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An ultraviolet absorbing pigment causes a narrow-band violet receptor and a single-peaked green receptor in the eye of the butterfly Papilio

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Abstract

The distal photoreceptors in the tiered retina of Papilio exhibit different spectral sensitivities. There are at least two types of short-wavelength sensitive receptors: an ultraviolet receptor with a normal spectral shape and a violet receptor with a very narrow spectral bandwidth. Furthermore, a blue receptor, a double-peaked green receptor and a single-peaked green receptor exist. The violet receptor and single-peaked green receptor are only found in ommatidia that fluoresce under ultraviolet illumination. About 28% of the ommatidia in the ventral half of the retina exhibit the UV-induced fluorescence. The fluorescence originates from an ultraviolet-absorbing pigment, located in the most distal 70 μm of the ommatidium, that acts as an absorption filter, both for a UV visual pigment, causing the narrow spectral sensitivity of the violet receptor, and for a green visual pigment, causing a single-peaked green receptor. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: Spectral sensitivity; Filtering; Retinol; Papilio xuthus

1. Introduction

The photoreceptors in the distal retina of the Japanese yellow swallowtail butterfly, Papilio xuthus, can be divided into four spectral types. Their spectral sensitivities, obtained by intracellular electrophysiological recording, peak in the UV, violet, blue, and green, respectively (Arikawa, Inokuma & Eguchi, 1987; Bandai, Arikawa & Eguchi, 1992). However, the sensitivity spectra show distinct and characteristic variations. For example, whereas the sensitivity spectrum of the ultraviolet receptor closely conforms to a normal absorbance spectrum of a visual pigment, that of the violet receptor is aberrantly narrow. Furthermore, green receptors either have the normal, secondary β-bond in the UV region, or have a distinctly low sensitivity in the UV (Bandai et al., 1992).

An explanation of these puzzling spectral characteris-
yellow and red ommatidia is random (Arikawa & Stavenga, 1997).

We argue in the present paper that the electrophysiological determination of sensitivity spectra of the photoreceptors can be understood from the anatomical and optical observations when we assume that the fluorescence originates from an ultraviolet-absorbing pigment that acts as an absorption filter for both a UV-visual pigment, causing the sharp-peaked violet receptor by depressing the β-band.

2. Materials and methods

2.1. Animals

Spring form males of the Japanese yellow swallowtail butterfly, *Papilio xuthus*, were used within 3 days after emergence. The butterflies were reared on fresh citrus leaves at 25°C under a light regime of 8 h light: 16 h dark. The pupae were stored at 4°C for at least 2 months and then allowed to emerge at 25°C.

2.2. Electrophysiology

The electrophysiological methods were as described previously (Bandai et al., 1992). Briefly, a butterfly was mounted in a Faraday cage and a glass micropipette, filled with a 5% lucifer yellow CH aqueous solution (resistance about 300 MΩ), was inserted into the retina through a hole made in the dorsal cornea. After a photoreceptor was impaled, first its spectral sensitivity was determined. The cell was stimulated on-axis light by a point source, delivering an equiquantal series (maximally 5.0 × 10¹¹ photons cm⁻² s⁻¹ at the corneal surface) of monochromatic flashes, in the wavelength range 290–700 nm. Then the polarization sensitivity of the cell was measured to identify the photoreceptor’s position in the ommatidium (Bandai et al., 1992; Arikawa & Uchiyama, 1996). After the measurements, the photoreceptor was filled with lucifer yellow CH by applying a hyperpolarizing DC current of 2–5 nA for 5–10 min. The butterfly was then unmounted from the Faraday cage and positioned under an epi-fluorescence microscope (BX-60, Olympus). The ommatidium containing the lucifer-filled photoreceptor was identified and photographed under violet excitation (dichroic cube U-MNBV: excitation band-pass filter at 420 nm and emission cut-off filter at 470 nm).

2.3. Fluorescence microscopy

The ommatidial autofluorescence was observed in vivo, also with the fluorescence microscope (BX-60, Olympus), equipped with the dichroic cube U-MWU (band-pass filter at 350 nm and cut-off filter at 420 nm). The UV-induced whitish emission was photographed in various regions of the eye. The localization of the fluorescing pigment within the ommatidia was observed in fresh sections, made with a cryostat.

2.4. Retinol extraction and HPLC

3-Hydroxyretinol was extracted from the *Papilio* retina with the formaldehyde method and chromatography was performed by HPLC (Hitachi 655) as described before (Seki, Isono, Ito & Katsuta, 1994).

2.5. Microspectrophotometry

The emission spectrum of 3-hydroxyretinol, precipitated onto a microscope slide, was measured with a microscope-attached photodiode array (USP-410, Unisoku), equipped with an image intensifier (V1366U, Hamamatsu photonics). The ommatidial fluorescence was measured in the living eye with the same equipment, with the microscope focused at the deep pseudopupil (Stavenga, 1979).

2.6. Modeling

We have calculated the absorption of light in the individual photoreceptors (R1-4) with an optical waveguide model for the rhabdom. The change in light flux along the rhabdom is described by (Snyder, Menzel & Laughlin, 1973):

\[
\frac{dI(z, \lambda)}{dz} = -\eta(\lambda) \left[ \sum f_j(z) \kappa_j(z) l_j(\lambda) + \kappa_4(z) z_j(\lambda) \right]
\]

where \( I(z, \lambda) \) is the light flux at a distance \( z \) from the tip of the rhabdom; \( \lambda \) is the light wavelength; \( f_j \) is the fraction of the rhabdom cross-section taken up by photoreceptor \( R_j \) (\( j = 1–4 \)); \( \kappa_j \) is the peak absorbance coefficient of visual pigment \( f \); \( \kappa_4 \) is the peak absorbance coefficient of the fluorescent (ultraviolet absorbing) pigment; and \( z_j \) and \( \kappa_4 \) are the (normalized) absorption spectra of the visual and fluorescent pigments, respectively. The light fraction absorbed by the visual pigment in each photoreceptor, integrated over the photoreceptor’s length, yields its absorbance spectrum. Normalization then yields the sensitivity spectrum.

The dependence of the absorption of light on waveguide effects is accounted for by \( \eta(\lambda) \), the fraction of the light flux propagated within the rhabdom boundary. A crucial parameter here is the waveguide parameter

\[
V = \pi d(n_1^2 - n_2^2)^{1/2} / \lambda
\]

where \( d \) is the waveguide diameter, and \( n_1 \) and \( n_2 \) are the refractive indices of the waveguide and its surroundings,
respectively. When mainly one mode is propagated, the fraction of light within the light guide can be approximated by:

\[ \eta(V) = a - b \exp(-cV) \]

with \( a = 0.96, b = 2.82, c = 1.27 \) (Smakman & Stavenga, 1986). The visual pigment can only absorb from this part of the light flux.

The assumptions for the absorbance spectra of the visual pigments are the following. A visual pigment spectrum is an algebraic sum of the \( \alpha \) - and \( \beta \) -absorbance bands (indices 1 and 2, respectively): \( \alpha_j = \alpha_{ij} + \alpha_{2j} \), where each band is described by:

\[ \alpha_{ij} = A_{ij} \exp(-a_{ij} x^2 (1 + a_{ij} x + a_{2j})) \]

with \( x = \log_{10}(\lambda/\lambda_{\text{max}}); \lambda_{\text{max}} \) the peak wavelength of the band; the \( \lambda_{\text{max}} \) of the \( \beta \) -band is assumed to be 360 nm; \( a_{01} = 380, a_{02} = 247, a_{11} = 6.09, a_{12} = 3.59, \) and \( a_{21} = 3 \) \( a_{i1/2} \) (\( i = 1, 2 \)); the amplitude of the \( \beta \) -band relative to that of the \( \alpha \) -band, \( A_{21}/A_{11} \), is 0.29 (Stavenga, Smits & Hoenders, 1993).

Anatomical data show that the rhabdom of *Papilio* is made up of the rhabdomerines of nine photoreceptor cells (Ribi, 1987; Bandai et al., 1992). In *Papilio xuthus*, the distal part of the rhabdom (ca. 260 \( \mu \)m), consists of the rhabdomerines of cells R1–4, which are ultraviolet, violet, blue or green receptors. Going from distal to proximal the rhabdomerines of cells R1–4, which are ultraviolet, violet, blue or green receptors. Most proximally (ca. 30 \( \mu \)m), the rhabdom fully consists of the rhabdomerines of photoreceptor cells R5–8, which are either green or red receptors. Most proximally (ca. 30 \( \mu \)m) are the rhabdomerinic microvilli of photoreceptor R9. The photoreceptors R3–8 in a single ommatidium appear to possess either yellow or red screening pigment clusters adjacent to the rhabdom (Arikawa & Stavenga, 1997). These pigments appear to have very little effect on the sensitivity spectra of R1–4, and therefore we neglect their presence in this paper (Arikawa, Scholten & Stavenga, 1996).

In the model calculations, we have simplified the anatomical situation by assuming that the distal part of the rhabdom is a cylinder of circular cross-section with length 300 \( \mu \)m and diameter \( d = 2.6 \) \( \mu \)m. For the refractive indices we have used the values: \( n_1 = 1.36 \) and \( n_2 = 1.34 \) (Stavenga, 1974; Nilsson, Land & Howard, 1988). The absorbance coefficient of the visual pigment containing tissue, \( K_\alpha \), in all rhabdomerines was conservatively assumed to be 0.005 \( \mu \)m \(^{-1} \) at \( \lambda_{\text{max}} \) (Stavenga, 1976). Further assumptions are: the rhabdomerines of R1 and R2 are identical in both size and visual pigment content; R3 and R4 are similarly identical; the fraction of the rhabdom cross-section taken up by R1,2 and R3,4 is 70 and 30\%, respectively, i.e. \( f_1 = f_2 = 0.35 \) and \( f_3 = f_4 = 0.15 \). R1,2 contain both either an ultraviolet or a blue rhodopsin, and R3,4 contain the same green rhodopsin. As a consequence of this assumption, the effect of lateral filtering between rhabdomeres was also considered. Each combination of visual pigments was considered in two situations; i.e. (i) the rhabdom contains also an ultraviolet absorbing screening pigment; and (ii) it does not contain the pigment.

### 3. Results

#### 3.1. Some Papilio photoreceptors have unusual sensitivity spectra

The photoreceptor set R1–4 of *Papilio*, distally in the retina, investigated by intracellular electrophysiology, consists of an ultraviolet (UV), a violet (V), a double-peaked green (DG) and a single-peaked green (SG) receptor (Fig. 1). Whereas the sensitivity spectra of the UV- and DG-receptors conform reasonably well to spectra expected for a normal visual pigment (Fig. 1a,e), the violet receptor’s spectrum is much narrower than normal (Fig. 1b). Furthermore, the spectral sensitivity of the single-peaked green receptor is much depressed in the ultraviolet with respect to the normal case (Fig. 1d). The difference between the two green receptor types suggests that the depression of the \( \beta \) -band is due to a UV-absorbing pigment, that acts as a filter for a normal green visual pigment. This suggestion immediately leads to another conjecture, namely that the sharp-peaked violet receptor is also due to the UV-absorption filter. The location of the hypothesized filter should be somewhere distal in the retina to be optically most effective. The anatomical and optical data, presented below, provide evidence for this view.

#### 3.2. A restricted set of ommatidia in the ventral eye exhibits UV-induced autofluorescence

Viewing the compound eye of *Papilio* under UV epi-illumination, we discovered that the dorsal part exhibits a weak, homogenous fluorescence (see further at the end of Section 4). More strikingly, some of the ommatidia in the ventral part emit a distinct, whitish fluorescence (Fig. 2a). The few fluorescing ommatidia are randomly distributed and thus look like stars in the dark night sky. Rotation of the eye (and the butterfly) clearly demonstrated that the fluorescing ommatidia only occur in a limited area, determined by the aperture of the microscope objective. Upon focusing the microscope up and down, around the corneal level, doughnut-like patterns appeared at certain levels. The patterns are very similar to the waveguide mode patterns seen in reflection with incident illumination in the eyes of, e.g. nymphalid and pierid butterflies (Nilsson et al., 1988; van Hateren, 1989). We recall here that these butterfly families possess...
a tapetum below the rhabdoms, which is absent in the papilionids (Bernard & Miller, 1970; Miller, 1979; Stavenga, 1979). The conclusion that the fluorescence patterns seen in the Papilio eye represent wave-guide mode patterns implies that the fluorescing substance is located in the retinal layer and that the emitted light is guided via the rhabdom.

The fluorescing ommatidia are found in the ventral half of the compound eye. We counted the number of fluorescing ommatidia in 20 photographs taken from five individuals, and thus found that about 28% of ventral ommatidia fluoresce (Arikawa & Stavenga, 1997).

To further investigate the localization of the fluorescing pigment, we observed fresh eye sections, made with a cryostat. Quite satisfactory, the fluorescing pigment is seen in only a number of scattered ommatidia. The fluorescence appeared to be restricted to a distal stretch of ca 70 μm of the photoreceptor layer (Fig. 2b,c,d).

The fluorescing pigment appeared to be rather labile. Prolonged UV-illumination with the microscope’s mercury lamp caused a rapid fading of the fluorescing stars, within half a minute. The fluorescence pattern fully regenerated after a dark adaptation time of several hours, however.

3.3. The ommatidial UV-induced emission corresponds to that of 3-OH-retinol

To approach the question of the nature of the fluorescing pigment in the ommatidia, the emission spectrum was measured from the deep pseudopupil in the eye of live animals, with a photodiode array attached to the microscope (Fig. 3, noisy curve). The spectrum exhibits a main, broad band in the wavelength region between 420 and 600 nm; the peak is at about 480 nm.

The bleaching experiments suggest that the UV-absorbing pigment is a retinoid. Indeed, the emission spectrum measured from a 3-hydroxyretinol extract of the Papilio retina, precipitated onto a microscope slide (Fig. 3, dotted curve), is very similar to that measured from the living eye. The correspondence of the two spectra indicates that 3-hydroxyretinol is a possible candidate for the fluorescing pigment.

3.4. The violet receptors and single-peaked green receptors are located in the fluorescing ommatidia and their spectral sensitivity is affected by the V-absorbing pigment

Preliminary modeling suggested that the aberrant sensitivity spectra of the violet and single-peaked green receptors might be due to the absorbing effect of a UV filter acting on a visual pigment with a normal shape. Consequently, the aberrant photoreceptors must be localized in the fluorescing ommatidia.

To test this hypothesis, electrophysiological experiments were performed with electrodes filled with lucifer yellow. After recording, the photoreceptor was filled and
Fig. 2. UV-induced fluorescence of the *Papilio* eye. (a) Intact. The ventral half exhibits little fluorescence, except for some of the ommatidia that have visual fields within the aperture of the microscope objective (Olympus 4x, NA 0.16). The fluorescing ommatidia are distributed randomly (arrowhead). The fluorescence emerges from the rhabdoms, as witnessed by mode patterns, best visible at a level slightly proximal to the cornea. Compared to the ventral half of the eye, a rather strong fluorescence is observed in the dorsal part of the eye (D). This is emitted by the corneal facet lenses. (b) The transverse section immediately below the cone tips, shows a central, circular area with scattered, fluorescent dots. (c) The deeper section shows the scattered fluorescent dots as an annulus, indicating that the fluorescent pigment exists only over a limited depth, as shown in d. (d) A longitudinal section. A fluorescing pigment exists in a restricted number of ommatidia in a limited, distal part, i.e. over a length of approximately 70 μm (arrowheads). The distal positioning is in line with the function of the fluorescing pigment, namely to act as an absorption filter for the photoreceptor cells. Co, cornea. Bars 500 μm (a) 200 μm (b, c), 100 μm (d).

subsequently observed under the fluorescence microscope. Invariably, violet and single-peaked green receptors happened to be colocalized with the ommatidial fluorescence (*n* = 9; Fig. 4a,b). UV- and double-peaked green cells were exclusively found in non-fluorescing ommatidia (*n* = 11; data not shown).

This result strongly motivated further modeling. A satisfactory fit to the spectral sensitivity measured for the violet receptor was obtained by assuming a rhodopsin with *λ*<sub>max</sub> = 360 nm in R1,2, filtered by an ultraviolet-absorbing pigment with a 3-hydroxyretinol spectrum, and a peak density of 0.1 μm<sup>−1</sup> (Fig. 5a). Similarly, the effect of the UV-filter on a green rhodopsin peaking at 520 nm in R3,4 is a loss in sensitivity in the β-band (Fig. 5b).

3.5. Fluorescence in the dorsal eye part is due to a pigment in the cornea

The main issue of the analysis so far has been the

Fig. 3. Emission spectrum measured from the deep pseudopupil in the compound eye under UV excitation (noisy curve) and the emission spectrum of 3-hydroxyretinol, extracted from *Papilio* eyes and deposited on a microscope slide (dotted curve). The correspondence suggests that 3-hydroxyretinol might be the fluorescing pigment in the butterfly eye.
Fig. 4. Sensitivity spectra and localization of violet (V, left column) and single-peaked green receptors (SG, right column). Middle photographs shows ommatidia containing photoreceptors stained with lucifer yellow (violet-induced green emission). Both photoreceptor types appear to be localized in ommatidia that fluoresce under ultrablue illumination (lower photographs). Bar = 200 μm.

Origin of the distinct UV-induced fluorescence, displayed in a restricted set of ommatidia in the ventral retina of the intact eye of Papilio, and the implications for the spectral sensitivity of the photoreceptors. Yet, the dorsal eye region displays a noticeable fluorescence also, though rather weak (Fig. 2a). This fluorescence did not have features clearly connected to the aperture of the microscope objective, suggesting that the
Fluorescing pigment in this case is within the corneal layer. To settle this question, the cornea was isolated from the underlying retina. Indeed, only the dorsal half fluoresces, and no ‘stars’ could be seen in the ventral half of the cleaned cornea. Clearly, some fluorescing pigment is located in the corneal facet lenses of the dorsal half only. Also this pigment is bleached by prolonged UV light, but no recovery was observed at least in several days.

The nature of the fluorescing pigment remains unclear. Whether it has any visual function seems to be doubtful. At least, transmission microspectrophotometry on the cleaned cornea (unpublished) showed that the absorption by the facet lenses is at most a few percent throughout the whole visual wavelength range, including the ultraviolet.

4. Discussion

The UV-induced fluorescence patterns observable in the intact *Papilio* eye (Arikawa & Stavenga, 1997), as well as in retinal sections, demonstrate that an ultraviolet-absorbing and whitish fluorescing pigment is distributed randomly in the ventral retina. The nature of the pigment is not yet convincingly established, although a good case can be made for 3-hydroxyretinol. The aldehyde, 3-hydroxyretinal, is the ubiquitous chromophore of butterfly visual pigments and the alcohol, a necessary component of the cyclic visual pigment metabolism is present in high concentration in the *Papilio* retina (Seki, Fujishita, Ito, Matsuoka & Tsukida, 1987; Shimazaki & Eguchi, 1993). Reflection microspectrophotometry on the eyes of butterflies with a tapetum have indicated that butterfly visual pigments have a high turnover rate (Bernard, 1983; Stavenga, 1975). The rapid fluorescence recovery after bleaching of the fluorescence in the *Papilio* eye suggests that membrane and/or visual pigment turnover underlies this phenomenon.

Fly visual pigments, like those of butterflies, use 3-hydroxyretinal as the chromophore. However, 3-hydroxyretinol is not an absorption filter in the fly eye, but rather it is exploited as a sensitizing pigment (Vogt, 1989). 3-hydroxyretinol intimately links to the blue-absorbing rhodopsin of R1–6 photoreceptors, absorbs energy of UV light, and transmits the energy to the main chromophore, 3-hydroxyretinal, which leads to phototransduction. When this intimate link does not exist, the remaining action is the filtering effect, which is probably the case in the *Papilio* eye.

Filtering pigments, embedded in the photoreceptor membrane, that modify the spectral sensitivity have been found also in fly photoreceptors (housefly *Musca domestica*) (Kirschfeld, 1986). The rhabdomere of central photoreceptor R7y contains a mixture of the carotenoids lutein and zeaxanthin, which have a main absorption band in the blue. The filtering mechanism works oppositely to that described above for the butterfly, as the fly filter hypsochromically shifts the spectrum of the R7y’s violet rhodopsin ($\lambda_{\text{max}} = 430$ nm) to a UV sensitivity spectrum (peak at 355 nm). The central fly photoreceptor R8y has a rhodopsin with $\lambda_{\text{max}} = 520$ nm, but here the spectral sensitivity peaks at 530 nm and thus is shifted slightly bathochromically. The fly has another set of central photoreceptor cells, R7p and R8p, with spectral sensitivities peaking at 340 and 460 nm, respectively (Hardie, 1986; Feiler, Bjornson, Kirschfeld, Mismer, Rubin, Smith, Socolich & Zuker, 1992). A colour opponent system is most likely mediated by the two pairs of central photoreceptors, R7y/R8y and R7p/R8p (Troje, 1993).

The fly and butterfly cases have a further correspondence in that the two pairs, R7y/R8y and R7p/R8p, are randomly organized in the fly retina (Hardie, 1986). The *Papilio* retina also has a basically random organization as can be observed in a most colourful way by transmission microscopy of eye slices. The ommatidia then appear either yellow or (more or less saturated) red. This is due to clusters of densely coloured pigment
near the rhabdom, which in the distal retina is restricted to R3.4. The red and yellow pigmented ommatidia are randomly distributed over the eye (Arikawa & Stavenga, 1997). Interestingly, a random organization of the butterfly retina is found in several butterfly families, suggesting that this is an important element of a colour discrimination system.

Butterflies use their ventral eye for food and mate search for which, presumably, color discrimination plays an essential role (Hidaka & Yamashita, 1975; Arikawa et al., 1987). The present study shows that the sharp-peaked violet receptor and the single-peaked green receptor are exclusively found in the ventral eye where the fluorescing pigment exist. This finding suggests that there is a functional link between an improved color discrimination ability and the sharpened spectral sensitivities.

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