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ORIGINAL PAPER

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Visual pigment spectra of the comma butterfly, *Polygonia c-album*, derived from in vivo epi-illumination microspectrophotometry

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Abstract The visual pigments in the compound eye of the comma butterfly, *Polygonia c-album*, were investigated in a specially designed epi-illumination microspectrophotometer. Absorption changes due to photochemical conversions of the visual pigments, or due to light-independent visual pigment decay and regeneration, were studied by measuring the eye shine, i.e., the light reflected from the tapetum located in each ommatidium proximal to the visual pigment-bearing rhabdom. The obtained absorbance difference spectra demonstrated the dominant presence of a green visual pigment. The rhodopsin and its metarhodopsin have absorption peak wavelengths at 532 nm and 492 nm, respectively. The metarhodopsin is removed from the rhabdom with a time constant of 15 min and the rhodopsin is regenerated with a time constant of 59 min (room temperature). A UV rhodopsin with metarhodopsin absorbing maximally at 467 nm was revealed, and evidence for a blue rhodopsin was obtained indirectly.

Keywords Color · Vision · Insect · Compound eye · Eye shine · Rhodopsin

Abbreviations DPP: Deep pseudopupil · UV: Ultraviolet

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Introduction

The prominent compound eyes of diurnal butterflies provide the input for a spatially acute as well as color vision system (Arikawa et al. 1987; Kinoshita et al. 1999; Land and Nilsson 2001). A unique feature of butterfly eyes is their colorful eye shine. In the past decades, combined anatomical and optical studies have yielded considerable insight into the origin and optical mechanisms underlying this intriguing phenomenon (Miller and Bernard 1968; Ribi 1979; Stavenga 2002a, b; Briscoe et al. 2003).

In a butterfly ommatidium, light is focused by a lens and cone into the rhabdom, a cylindrical structure consisting of the rhabdomeres of nine photoreceptor cells, the microvilli of which contain the visual pigment molecules. The rhabdom acts as a light guide, so to realize a high absorption of incident light by the visual pigments. Light that has not been absorbed and reaches the proximal end of the rhabdom is reflected at the tapetum, a multilayered structure created by intricately folded tracheoles. After a second passage through the rhabdom, part of the reflected light leaves the eye, giving rise to the phenomenon of eye shine. The observed color of the eye shine is attributed to the combined effect of the selective spectral absorption by visual pigments and the interference reflection properties of the tapetum (Miller and Bernard 1968; Stavenga 2002b). An additional spectral effect can result from colored screening pigments, which in several occasions surround the rhabdom (Ribi 1979; Arikawa and Stavenga 1997; Qiu et al. 2002). The eye shine is only seen in dark-adapted eyes. Illumination rapidly extinguishes the eye shine, because pupillary pigments inside the photoreceptor cells migrate towards the rhabdom upon light adaptation and thus effectively reduce the propagating light flux (Stavenga et al. 1977; Qiu et al. 2002).

Recent research on the Japanese swallowtail butterfly, *Papilio xuthus*, applying molecular biology and electrophysiology, revealed that the ommatidial

composition of the eyes is heterogeneous. The eyes contain a few classes of randomly distributed ommatidia, with often a marked dorso-ventral regionalization (Arikawa and Stavenga 1997; Kitamoto et al. 1998). A similar situation exists in the pierid butterfly *Pieris rapae* (Qiu et al. 2002; Wakakuwa et al. 2004) and the nymphalid *Vanessa cardui* (Briscoe et al. 2003). Heterogeneity and regionalization seem to be general characteristics of butterfly eyes (Stavenga et al. 2001; Warrant et al. 2003). So far, the eye heterogeneity has been documented for a restricted number of butterfly species using large aperture epi-illumination microscopy (Stavenga 2002a, b; Vanhoutte et al. 2003), complemented by recordings of reflection spectra by microspectrophotometry from individual facets (Vanhoutte 2003).

The main challenge of the present research is to understand the basic principles of eye design and their relation with butterfly sensory ecology, especially concerning the spectral properties of the retina determining color discrimination (Bernard and Remington 1991; Arikawa and Stavenga 1997). In this paper, we use optical techniques to determine the spectral properties of the visual pigments. We have calculated absorbance difference spectra from measured reflection spectra during photochemical conversions of the visual pigments, as well as during long-term dark processes, revealing metarhodopsin degradation and rhodopsin regeneration (Bernard 1983a, b).

For the present study, we selected the comma, *Polygonia c-album*, because of its homogeneous red-orange eye shine that extends far into the red region of the spectrum (halfwidth cut-off value at 700 nm), indicating a strongly homogeneous population of ommatidia (Stavenga 2002a; Vanhoutte 2003). This allows in vivo photochemical characterization of the visual pigments.

Materials and methods

Animals and preparation

The comma butterfly, *Polygonia c-album*, captured in Groningen, the Netherlands, was mounted in a plastic tube and positioned on the goniometer of a microspectrophotometric set-up. The head and thorax were sealed to the plastic tube with wax, to avoid eye movements. The animal was fed with sugar solution before the experiment.

Optical set-up

Reflection spectra were recorded with the optical set-up of Stavenga (2002b), which was slightly modified for the present experiments (Fig. 1). Briefly, this set-up is an assembly of lenses arranged pairwise as telescopes, that is, the lens pairs L0 and L3, L0 and L2, and L1 and L2, all have coinciding focal points. A half-mirror in between L0 and L2 is positioned under 45°. D1 and D2 are diaphragms. The test light (TL) source was a 50 W halogen lamp. The light beam was equipped with a lightly blue-colored filter to suppress the light flux at the longer wavelengths, where the eye shine generally is the strongest. The light reflected by the butterfly eye was projected by objective L0 and lens L3 in the plane of diaphragm D2, where it was captured by a quartz fiber (diameter 0.6 mm) and guided to a spectrophotometer (SD2000, Avantes, Eerbeek, the Netherlands), consisting of a monochromator and a linear diode array, with spectral range 178 to 890 nm and resolution 0.3 nm. Data-acquisition, operating in the Labview 6.0 environment (HSD drivers, Avantes), was externally triggered in combination with the shutters in the light path, which were driven by TTL pulses of DAC's of a CED 1401 interface under control of Signal2 software (Cambridge Electronic Design, Cambridge). A 100 W

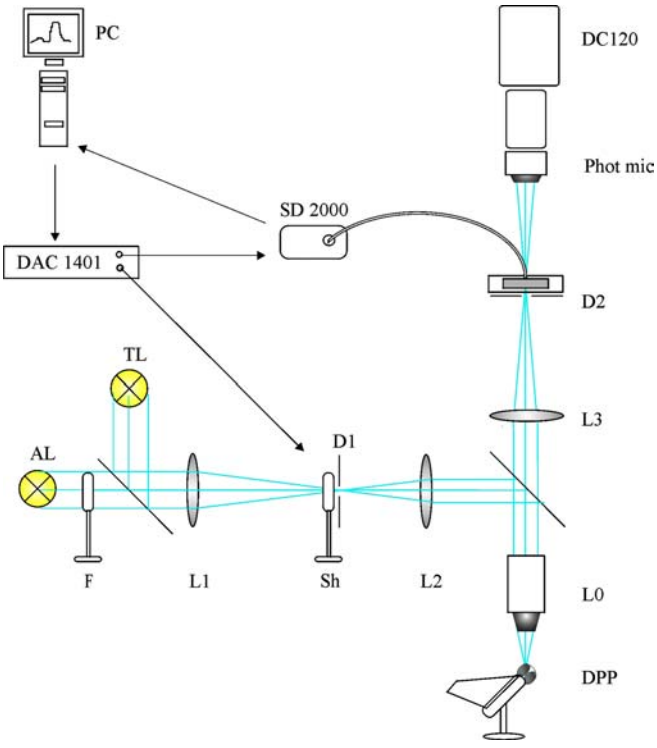

Fig. 1 The optical set-up used to record reflection spectra. Central to the set-up is the spectrometer (SD2000), which can be triggered to start data-acquisition by a signal from the DAC 1401. Data-acquisition and opening of the shutters (Sh) in the light path are triggered simultaneously by TTL pulses of DAC's of the CED 1401 on a Pentium computer (PC). Arrows indicate signals from DAC and PC. Density or interference filters (F) may be placed in the light beam. A parallel beam enters the microscope objective (L0) and is focused on the deep pseudopupil (DPP), which can be seen in the center of the eye. The telescopic lens pair L0 and L3 images the DPP on the secondary focal plane of L3, where the tip of an optical fiber is positioned. Light entering the fiber is guided to the spectrometer. In an alternative arrangement, a photomicroscope (Phot mic), equipped with a digital camera (DC120) is used to photograph either the DPP image or the eye at the level of the cornea (see Fig. 2). Diaphragms D1 and D2 minimize stray light. TL and AL are the test and adapting light sources, respectively

mercury lamp served as the adapting light (AL) source. The light beam was equipped with various interference filters (Schott, DAL, bandwidth ~15 nm) or a long-pass orange filter (Schott, OG570), and thus provided selective spectral irradiation. The irradiance at the level of the focal plane of the microscope objective (Zeiss 10×, 0.3), was measured with a calibrated photometer.

Eye shine and pseudopupils

The epi-illumination microscope with the spectrophotometer fiber removed allows visualization of the butterfly eye shine by using a photomicroscope (Phot mic, Fig. 1) and a digital camera (Kodak, DC120). When a dark-adapted eye is observed with the microscope focused at the level of the eye's cornea, i.e., at the level of the facet lenses, one sees a bright eye shine emerging from ommatidia having a visual field within the aperture of the microscope (Fig. 2a). The number of shining facets thus depends on the numerical aperture of the microscope objective. The set of shining facets, called the luminous corneal pseudopupil (Franceschini and Kirschfeld 1971; Stavenga 1979), merges into a bright spot when the microscope is focused at the level of the center of curvature of the eye (Fig. 2b). This spot is called the luminous deep pseudopupil. For the observations of the eye shine as well as the measurements of the spectra, the butterfly was positioned so that the eye's deep pseudopupil (DPP) coincided with the focal plane of objective L0 (Fig. 1). Lens L3 then images the DPP in its secondary focal plane, that is, the plane of diaphragm D2.

The eye shine is only seen in a properly dark-adapted eye and vanishes within a few seconds of illumination, because pigment granules existing in the soma of the photoreceptors migrate towards the rhabdom and there absorb the light flux in the boundary wave. The assembly of photoreceptor pigment granules is therefore called the pupil (Stavenga 1979). The pupil mechanism, in addition to controlling the light absorbed by the visual pigments, can strongly disturb photochemical experiments, the theme of this paper. This disturbance can be largely avoided by an appropriate protocol that allows enough time for the pigment granules to be withdrawn from the rhabdom boundary.

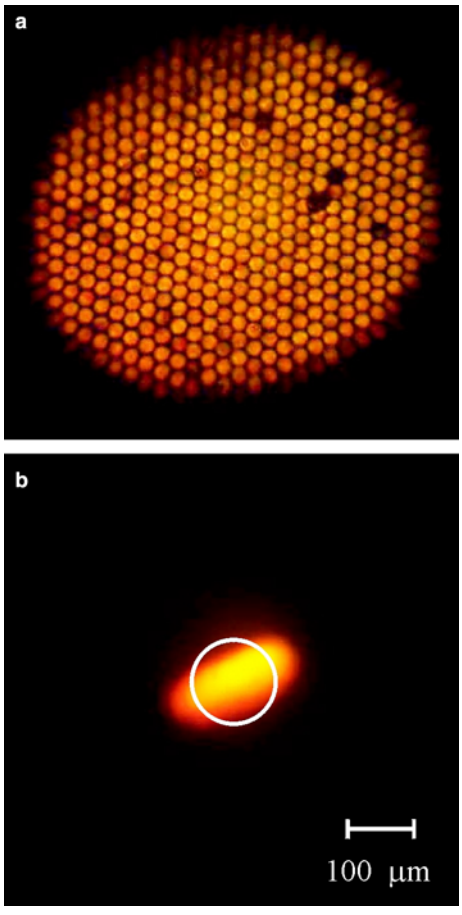

Fig. 2 Eye shine of the comma butterfly. **a** Magnified image of the cornea obtained with the epi-illumination set-up of Fig. 1. The eye shine, being light reflected from the tapeta in the individual ommatidia, has a nearly homogeneous orange-red color. *Black dots* are poorly reflecting facets. Nearly 400 facets, average diameter 25 µm, contribute to the eye shine at the corneal level. The acceptance angle of the objective (Zeiss 10×, 0.3) is 35° and the cross-section of the corneal pseudopupil is made up of 23 ommatidia, and thus the interommatidial angle is about 1.5°. **b** Focusing the photomicroscope at the level of the center of curvature of the eye yields the luminous deep pseudopupil (DPP), which is slightly oval, due to the somewhat ellipsoidal shape of the eye. In the microspectrophotometrical experiments, the tip of the optical fiber of the spectrophotometer (accepting field indicated by the *solid circle*) is positioned in the magnified image of the DPP (see Fig. 1, D2)

Photochemical protocol and spectrophotometric analysis

Invertebrate visual pigments have two thermostable states, the native rhodopsin and its metarhodopsin state, which can be converted into each other by photon absorption (Hamdorf 1979). We have investigated the visual pigment photochemistry based on the present knowledge, (1) that intense illumination rapidly creates a photosteady state, with a rhodopsin-metarhodopsin ratio depending on the spectral content of the illumination, and (2) that metarhodopsin is rather rapidly degraded, with a time course of several minutes at room temperature, and rhodopsin is more slowly reconstituted, within a few hours (Bernard 1983b). The dark processes were monitored with a test light beam equipped with grey filters so that the test light negligibly changed the visual pigment composition. The unfiltered test light was sufficiently bright for substantial photochemical conversions within a time span of a few seconds. Time series of spectra during photoconversions or dark periods were recorded with a minimum integration time of 10 ms for a single spectrum. The spectra were analyzed off-line in MATLAB5.3 (MathWorks) on a Pentium PC. The standard analysis comprised the

following steps: (1) spectral data from the spectrophotometer were stored as raw reflection spectra; (2) the recorded spectral data were averaged and/or smoothed with a Savitzky–Golay polynomial smoothing filter with polynomial order 2 and frame size less than 25 nm (75 data points) and then converted into normalized reflectance spectra by dividing the spectra with a spectrum measured from a white reference reflector (MgO); (3) the reflectance spectra were converted to absorbance difference spectra by taking the common logarithm (base 10) and then subtracting the resulting spectra from a certain reference spectrum (usually the spectrum obtained after prolonged dark regeneration, where one may assume to have 100% rhodopsin); (4) the time course of the absorbance changes was calculated; mean values of the normalized spectra then indicate the relative fractional concentration change in time.

Results

Photoconversion of green visual pigment

A comma, *Polygonia c-album*, was dark adapted for a prolonged time, i.e., overnight, and then, during application of broadband illumination (Fig. 3a), reflectance spectra were recorded using an integration time of 10 ms. The averages of 15 sequentially measured spectra were calculated, yielding an interval time of $\Delta t = 0.15$ s. Fig. 3b presents the resulting spectra normalized at the maximal reflectance value. The illumination caused a reflectance increase in the long-wavelength region, above 525 nm, and a reflectance decrease below that wavelength. The light-induced changes are more clearly seen in the common logarithm of the reflectance spectra (Fig. 4a). Figure 4b shows the absorbance difference spectra, calculated by subtracting all spectra of Fig. 4a

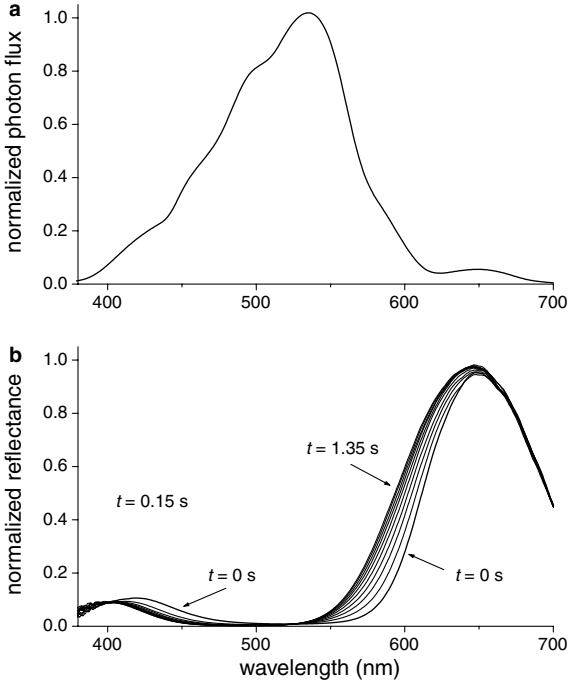

Fig. 3 Reflectance changes caused by photoconversion of green visual pigment by broadband illumination. **a** Spectral distribution of the halogen lamp at the level of the objective focal plane. **b** Reflection spectra recorded with an integration time of 10 ms during 2 s illumination. Here, ten reflectance spectra, averages of 15 sequential spectra ($\Delta t = 0.15$ s) normalized at the peak value of the last spectrum, are plotted together with the first spectrum after dark regeneration (*bold line*)

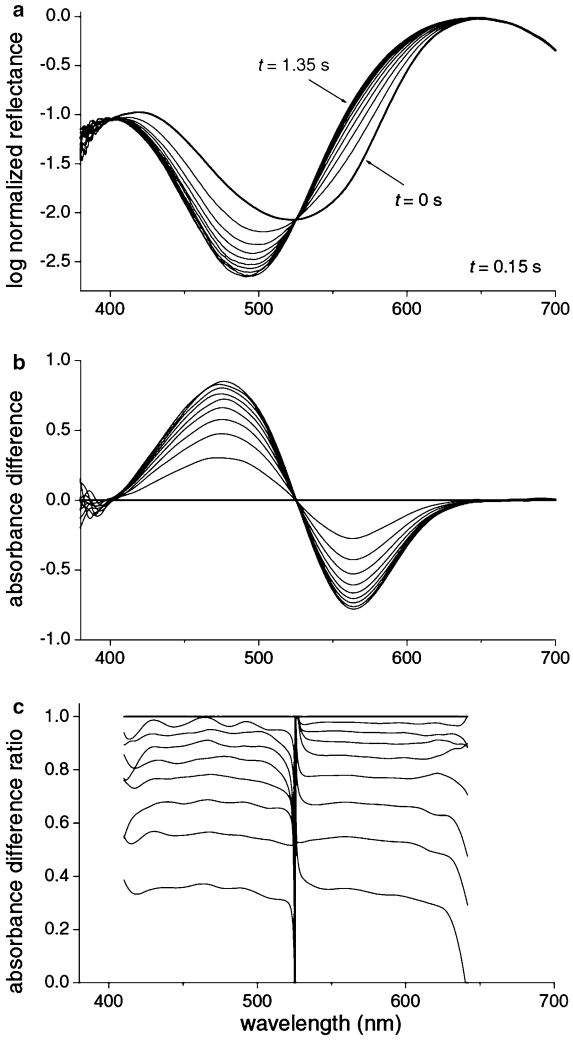

Fig. 4 Spectral changes due to photoconversion of green visual pigment. **a** The common logarithm of the normalized reflectance spectra of Fig. 3b. **b** Absorbance difference spectra calculated from the spectra of **a** with the first (dark-adapted) spectrum used as a reference (*bold line*, $t = 0$ s). During the 1.35 s broadband illumination, the green visual pigment, initially 100% rhodopsin, is partly converted to metarhodopsin. The absorption decreases above 525 nm, the isosbestic point, and increases between 400 nm and 525 nm. **c** Absorbance difference ratio of the spectra given in **b**, obtained by dividing all difference spectra by the last difference spectrum ($t = 1.35$ s). The spectra became noisy below 420 nm and above 620 nm due to the small absorbance differences and therefore these wavelengths ranges were omitted. The absorbance difference ratio spectra are approximately flat except near the isosbestic point

from the first spectrum ($t = 0$ s, bold line; see Appendix 1, 2). The spectra are characteristic for a green-absorbing rhodopsin that is interconvertible with a blue-absorbing metarhodopsin (Hamdorf 1979).

The difference spectra are approximately zero near 525 nm, the isosbestic point. When exclusively only one, green visual pigment was involved all difference spectra should be proportional to each other and zero at one and the same wavelength. The difference spectra of Fig. 4b divided by the maximal absorbance difference spectrum, obtained at $t = 1.35$ s, yields approximately flat spectra with slight anomalies near the isosbestic point, indicating the participation of more than one visual pigment (Fig. 4c, see Discussion).

The time course of the conversion of the green rhodopsin was estimated by taking for each spectrum of Fig. 4b the average of the absorbance difference at two wavelength ranges, 440–500 nm and 540–600 nm, respectively. Normalization of the obtained values to the final value (at $t = 1.35$ s) yielded two approximately exponential curves with time constants 0.413 s and 0.507 s, respectively (Fig. 5), or, an average time constant of 0.460 s. The photochemical conversion was virtually completed within the response time of the dark-adapted pupil, being 1.5–2.0 s (Stavenga et al. 1977). Contamination of the photochemical experiments by the pupil mechanism hence must be negligible. Nevertheless, the difference in the two time constants shows that the difference spectra are not fully proportional to each other, presumably due to non-trivial properties of the optical system, consisting of a light focusing facet lens and cone, and a waveguiding rhabdom.

Fast metarhodopsin degradation and slower rhodopsin regeneration

The metarhodopsin that is created by photoconversion of rhodopsin is removed from the rhabdom and probably degraded (Bernard 1983b; Schwemer 1989). This can be appreciated from the eye shine photograph of Fig. 6. First a dark-adapted eye was subjected for a few minutes to a series of intense red illuminations applied via a small aperture objective (Olympus 4×, 0.1). The intense red light created initially a large amount of blue-green absorbing metarhodopsin in the central ommatidia, causing an increase in yellow-orange eye shine. A distinct yellowing occurred subsequently, due to an overall loss in absorption, i.e., a decrease in visual pigment content. The eye shine was then photographed using a larger aperture objective (Zeiss 10×, 0.3). The result shows a central set of ommatidia with yellow eye shine, surrounded by ommatidia with reddish eye shine.The central ommatidia regained their original reddish color after a few hours darkness, clearly due to rhodopsin regeneration.

We further studied the processes of metarhodopsin decay and rhodopsin regeneration by first adapting the eye with bright white light and then measuring every 5 min a reflectance spectrum during 4 h, using the broadband test light (Fig. 3a). The measured reflectance spectra were converted into absorbance difference spectra (Fig. 7a), with the spectrum measured after 240 min in the dark as the reference spectrum (in this final situ-

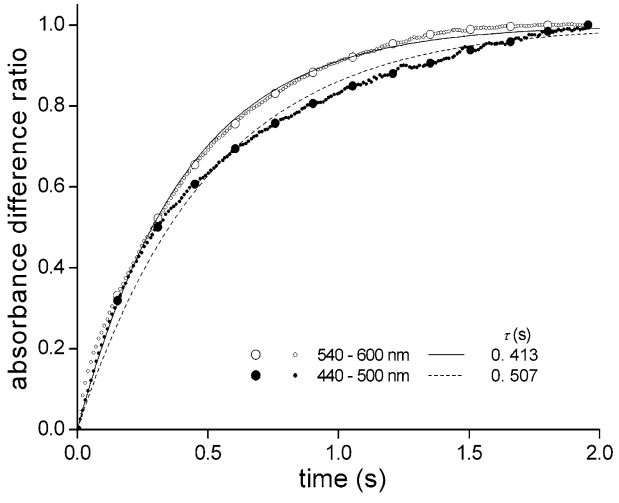

Fig. 5 Time course of the conversion of initially 100% green rhodopsin (R) to a new photosteady state with its metarhodopsin (M). The normalized mean absorbance difference in the spectral intervals 540–600 nm and 440–500 nm (Fig. 4b) approximate first order exponential time courses with time constant 0.413 s and 0.507 s, respectively

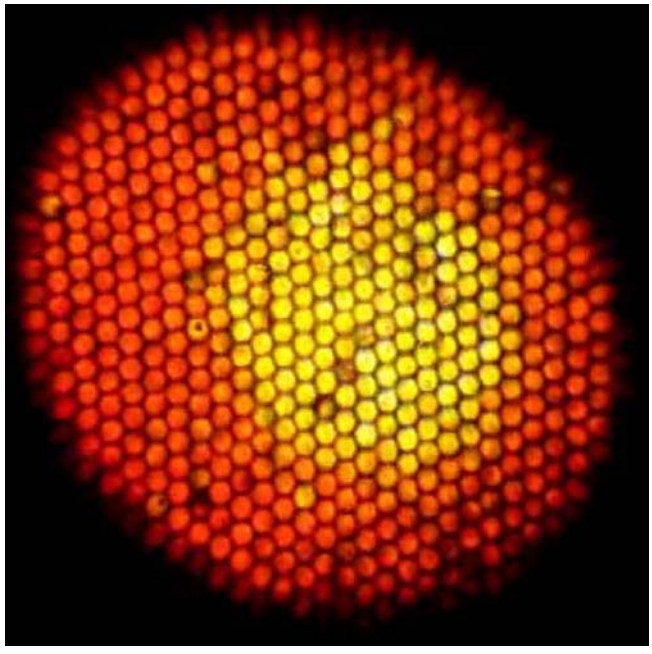

Fig. 6 The luminous corneal pseudopupil of the comma, *Polygonia c-album*, photographed with a wide aperture microscope objective (10×, 0.3), after a bleaching procedure with a repetitive red light stimulation protocol using a smaller aperture objective (4×, 0.1)

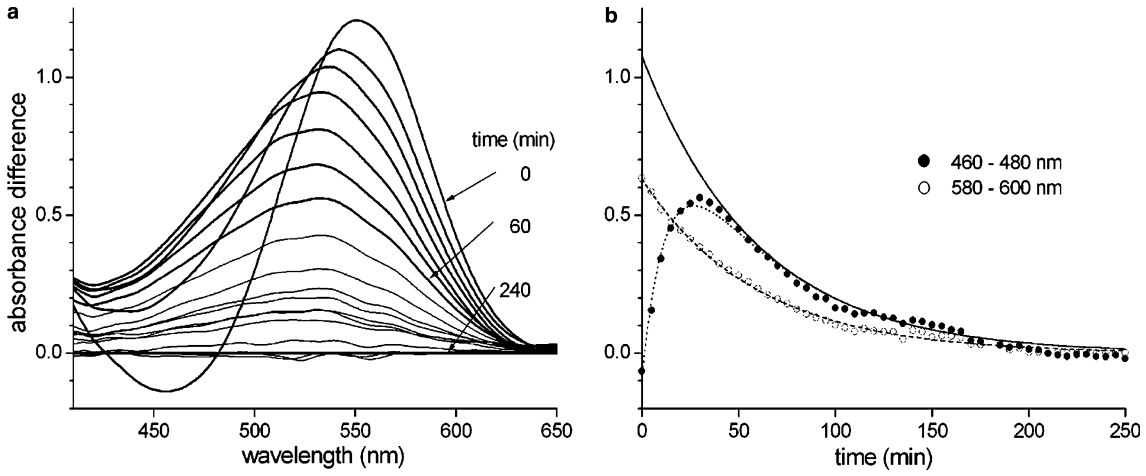

Fig. 7 Kinetics of dark regeneration of green visual pigment measured with a weak test light at intervals of 5 min during 240 min. **a** Difference spectra calculated from reflectance spectra with the last spectrum ($t = 240$ min) as a reference. A total of 19 spectra are shown, the first hour every 10 min, and after 1 h only every 15 min. **b** The time course of the rhodopsin regeneration is calculated from the mean of the absorbance difference over the spectral interval 580–600 nm (*opened circle*, $\Delta t = 5$ min), because metarhodopsin absorption is minor in that wavelength range (Fig. 6a). The time course of regeneration approximates a single exponential function with a time constant of 59 min (*dashed line fit*). The time course of the absorbance difference in the interval 460–480 nm (*filled circle*, $\Delta t = 5$ min) can be approximated with two exponential functions (*dotted line*), one representing the rhodopsin regeneration (time constant 59 min, *solid line*) and the other for the metarhodopsin degradation, yielding a time constant of 15 min (see Appendix 1)

ation the rhodopsin concentration is high). The decrease in absorbance in the long wavelength range with respect to the final situation, occurring during the 4 h dark period, demonstrated the reconstitution of rhodopsin. The average absorbance difference in the wavelength range 580–600 nm followed an exponential time course, with a time constant of 59 min (Fig. 7b). This is the time constant of rhodopsin regeneration, because metarhodopsin absorption is minor above 580 nm (see Fig. 8a).

The average absorbance difference in the wavelength range 460–480 nm had a biphasic time course (Fig. 7b), which is well approximated by two exponential functions with time constants 15 min and 59 min (see Appendix 1). Both metarhodopsin and rhodopsin absorb in the 460–480 nm wavelength range, and therefore the first time constant, 15 min, is that of metarhodopsin degradation. The degradation of metarhodopsin that is created by the initial bright illumination is virtually complete after 40 min, and therefore the difference spectra obtained at later times are fully due to changes in the rhodopsin concentration. The later spectra indeed approximate a rhodopsin template peaking at 532 nm.

We studied the spectral properties of the metarhodopsin by applying 2 s red flashes with an interval of 5 min during a total period of 210 min. Bright red light effectively converts rhodopsin and very little photoreconverts the metarhodopsin. A rather long intermittent period of 5 min darkness was chosen to be sure that the pupil was fully open. The reflection spectrum was measured with a brief broadband test light shortly before applying a red flash. The last reflectance spectrum, i.e., the one resulting after the 210 min measurement period, was used as a reference in the calculations of the absorbance difference spectra. The spectra reveal that the red flashes converted the green-absorbing rhodopsin into a blue-absorbing metarhodopsin, which then gradually vanished. The metarhodopsin absorbs maximally at 492 ± 1 nm (Fig. 8a).

The mean of the absorbance difference in the wavelength range 440–500 nm spectra followed an exponential time course, with a time constant of 56 min (Fig. 8a). The initial trend deviates from the later exponential process, possibly due to a rapid reconstitution of rhodopsin from a readily available store. Reconstitution presumably cannot keep up with metarhodopsin removal, as this occurs at a more rapid pace. At the end of the measurement period of 3.5 h, bleaching apparently was not yet complete, as the amplitude of the reflectance spectra still increased very slowly. The actual time course of the spectral changes will be determined by the interplay of metarhodopsin removal as well as rhodopsin reconstitution allowed by the 5 min intermittent dark periods.

Photoconversion of a UV rhodopsin into a blue-absorbing metarhodopsin

We investigated the presence of a UV visual pigment, following the experiment of Fig. 8 during which removal of the green visual pigment had occurred. Prolonged illumination times appeared to be necessary for substantial photoconversions of visual pigment by UV light, because the substantial amount of glass in the set-up considerably reduced the light flux in the ultraviolet. Prolonged illumination times activated the pupil mechanism, however. In order to avoid contamination of the reflection measurements, long dark adaptation times were needed for sufficient relaxation of the pupil. We applied monochromatic 364 nm light

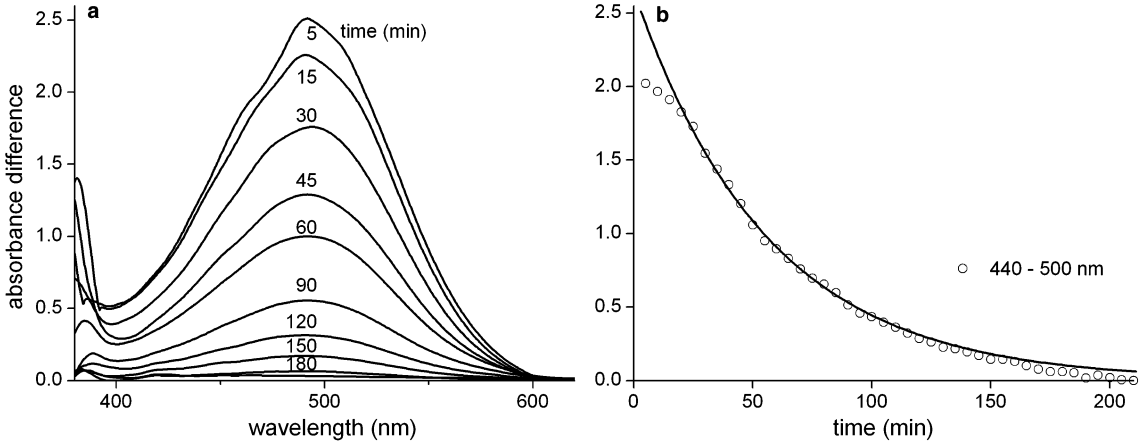

Fig. 8 Bleaching of green visual pigment. **a** After prolonged dark adaptation, the eye was first white-light adapted, causing partial conversion of the green rhodopsin to metarhodopsin (as in Figs. 3–4). Then each 5 min during a period of 210 min a reflectance spectrum was measured, which was followed by a 2 s red adapting flash. Absorbance difference spectra were calculated from the reflectance spectra by taking the reflectance spectrum measured after 210 min 'bleaching' as the reference spectrum. The difference spectra show intrinsic bleaching of the green rhodopsin's photoproduct, metarhodopsin, which absorbs maximally at 492 ± 1 nm. **b** The time course of the mean of the absorbance difference over the spectral interval 440–500 nm approximated a first order exponential, with time constant 56 min. Only data from 15 min after the first red light pulse were incorporated in the exponential fit, because the data there clearly deviated from an exponential process, probably due to rapid rhodopsin reconstitution

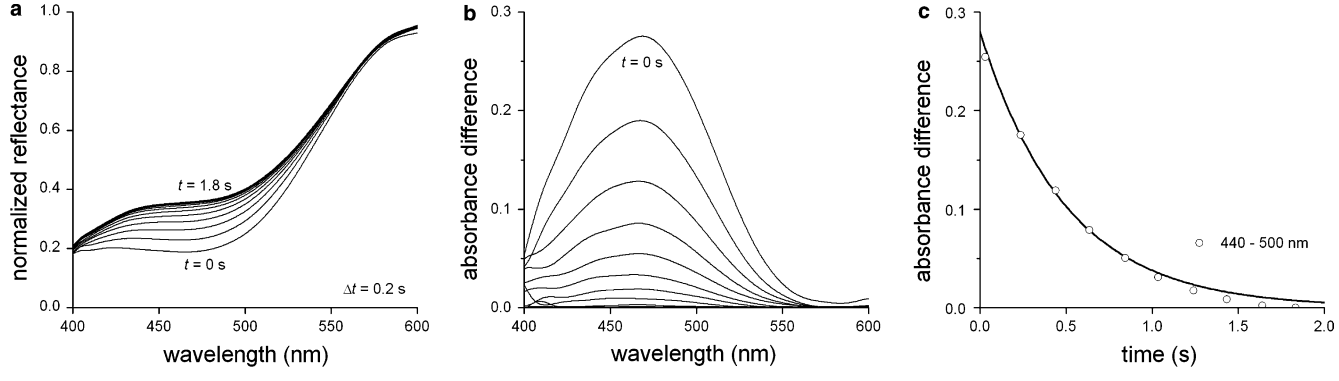

Fig. 9 Photochemical conversion of the metarhodopsin of UV rhodopsin. **a** The green visual pigment was first bleached and then the eye was irradiated during 5 s with intense monochromatic 364 nm light. After a dark period of 3 min, allowed for full pupil relaxation, the eye was illuminated for 1.8 s with broadband test light and reflection spectra were recorded with an integration time of 10 ms. Here 10 spectra, normalized at the peak value of the last spectrum (at $t = 1.8$ s), are plotted with a time interval of 0.2 s. The reflectance increase between 400 nm and 500 nm demonstrates the reconversion of metarhodopsin to the rhodopsin state. **b** Difference spectra calculated from the reflectance spectra of **a** with the final spectrum ($t = 1.8$ s) as the reference spectrum. The difference spectra are nearly proportional, peaking at 467 ± 2 nm. **c** The mean absorbance difference was calculated over the spectral interval 440–500 nm. The time course of metarhodopsin conversion approximates a first order exponential with time constant 0.5 s

for 5 s, followed by a dark-adaptation period of 3 min, and then recorded reflection spectra using broadband illumination during 1.8 s (integration time 10 ms), from which normalized reflectance spectra were calculated (Fig. 9a).

The initial blue reflectance, between 400 nm and 550 nm, was low, but the bright broadband test light caused a gradual reflectance increase. Clearly, the 364 nm light had created a blue-absorbing metarhodopsin, which the test light reconverted back into its UV-absorbing rhodopsin. The measured reflectance spectra were converted into absorbance difference spectra (Fig. 9b) by taking the last spectrum ($t = 1.8$ s) as the reference spectrum. The absorbance difference spectra yield a blue-absorbing metarhodopsin with peak wavelength 467 ± 2 nm. The absorbance differences in the blue followed an exponential time course with a time constant of 0.50 s (Fig. 9c), which is very similar to the time constant obtained for the photoconversion of the green visual pigment into a new photosteady state (0.46 s; Fig. 5). Because in both cases the same illumination was applied, this indicates that the photosensitivities of the ultraviolet and green visual pigments are similar.

Application of monochromatic illuminations of wavelengths 374 nm and 390 nm, respectively, yielded photosteady states with reflectance spectra differing from that induced by the 364 nm light (Fig. 10a). Using the reflectance spectrum resulting after intense red light as a reference (Fig. 10a, > 570 nm) yielded the

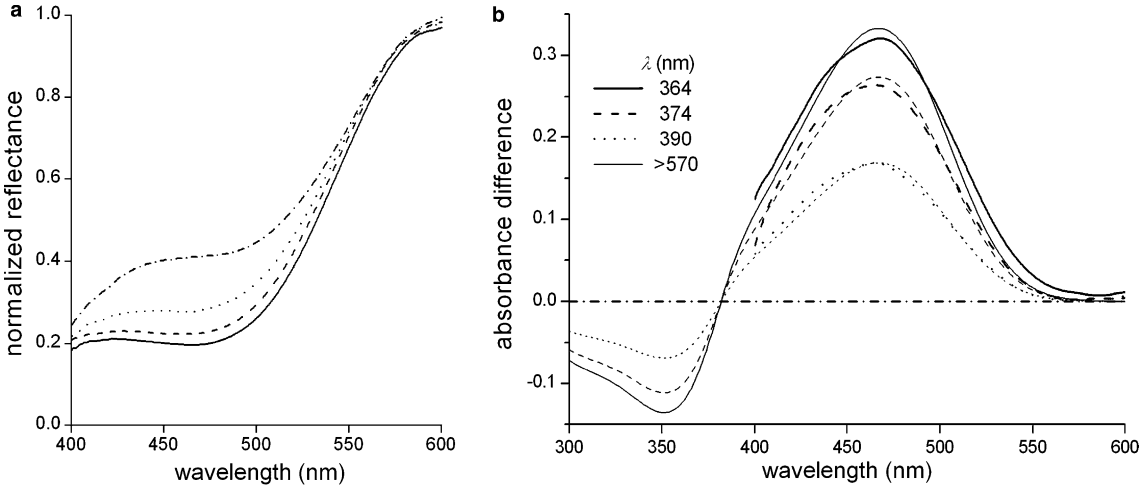

Fig. 10 Photosteady states of the UV visual pigment established by different illuminations. **a** Reflectance spectra obtained after actinic light flashes at 364, 374 and 390 nm, and long pass filtered, red (> 570 nm) light. **b** The difference spectra corresponding to the reflectance spectra of **a** were calculated with the spectrum measured after the red adaptation as the reference spectrum. The peak of the UV difference spectra is 467 ± 2 nm. The experimental difference spectra are approximated with difference spectra calculated with a UV visual pigment R350/M467 (*thin lines*)

absorbance difference spectra of Fig. 10b. The amplitudes of the difference spectra decrease with increasing wavelength of the illumination, in accordance with the photochemistry of a UV-absorbing rhodopsin and a blue-absorbing metarhodopsin.

Blue visual pigment

We also investigated whether a blue visual pigment was present. We illuminated the eye with various monochromatic lights in the blue to green wavelength range, but the obtained difference spectra did not unequivocally reveal a photointerconvertible blue visual pigment. We will argue below that the comma retina nevertheless contains a minor amount of blue rhodopsin.

Discussion

Green visual pigment

Microspectrophotometry of visual pigments in the eyes of completely intact, living animals is possible in butterflies due to the unique presence of a tapetum that is proximal to the rhabdoms (Miller and Bernard 1968). Difference spectra derived from reflectance spectra measured in vivo then allow the estimation of the spectral characteristics of the rhodopsin and metarhodopsin state (Bernard 1979, 1983b). Here, we performed spectrophotometric recordings in a novel telemicroscopic set-up, allowing usage of a large-aperture microscope objective whilst obliterating disturbing background reflections (Stavenga 2002b).

In his bleaching protocol for green visual pigments, Bernard (1979, 1983b) used a red flash or a series of red flashes, followed by long dark periods (60–90 min) during which a few sample spectra were taken. In our experiments the prolonged dark periods lead to substantial incorporation of new rhodopsin, however. In the experiments with the repeated red flashes, any newly created rhodopsin (R) was promptly converted to the metarhodopsin (M) form, which then degraded in the dark, yielding a better spectral separation of the metarhodopsin. The bleaching and regeneration experiments yielded the λ_{max}-value 492 nm for M and 532 nm for R, respectively, i.e., the green visual pigment is characterized by R532/M492. Very similar values were reported for the green visual pigment of the closely related nymphalid *Vanessa cardui*: R530/M490 (Briscoe et al. 2003).

UV visual pigment

Bleaching of the green visual pigment enabled the identification of a UV-absorbing pigment by applying monochromatic ultraviolet light, which created a blue-absorbing metarhodopsin peaking at 467 nm. Due to limitations of the experimental setup, reflectance changes in the ultraviolet wavelength range could not be measured. Nevertheless, the dependence of the amount of metarhodopsin formed by the ultraviolet illuminations clearly demonstrated the involvement of a UV absorbing rhodopsin. The electrophysiological recordings on *Polygonia c-aureum* by Kinoshita et al. (1997) revealed UV receptors with peak sensitivity at about 350 nm. Presumably this is the λ_{max} of the UV rhodopsin of *Polygonia*. The UV visual pigment then is characterized by R350/M467. Difference spectra derived from such a UV visual pigment indeed well approximate the difference spectra obtained in the photoconversion experiments (Fig. 10b).

Relative distribution of visual receptor types

The photochemical and regeneration experiments in the comma show that the rhabdoms normally contain a high concentration of green visual pigment. Figure 8 shows a maximal absorbance of 2.5 when virtually all green visual pigment molecules are in the metarhodopsin state. Figure 9 shows a maximal absorbance of ~0.28 due to creation of the blue-absorbing metarhodopsin of the UV visual pigment by 364 nm light. That wavelength establishes a photosteady state of the UV visual pigment R350/M467 with a metarhodopsin fraction of roughly 0.70, assuming a relative quantum efficiency $\gamma_M/\gamma_R = 1$ (see Appendix 2; Stavenga and Schwemer 1984), yielding an absorbance of ~0.40 when all UV visual pigment molecules are in the metarhodopsin state. Assuming that the peak molecular absorption coefficients of the different visual pigments are approximately the same, we conclude that the ratio of the UV and green visual pigments is about 1:6.

These values are in good agreement with the expression patterns of the visual pigments identified in other lepidopteran species with three visual pigment types. The majority of the photoreceptors in *Vanessa cardui* (72%, Briscoe et al. 2003) and *Manduca sexta* (71.4%, White et al. 2003) contains green visual pigment. Microspectrophotometry showed that 15% of the total visual pigment content in *Vanessa cardui* is UV pigment (Briscoe et al. 2003), and histological studies of the UV-opsin in the retina of *Manduca sexta* yielded a very similar value: 14.3% (White et al. 2003). This is also in line with electrophysiological recordings on the Japanese nymphalids *Polygonia c-aureum* and *Sasakia charonda* which yielded a majority of green receptors, with few blue and UV receptors. Of the 80 receptors recorded in *Polygonia c-aureum* 85% were green receptors, against 11% blue and 4% UV receptors (Kinoshita et al. 1997).

Blue visual pigment

The measured reflectance spectra (Figs. 9, 10) showed a pronounced trough in the blue wavelength range, even after bleaching of the green visual pigment, suggesting the presence of a blue-absorbing rhodopsin. Furthermore, the difference spectra obtained during photoconversion of the green rhodopsin of the comma, *Polygonia c-album*, with broadband light showed anomalies around the isosbestic point of the green visual pigment, indicating the participation of a blue visual pigment (Fig. 4c).

We therefore investigated the possible optical effects of a blue rhodopsin in the photoconversion experiments with a computational model, where a butterfly rhabdom

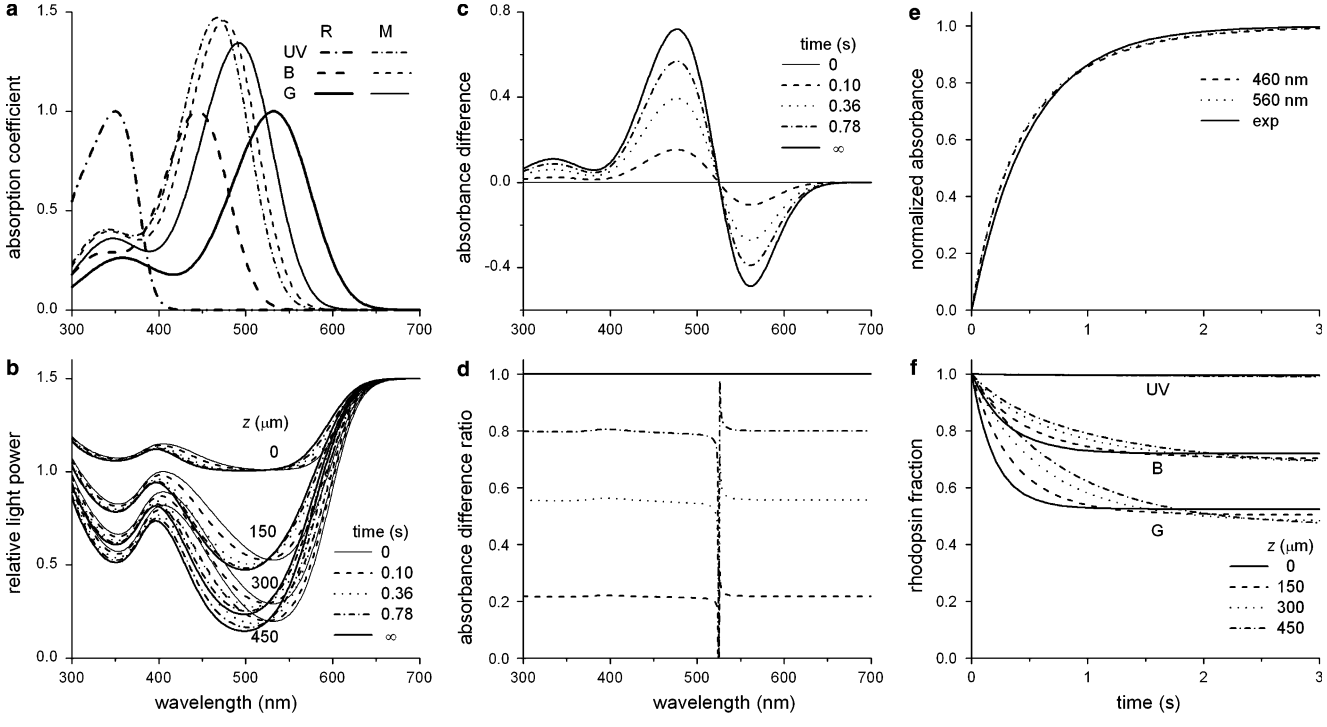

Fig. 11 Light-induced visual pigment processes in the butterfly rhabdom. **a** Absorption spectra of the rhodopsin (R) and metarhodopsin (M) state of the three visual pigments (UV, B, and G), assumed to populate the rhabdom in the ratio UV:B:G = 1:1:6. **b** Relative light power resulting at different spatial locations, given by the length coordinate z of the rhabdom, at a few time points during the photoconversion process. The photosteady state is reached at $t = \infty$. **c** The absorbance difference at a few time points calculated from the light flux that, after reflection at the tapetum, emerges from the rhabdom (see Appendix 1, 2). **d** The absorbance difference spectra of **c** divided by the extreme spectrum (for $t = \infty$; for legends of the lines, see c). **e** Time course of the absorbance differences at wavelengths 460 and 560 nm (see **c**), compared with an exponential function (exp) with time constant $\tau = 0.5$ s. **f** Time course of the rhodopsin fraction of the three visual pigments at four locations in the rhabdom

contains three visual pigments, an ultraviolet, blue and green visual pigment given by R350/M467 (UV), R445/M475 (B) and R532/M492 (G), respectively (Fig. 11a; see Appendix 2). The visual pigments were assumed to occupy the rhabdom in the ratio 1:1:6, i.e., by eight photoreceptors that equally share the cross-section of a rhabdom over the whole length of 450 μm (we neglected the short basal photoreceptor R9). The peak molecular absorption coefficient, $\alpha_{max} = 1.56 \cdot 10^{-8}$ μm^2 (Dartnall 1972), and the concentration of the visual pigment molecules, $C_0 = 3.85 \cdot 10^5$ μm^{-3}, based on a peak absorption coefficient of rhabdomeral tissue $\kappa_{max} = 0.006$ μm^{-1}, were assumed to be the same for all rhodopsins. In Fig. 11b, all visual pigment molecules are in the rhodopsin state at time $t = 0$ s, and then a constant light flux, with peak value $I_0 = 9 \cdot 10^6$ photons μm^{-2} and spectral distribution given in Fig. 3a, enters the rhabdom. The light causes photoconversion of rhodopsin to metarhodopsin, until a photosteady state is reached. (The value of I_0 was chosen such that the time course of photoconversion conformed with that of the experiment of Figs. 3–5, i.e., yielding a time constant $\tau \sim 0.5$ s.) The light propagates through the rhabdom and is partly absorbed there, depending on the absorption coefficient summed over all visual pigments and the reflectance of the tapetum (Fig. 11b). The tapetum reflectance was taken to be 0.5. The light power at the rhabdom entrance relative to the incident light power then becomes 1.5 at the longer wavelengths where visual pigment absorption is negligible. The reflectance, i.e., the relative light flux emerging from the rhabdom after having traveled twice through the rhabdom length, changes due to the photochemical processes. Fig. 11c shows the absorbance differences calculated from the reflectance changes at a few time points. The absorbance difference spectra divided by the final spectrum, that of the photosteady state (Fig. 11d), reveals anomalies near the isosbestic point of the green visual pigment. The anomalies are due to photoconversions of the blue visual pigment, because they do not occur when the blue visual pigment is replaced by a green rhodopsin.

The speed of the conversion process decreases with increasing z, the length coordinate of the rhabdom, because the light power diminishes due to the fact that the visual pigments in the rhabdom absorb from the propagating light flux and thus act as optical filters (Fig. 11b). The conversion time courses, as calculated from the absorbance changes at the wavelengths where the absorbance changes are large (460 and 560 nm, Fig. 11c), nevertheless approximate a simple exponential function (Fig. 11e). The time course of the local changes in visual pigment composition strongly varies along the rhabdom, due to the gradient in the light power, but the rhodopsin fractions in the steady state are surprisingly independent of the location (Fig. 11f). The broadband illumination creates photosteady states with approximate rhodopsin fractions UV:B:G = 1.0:0.7:0.5, i.e., the blue-green illumination negligibly affects the composition of the ultraviolet visual pigment, but it shifts half of the green rhodopsin molecules into their metarhodopsin state.

The rhodopsin and metarhodopsin spectra used in the calculations were obtained with the visual pigment template of Govardovskii et al. (2000), where spectral shape is fully determined by the wavelength of maximal absorption. Calculations with visual pigment spectra peaking at slightly different wavelengths demonstrated that the anomalous effects of Fig. 11d were insensitive to the exact choice of the spectral locations of the rhodopsin-metarhodopsin pairs. We conclude that the anomalies in the spectra of Fig. 4c are due to the photoconversion of blue visual pigment simultaneous with the photoconversion of green visual pigment in the rhabdoms of the comma.

We note here that we have explored a simplified model of the butterfly rhabdom. We have neglected optical waveguide effects and also the heterogeneity of the rhabdoms in butterfly eyes. Presumably the differences in the expression of the UV and blue visual pigments (Briscoe et al. 2003; White et al. 2003; see also Arikawa and Stavenga 1997) will not have major effects on the spectral changes measured via the eye shine.

Visual pigment degradation and regeneration

The kinetics of the dark processes of the green visual pigment of the comma, *Polygonia c-album*, corroborates the general picture outlined by Bernard (1983b). The time constant of 15 min of metarhodopsin degradation (Fig. 7, at ~21°C) is similar to the 18 min measured by Bernard (1983b) in *Vanessa cardui* (at 23°C). Rhodopsin regeneration, with a time constant of ~60 min (Fig. 7), is much slower (see Bernard 1983a, b).

The molecular mechanisms behind the selective metarhodopsin degradation and rhodopsin regeneration in butterflies remain to be elucidated, but presumably are similar to those of flies and bees (Schwemer 1989, 1993; Smith and Goldsmith 1991).

Butterfly visual pigments in natural conditions

The kinetics of the dark conversions of butterfly visual pigments is fast compared to that of fly visual pigment, where the time constant for metarhodopsin removal is 3.3 h and that of rhodopsin regeneration 200 h (blowfly; Schwemer 1984). Why are the processes in the fly so much slower? The main fly visual pigment is a rhodopsin absorbing in the blue-green, which is photoconverted into a bathochromic-shifted metarhodopsin, absorbing maximally at 570 nm (Schwemer 1989). The red pigment of the screening pigment cells serves as a selective red filter, so that red stray light pervading the eye can efficiently reconvert existing metarhodopsin molecules into their native rhodopsin state. Consequently, the steady state metarhodopsin concentration in fly eyes is very low (Stavenga 1979, 2002a). Red stray light would be

detrimental in butterfly eyes, as it would cause photoconversion of the green rhodopsin into its blue-absorbing metarhodopsin, and therefore butterfly eyes have very dense, black screening pigment that efficiently absorbs stray light.

The broadband illuminants in natural habitats will expectedly establish photosteady states with a substantial metarhodopsin concentration (Fig. 4, 11f), possibly leading to loss of visual pigment, due to the rapid metarhodopsin degradation and slow rhodopsin regeneration. However, conversion speeds will be slow compared to those in Figs. 3–5, 9, as the well focused halogen lamp is orders of magnitude brighter than natural light (except for that of the direct sun). The actual photoconversion speeds will be further slowed down, because photoconversion is proportional to the light flux in the rhabdom, and that is under control of a pupil mechanism, which is activated by bright light (Stavenga et al. 1977). The pupil-controlled light-flux may be so low that the rate of photoconversion, and the resulting metarhodopsin decay, can be well compensated by the rate of regeneration of the rhodopsins. This notion leads to the conjecture that the speed of the pupil-controlled photoconversion and the enzymatic visual pigment turnover are tuned to the radiance of the light in the habitat of a butterfly. It will be interesting to put this hypothesis to a critical test.

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Appendix 1

Time course of absorbance difference spectra during metarhodopsin degeneration and rhodopsin regeneration

When light travels through a rhabdom with length L and absorption coefficient κ, the transmittance, T, of the rhabdom equals

$$T = \exp(-\kappa L) \tag{1}$$

When M is the tapetal reflectance, the reflectance of the rhabdom is $R = MT^2$. Because the absorbance is the negative common logarithm of the transmittance, light that has traveled twice the rhabdom length experiences an absorbance, A, given by

$$A = -\log_{10}(T^2) = 0.87\kappa L \tag{2}$$

The difference in absorbance between two time points t_1 and t_2 is:

$$\begin{aligned} A(t_2) - A(t_1) &= 0.87L[\kappa(t_2) - \kappa(t_1)] \\ &= -\log_{10}[R(t_2)/R(t_1)] \end{aligned} \tag{3}$$

In other words, the absorbance differences due to photochemical conversions of visual pigments can be derived from reflectance measurements.

Considering only one visual pigment, the absorption coefficient, κ, equals

$$\kappa = \alpha_R C_R + \alpha_M C_M \tag{4}$$

where α_R and α_M are the molecular absorption coefficients of the rhodopsin state, R, and metarhodopsin, M, respectively, and C_R and C_M are the concentrations of the two visual pigment states. With the total concentration $C_0 = C_R + C_M$ and fractions $f_R = C_R/C_0$ and $f_M = C_M/C_0$, it follows that the absorption coefficient at time t is

$$\kappa(t) = C_0[\alpha_R f_R(t) + \alpha_M f_M(t)] \tag{5}$$

Dark adaptation during a sufficiently long period t_∞ yields a pure rhodopsin fraction, or $f_R(t_\infty) = 1$. The rhabdom absorbance at time t differs from that at time t_∞ by (see Figs 7a, 8a):

$$\begin{aligned} \Delta A(t) &= A(t_\infty) - A(t) \\ &= 0.87L[\kappa(t_\infty) - \kappa(t)] \\ &= 0.87C_0 L f_M(t)(\alpha_R - \alpha_M) \end{aligned} \tag{6}$$

When the metarhodopsin concentration at $t = 0$ s, the start of a dark period, equals $f_{M,0}$, and the processes of rhodopsin regeneration and metarhodopsin degradation are first order processes, with time constants τ_r and τ_d, the time course of the rhodopsin fraction is described by

$$f_R(t) = 1 - f_{M,0}\exp(-t/\tau_r) \tag{7a}$$

and the time course of the metarhodopsin fraction is given by

$$f_M(t) = f_{M,0}\exp(-t/\tau_d) \tag{7b}$$

It follows that the time course of the absorbance difference spectra, $\Delta A(t)$, is then described by (see Fig. 7b):

$$\Delta A(t) = 0.87C_0 L f_{M,0}[\alpha_R \exp(-t/\tau_r) - \alpha_M \exp(-t/\tau_d)] \tag{8}$$

Appendix 2

Visual pigment conversions in a butterfly rhabdom with three visual pigments

Butterfly visual pigments are bistable and photointerconvertible, and therefore illumination of a dark-adapted eye causes partial conversion of rhodopsin into metarhodopsin until a steady state is reached. The photochemistry of the photoconversion process is given by (see e.g. Hamdorf 1979; Stavenga and Schwemer 1984):

$$R \Leftrightarrow M, \text{or}, \frac{\mathrm{d}f_{\mathrm{R}}}{\mathrm{d}t} = -k_{\mathrm{R}} f_{\mathrm{R}} + k_{\mathrm{M}} f_{\mathrm{M}} \quad (9)$$

where the sum of the fractions f_{R} and f_{M} is 1, and the rate constants k_{R} and k_{M} are given by

$$k_{\mathrm{R,M}} = \int I(\lambda)\alpha_{\mathrm{R,M}}(\lambda)\gamma_{\mathrm{R,M}}\mathrm{d}\lambda \quad (10)$$

where λ is the wavelength, I is the light power, $\alpha_{\mathrm{R,M}}$ are the molecular absorption coefficients, and $\gamma_{\mathrm{R,M}}$ the quantum efficiencies of the R and M states.

We have performed calculations of the photoconversions for a butterfly rhabdom with three visual pigments, given by: R350/M467 (UV), R445/M475 (B), and R532/M492 (G), with isosbestic wavelengths 382, 435 and 523 nm, respectively. We assumed eight photoreceptors that equally share the cross-section of a rhabdom over the whole length, i.e., the assumed ratio was UV:B:G = 1:1:6. The spectral shape of both rhodopsins and metarhodopsins was obtained with the template of Govardovskii et al. (2000), and the maximal absorption coefficient of the rhabdom when all molecules would be of the same rhodopsin type was taken to be $\kappa_{\max} = 0.006\ \mu\mathrm{m}^{-1}$; with more than one type the absorption coefficient of each type is multiplied with the volume ratio that the specific rhabdomere contributes to the rhabdom. The length of the rhabdom was assumed to be 450 μm (Briscoe et al. 2003), and the tapetal reflectance was assumed to be 0.5 and spectrally flat.

The local light power is the sum of the light power travelling in the forward direction and that travelling back after reflection at the tapetum:

$$I(\lambda, z) = I_0(\lambda)\left[\exp\left(-\int_0^z \kappa_{\mathrm{eff}}(\lambda, z)\mathrm{d}z\right) + M(\lambda)T(\lambda)\int_{L-z}^{L} \kappa_{\mathrm{eff}}(\lambda, z)\mathrm{d}z\right] \quad (11)$$

where I_0 is the incident light power, z is the location in the rhabdom, κ_{eff} is the sum of the absorption coefficients (Eq. 4) over the contributing visual pigments, whilst taking into account the relative proportions that the different photoreceptors contribute to the rhabdom cross-section, and the transmittance T (Eq. 1) now is:

$$T(\lambda) = \exp\left(-\int_0^L \kappa_{\mathrm{eff}}\mathrm{d}z\right) \quad (12)$$

The eye shine is the light flux that leaves the eye after having travelled forth and back through the rhabdom. As indicated in Appendix 1, a family of absorbance difference spectra can be straightforwardly calculated as the $-\log_{10}$ of the ratio of two reflection spectra, where a certain chosen reflection spectrum serves as a standard reference.

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