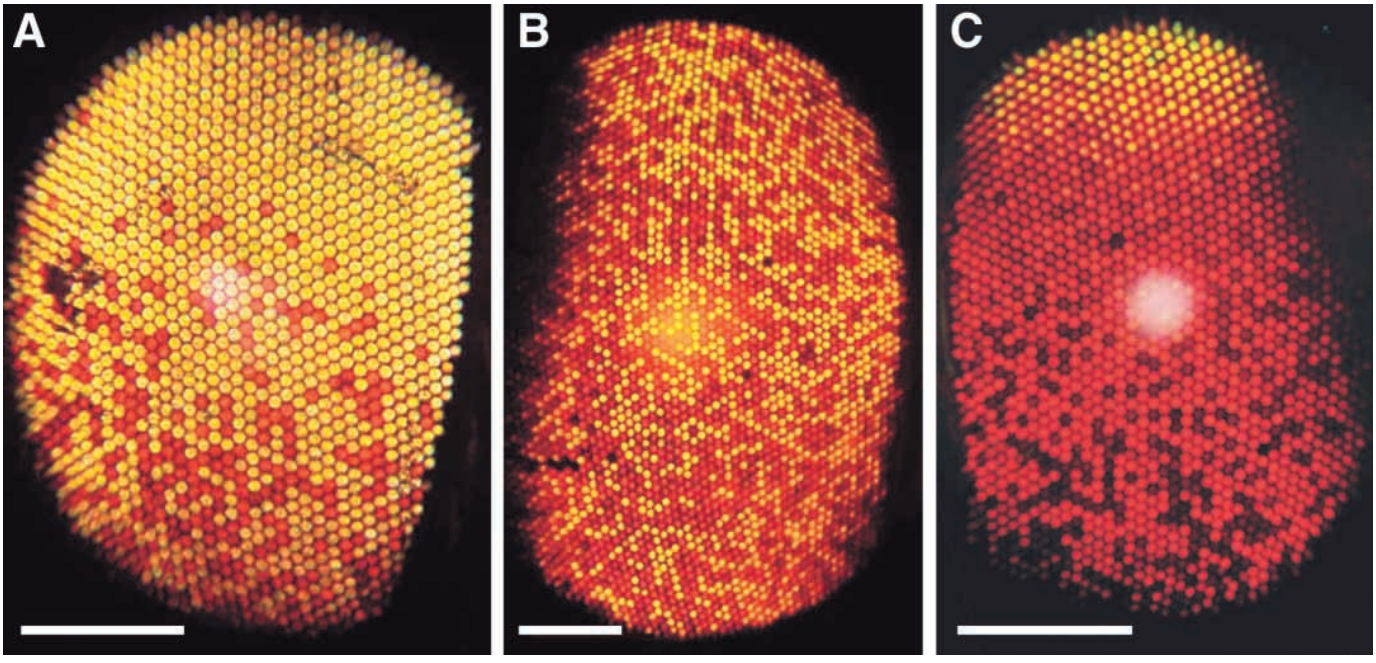

Fig. 2. The eye shine patterns in the eyes of the satyrine *Bicyclus anynana* (A), the heliconian *Heliconius melpomene* (B) and the small white *Pieris rapae* (B) observed with the large-aperture optical apparatus depicted in Fig. 1. The ommatidia in the three species reflect either predominantly yellow or predominantly red light. The red reflection is absent from a large dorsal area of the eye of *Bicyclus anynana* and from a small dorsal area of the eye of *Pieris rapae*; in *Heliconius melpomene*, both reflection types co-exist throughout the eye. The central 'hot spot' is due to reflection on the lens surfaces of the microscope objective. The dark areas in A and B are caused by specks of dust; the dark facets in C have a strong deep-red reflection. The scale bars, 300 μm in A–C, refer to the central part of the figures only because the optical apparatus suffers from slight barrel-type distortion.

exists, but red-reflecting ommatidia are completely absent dorsally.

Reflectance spectra

The different reflection colours in Figs 2 and 3 indicate that the ommatidia of butterfly eyes can be divided into distinct classes. This is confirmed by measurements of the reflectance spectra of individual ommatidia. Fig. 4 presents the spectra of two members of the two classes, yellow and red, distinguishable in the eye of *Bicyclus anynana* (see Fig. 2A). The reflectance spectra of the ommatidia within the same class appear to scatter slightly, but by no more than 5–10 nm. The reflectance spectra peak at around 580 and 650 nm and differ distinctly in shape. The reflectance of the yellow class covers a broad wavelength range, with a cut-off wavelength at approximately 600 nm, whereas that of the red class is negligible at wavelengths below 560 nm and its cut-off is at approximately 700 nm.

Discussion

Heterogeneity and regionalization of butterfly eye shine

Butterfly eye shine can be observed from a large number of ommatidia using a setup that exploits the approximately spherical architecture of the compound eye (Figs 1–3). In conventional epi-illumination microscopy, background reflections usually obscure the eye shine, mainly because the field diaphragm is imaged at the plane of observation. The image contrast is considerably improved when incident light is effectively channelled into the individual rhabdoms, which is achieved by careful diaphragming and focusing the light beam on the DPP. A further improvement is realized by diaphragming the image of the DPP. In this way, one can largely remove light scattered by pigments in the pigment cells and suppress the reflections from lens surfaces, e.g. from the corneal facet lenses and the optical components of the microscope.

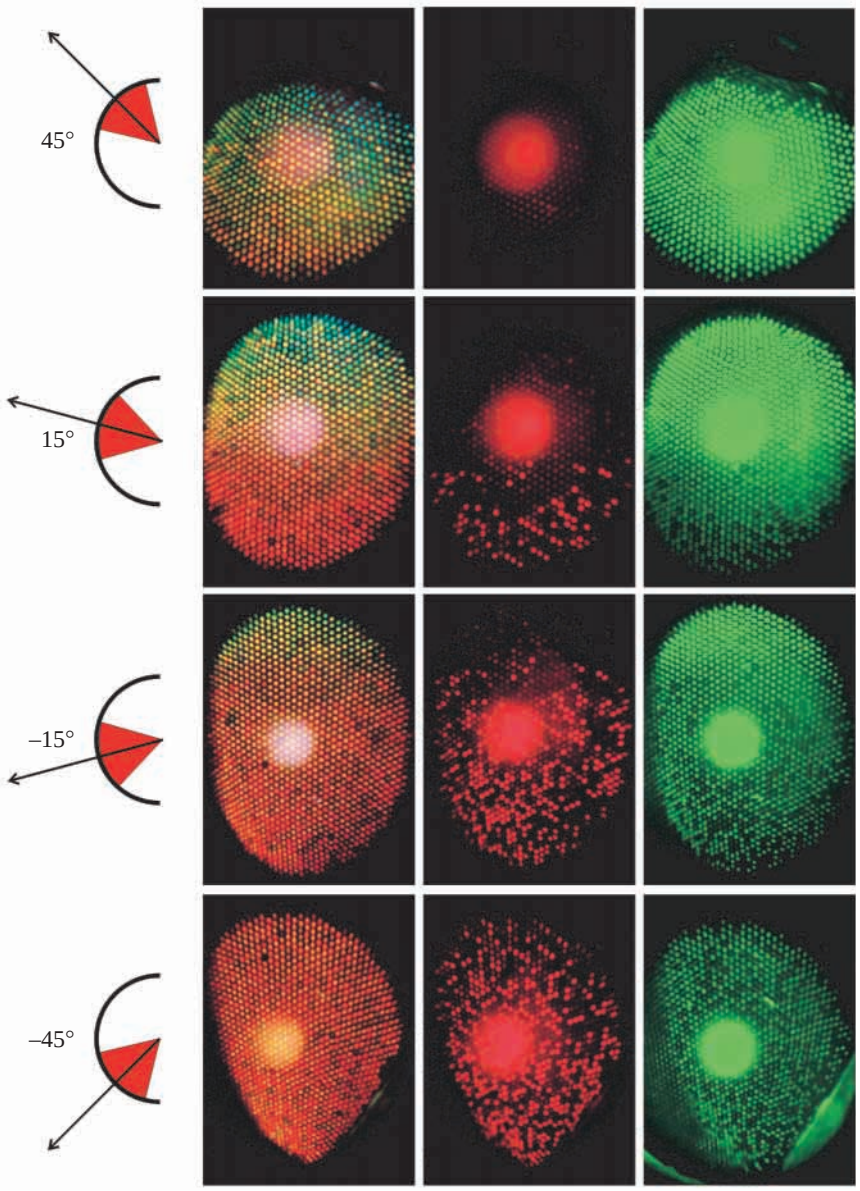

Fig. 3. The eye of the small copper *Lycaena phlaeas* photographed from four dorsal to ventral directions, differing by 30° from each other, with broad-band, white light (halogen lamp) and monochromatic red (670 nm) and green (550 nm) light. The effective aperture of the objective is approximately 60°. The frontal and ventral parts of the eye contain a mixture of red- and yellow-green-reflecting ommatidia, but dorsally the reflection colours are a mixture of blue and green; red reflection is absent from the dorsal region. The central 'hot spot' is due to reflection on the lens surfaces of the microscope objective. 0° is approximately horizontal.

Visualizing the eye shine with a large aperture gives an immediate impression of the distribution of the various classes of ommatidium over the eye. The striking feature of most butterfly eyes is the large degree of heterogeneity of the eye shine pattern. A survey of different species from the families Nymphalidae, Lycaenidae and Pieridae indicates that the eye shine emerging from individual facet lenses is characteristic of the species. The typical yellow/red pattern of Heliconinae (Fig. 2B) also exists in certain Nymphalinae (e.g. *Euphaedra christyi*), Charaxinae (e.g. *Charaxes fulvescens*) and Lycaenidae (e.g. *Polyommatus icarus*), but very different patterns also occur.

Eye regionalization is apparent when a specialized dorsal area exists. Its extent can be large as in *Bicyclus anynana* (Fig. 2A), rather minor, as in *Pieris rapae* (Fig. 2C), or it can even be absent, as in *Heliconius melpomene* (Fig. 2B). In a comparative study of a number of heliconian species that all lacked a distinct dorsal area, we found that the ratio of the

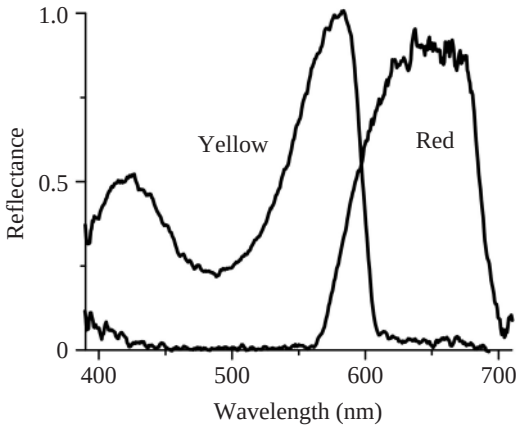

Fig. 4. Reflectance spectra measured from single-facet lenses in the eye of the satyrine *Bicyclus anynana*. The spectra fall into two classes, yellow and red. The reflectance spectrum of the yellow class is broad, extends into the ultraviolet, is very minor above 600 nm and peaks at around 580 nm; the reflectance spectrum of the red class is a restricted band around 650 nm and is negligible below 560 nm. The spectra are normalized to the peak value of the yellow class.

differently coloured facets can change markedly across the eye (M. Joron and D. G. Stavenga, unpublished observations), suggesting that heterogeneity and regionalization exist universally in butterfly eyes (Stavenga et al., 2001).

Eye regionalization suggests that different eye areas have special functions (Bernard and Remington, 1991). A plausible interpretation for the function of a distinct dorsal eye area is that it is specialized for discriminating objects against the sky, where short-wavelength light is dominant (Stavenga, 1992). This explanation probably holds for several butterfly species, as is suggested by the commonly shorter wavelengths of the eye shine dorsally compared with the eye shine of the ventral areas of the eye (Stavenga et al., 2001). The usual absence of red-reflecting ommatidia from the dorsal eye area (Figs 2A,C, 3) also indicates that red sensitivity is not at a premium there. However, there is substantial interspecies diversity. For example, the reflection pattern of *Hypolimnas anthedon* (Nymphalinae) does not comply with the general rule of shorter-wavelength reflections dorsally because the eye shine in a large dorsal area is homogeneous yellow-orange and that in the ventral area is either yellow or variable-green. Also, electroretinogram recordings in *Papilio xuthus* suggest that short-wavelength sensitivity is prominent ventrally (Arikawa et al., 1987).

Tapetum and photoreceptor screening pigment

The reflectance spectra measured from single facets of the two classes in *B. anynana* show distinct differences (Fig. 4). How can these spectra be interpreted? In general, three main variables determine the reflectance: the tapetum, the visual pigments inside the rhabdom and the screening pigments in the photoreceptor cell, when granules containing the latter pigments occur in the immediate surroundings of the rhabdom. The influence of visual pigment absorption on the spectra of Fig. 4 can be neglected because the spectra were measured after repeated bright illumination so that the green rhodopsin was virtually fully bleached (Bernard, 1983). Prolonged dark adaptation, for a few hours, yielded a spectrum with much lower reflectance for the yellow class and with a slightly different shape (Stavenga et al., 2000a).

In the case of the red ommatidia, reflectance is negligible below 560 nm. This is probably due to a strongly short-wavelength-absorbing, red-transmitting screening pigment sequestered in certain photoreceptor cells near the rhabdom. In the yellow ommatidia, a similar, strongly short-wavelength-absorbing pigment is clearly absent because reflection is considerable at all wavelengths below 600 nm. The long-wavelength cut-off values of the reflectance spectra, which are approximately 600 and 700 nm for the yellow and red class, respectively, must be determined by the tapetal mirrors. In other words, the yellow-reflecting ommatidia have a tapetum that reflects up to approximately 600 nm and no screening pigment, and the red-reflecting ommatidia have a tapetum that reflects up to approximately 700 nm together with a red photoreceptor screening pigment that absorbs up to approximately 560 nm. Tapetum and screening pigments are expressed together in unique combinations, thus determining the ommatidial classes. The reflectance spectra measured from single facets in the eyes of several other butterfly species (Qiu et al., 2002; K. J. A. Vanhoutte and D. G. Stavenga, unpublished observations) suggest that this conclusion holds quite generally.

Spectral shifts induced by red photoreceptor pigment

The sensitivity spectrum of a photoreceptor cell that receives light filtered by a red screening pigment depends on the absorption spectrum of the visual pigment and that of the screening pigment and its effective density. A distinct red sensitivity, with spectra peaking even above 600 nm, has been noted in several butterfly species (Swihart and Gordon, 1971; Bernard, 1979; Steiner et al., 1987; Scherer and Kolb, 1987a). In principle, this sensitivity could be based exclusively on red-absorbing rhodopsins (Bernard, 1979). However, the longest peak wavelength of an insect rhodopsin determined so far is 600 nm (Bernard, 1979; Bernard et al., 1988), and the often aberrant spectral shape of the sensitivity spectra indicates that red pigment filters play a central role in butterfly red sensitivity. A red filter can shift the sensitivity spectrum of a photoreceptor, which in the unfiltered situation peaks in the green or orange, towards longer wavelengths, i.e. into the red (Arikawa et al., 1999b).

The spectral shift will be especially prominent in the basal photoreceptor, R9. This photoreceptor fills a short, basal part of the rhabdom, as has been demonstrated in Nymphalinae (Kolb, 1985), Papilionidae (Arikawa and Uchiyama, 1996) and Pieridae (Qiu et al., 2002). To investigate the effect of red screening pigments on the sensitivity spectrum of R9, I have made a simple computational model (Fig. 5). Fig. 5A treats the case of the red-reflecting ommatidia of *Bicyclus anynana*, where a red pigment filter is inferred. The transmittance spectrum of the red filter can be derived from the measured

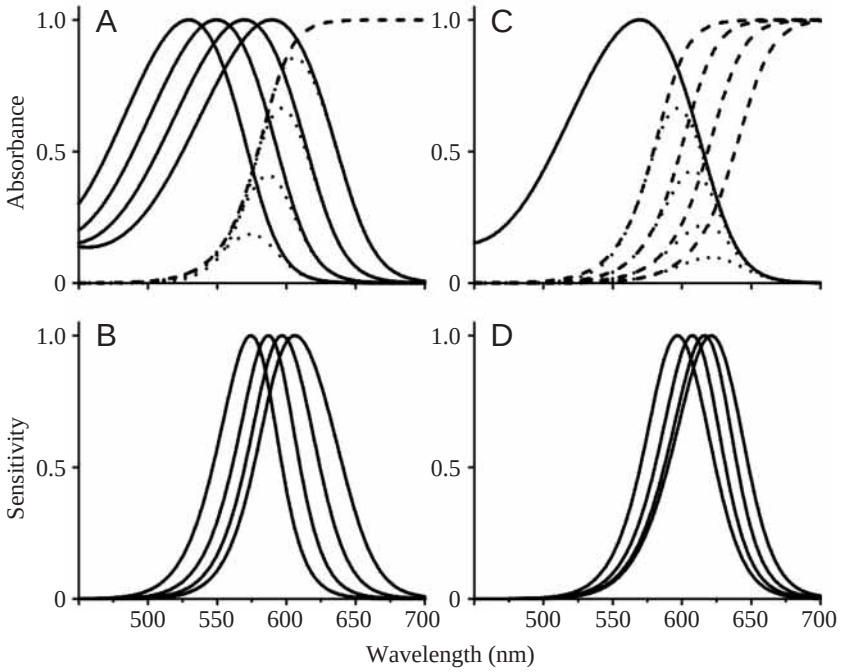

Fig. 5. Modelling of the shift in spectral sensitivity induced by photoreceptor screening pigment when accumulated near the rhabdom on the basal photoreceptor R9. (A) The screening pigment, with a transmittance spectrum given by the dashed curve (compare the red reflectance spectrum in Fig. 4 and see text), acts on four different visual pigments, peaking at 530, 550, 570 and 590 nm (continuous curves). The absorption by the visual pigments (dotted curves) is obtained by multiplying the absorption spectrum of rhodopsin by the filter transmittance spectrum. (B) Normalizing the resulting spectra yields the sensitivity spectra. (C) Four different red filters (dashed curves) act on a visual pigment peaking at 570 nm (continuous curve), giving rise to absorption spectra of lower magnitude (dotted curves). (D) Normalization yields the sensitivity spectra.

reflectance spectrum by assuming that the absorption of the visual pigment can be neglected and that the reflectance spectrum of the tapetum is flat in the wavelength range 550–650 nm. The normalized reflectance in that range then approximates a modified hyperbolic curve: $R(\lambda)= 1/[1+(\lambda_h/\lambda)^n]$; where λ is the wavelength of the light. The wavelength of half-reflectance, λ_h, and the exponent, n, are obtained by fitting the experimental data, yielding values of 590 nm and 60, respectively. The normalized transmittance is then calculated by taking the square root of $R(\lambda)$. The visual pigment in R9 is unknown, so four different rhodopsins are considered, peaking at 530, 550, 570 and 590 nm. Because the rhabdom length of R9 is short, the sensitivity spectrum in the unfiltered situation is virtually identical to the (normalized) absorption spectrum of the rhodopsin, whose shape can be assumed (Stavenga et al., 2000b). Multiplying the filter transmittance spectrum by the rhodopsin absorption spectrum and subsequent normalization yields the sensitivity spectrum of R9 (Fig. 5B). The induced spectral shift depends on the rhodopsin spectrum. The sensitivity peak wavelengths are bathochromic-shifted relative to the rhodopsin peak wavelengths by 44, 37, 27 and 16 nm, respectively. Fig. 5A shows that the resulting sensitivity depends strongly on the overlap between the rhodopsin and filter spectra. Of course, the absolute sensitivity is enhanced by the reflecting tapetum, but this will never amount to more than a factor 2. Fig. 5C depicts a photoreceptor with a rhodopsin peaking at 570 nm and the spectral shifts induced by four red filters with λ_h values of 590, 610, 630 and 650 nm (n=60). The induced spectral shifts are 27, 38, 46 and 51 nm, respectively. It is again clear that rhodopsin and filter spectra should have at least some overlap, as both the increment in spectral shift and the absolute sensitivity progressively drop when the overlap severely declines.

The small white *Pieris rapae* has two types of red-filtering pigment (Qiu et al., 2002), with λ_h values of approximately 610 and 640 nm. The induced spectral shifts in the red- and deep-red-pigmented ommatidia will be distinctly different. As indicated by Fig. 5, the shifts will depend strongly on the visual pigments in the corresponding R9 photoreceptors. We would expect *P. rapae* to have rhodopsins peaking near 600 nm because the rhodopsin and filter spectra will then overlap. Of course, the screening pigments will also induce spectral shifts in the proximal photoreceptors, R5–R8, but these shifts may be less pronounced because the pigment is distributed along an extended part of the rhabdom. As shown for *Papilio xuthus*, a more detailed analysis of the spectral effects of the screening pigments will be greatly facilitated when the sensitivity spectra of the various photoreceptor types are known (Arikawa et al., 1999a,b); such an analysis will then also allow the function of the tapetal mirrors to be assessed.

Function of red sensitivity

Shifting the sensitivity spectrum of a photoreceptor with a short-wavelength-absorbing filter is well known, e.g. the oil droplets in bird cones (Govardovskii, 1983) and the carotenoid filters in stomatopod rhabdoms (Marshall et al., 1991; for a review, see Douglas and Marshall, 1999). Filtering inevitably causes a reduction in absolute sensitivity, but this cost can be reduced by the tapetal mirror, and it can be easily worth the benefit of enhanced colour contrast discrimination (Govardovskii, 1983). The red receptors of butterflies may be of special importance during oviposition for discriminating suitable leaves for the larvae (Bernard and Remington, 1991; Chittka, 1996; Kelber, 1999). The extremely dense red pigmentation in the Pieridae and the apparently dual system for enhancing red sensitivity strongly suggest that spectral discrimination in the red part of the spectrum is especially

well-developed in this family (Kolb and Scherer, 1982; Scherer and Kolb, 1987a). However, red sensitivity is probably common among butterflies and may serve several functions, including feeding and mate recognition (Bernard, 1979; Scherer and Kolb, 1987b; Kinoshita et al., 1997).

Creating red receptors *via* selective red filtering by photoreceptor screening pigments is not restricted to butterflies; for example, sphecid wasps apply the same principle (Ribi, 1978b). It is intriguing that sphecids, like butterflies, also arrange their red pigments in four clusters in one class of ommatidium, this class being randomly distributed within a rather crystalline ordered ommatidial lattice (Ribi, 1978a).

Heterogeneity and colour vision

The design concepts underlying the ubiquitous heterogeneity in butterfly eyes are not understood. The available evidence, coming from different insect orders, suggests that heterogeneity and colour vision are somehow connected. For example, the central photoreceptors, R7 and R8, of fly ommatidia exist in two fixed combinations. The two classes of R7/8 pairs, which are distributed in a random pattern in the retina of flies (Franceschini et al., 1981; Hardie, 1986; Salcedo et al., 1999), probably together mediate colour vision (Fukushi, 1989; Troje, 1993).

Recent anatomical and molecular biological work on the moth *Manduca sexta* (R. H. White, unpublished results) describes a heterogeneous organization of the spectral receptor types in the ommatidial lattice strikingly similar to that of diurnal butterflies, e.g. the papilionid *Papilio xuthus* (Arikawa and Stavenga, 1997; Kitamoto et al., 2000) and the nymphalid *Vanessa cardui* (A. D. Briscoe and A. S. Szeto, unpublished results). The local heterogeneity of the spectral photoreceptor types in the eye of *Papilio xuthus* (Arikawa and Stavenga, 1997) and the possession of colour vision by this butterfly (Kinoshita et al., 1999; Kinoshita and Arikawa, 2000) may indeed indicate that heterogeneity and colour vision are intimately related.

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