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RANDOM ARRAY OF COLOUR FILTERS IN THE EYES OF BUTTERFLIES

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Summary

The compound eye of the Japanese yellow swallowtail butterfly *Papilio xuthus* is not uniform. In a combined histological, electrophysiological and optical study, we found that the eye of *P. xuthus* has at least three different types of ommatidia, in a random distribution. In each ommatidium, nine photoreceptors contribute microvilli to the rhabdom. The distal two-thirds of the rhabdom length is taken up by the rhabdomeres of photoreceptors R1–R4. The proximal third consists of rhabdomeres of photoreceptors R5–R8, except for the very basal part, to which photoreceptor R9 contributes. In all ommatidia, the R1 and R2 photoreceptors have a purple pigmentation positioned at the distal tip of the ommatidia. The R3–R8

photoreceptors in any one ommatidium all have either yellow or red pigmentation in the cell body, concentrated near the edge of the rhabdom. The ommatidia with red-pigmented R3–R8 are divided into two classes: one class contains an ultraviolet-fluorescing pigment. The different pigmentations are presumably intimately related to the various spectral types found previously in electrophysiological studies.

Key words: Japanese yellow swallowtail, butterfly, *Papilio xuthus*, colour vision, retina, visual pigment, vision, ommatidia, photoreceptor, spectral receptor type.

Introduction

Butterflies are often admired for their spectacular colour patterns. They are therefore generally assumed to possess colour vision. Behavioural experiments on butterflies have indicated that they can indeed perceive colour contrast (for example Hidaka and Yamashita, 1975; Ilse, 1928, 1937, 1941; Kolb and Scherer, 1982; Wehner, 1981). Compared with bees, in which extensive behavioural studies have demonstrated the ability to see colour (Menzel and Backhaus, 1989), and flies, which also possess a clear colour discrimination system (Fukushi, 1989; Troje, 1993), convincing behavioural evidence for colour vision in butterflies is lacking.

A basic physiological requirement for colour vision is the existence of a set of different spectral receptor types in the retina. This is certainly fulfilled in a number of butterfly species, as shown by anatomical (Ribi, 1978, 1987), optical (Bernard, 1979; Bernard and Remington, 1991) and electrophysiological (Eguchi *et al.* 1982; Horridge *et al.* 1984; Kinoshita *et al.* 1997; Matić 1983; Shimohigashi and Tominaga, 1991; Steiner *et al.* 1987) studies. Presently, the best characterized retina of a butterfly, in terms of the spectral properties of the photoreceptors, is that of the Japanese yellow swallowtail butterfly *Papilio xuthus*. We have demonstrated previously that the retina contains at least five types of spectral receptors, peaking in the ultraviolet, violet, blue, green and red wavelength regions (Arikawa *et al.* 1987). Subsequent work

has demonstrated that the nine photoreceptors of an ommatidium, R1–R9 (see Fig. 1), are spectrally heterogeneous. R1 and R2 are ultraviolet, violet or blue receptors, R3 and R4 are green receptors (Bandai *et al.* 1992) and R5–R9 are either green or red receptors (Arikawa and Uchiyama, 1996).

Within this diverse population of photoreceptors, the nature and composition of spectral receptors in the ommatidia has yet to be elucidated. A more complete characterization of the ommatidia and their arrangement over the retina is the aim of the present paper. Histological, electrophysiological and optical experiments on the compound eye of *P. xuthus* have identified three different types of ommatidia, randomly distributed over the retina. This random distribution may be crucial for colour vision.

Materials and methods

Animals

The Japanese yellow swallowtail butterfly *Papilio xuthus* Linnaeus was held as a laboratory stock culture. Other species were collected in Yokohama, Japan.

Anatomy

For light microscopy, the compound eyes were isolated from

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the head, and fixed in 4% paraformaldehyde in 0.1 mol l⁻¹ sodium cacodylate buffer at pH 7.2 (CB) for 30 min. The eyes were subsequently dehydrated through an acetone series, embedded in Epon, and sectioned at 10 µm using a rotary microtome. The sections were observed without staining.

For electron microscopy, the eyes were fixed in 2% glutaraldehyde and 2% paraformaldehyde in CB for 2 h, and further fixed in 2% OsO₄ in CB for 2 h. After being dehydrated, the tissues were embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate.

To observe ommatidial transmission, a fresh compound eye was cut at the level of the proximal photoreceptors using a vibrating microtome. The slice was then mounted, with the corneal side up, on the stage of an epifluorescent microscope (BX-60, Olympus). The focus was at the corneal surface. First, the transmitted light was photographed with white light illumination, from below. Subsequently, the ultraviolet-induced fluorescence was photographed (objective 4×, numerical aperture 0.16).

Electrophysiology

A butterfly was mounted in a Faraday cage, and a glass micropipette, filled with 5% Lucifer Yellow CH aqueous solution (resistance 150 MΩ), was inserted into the retina through a hole made in the dorsal cornea. After impalement of the electrode into a single photoreceptor, the spectral sensitivity of the cell was determined by axial stimulation with a point source delivering an equiquantal series of monochromatic lights with wavelengths ranging from 290 to 700 nm (maximal intensity 5.0 × 10¹¹ photons cm⁻² s⁻¹ at the corneal surface). After injecting Lucifer Yellow into the photoreceptor, by applying 2–5 nA of hyperpolarizing d.c. current for 5–10 min, the eye was prepared for light microscopy as described above. The Lucifer-Yellow-injected photoreceptors were then identified in 10 µm sections under violet epi-illumination.

Results and discussion

Red and yellow pigmentation of ommatidia

To study the histological relevance of the distribution of spectral receptors, we first prepared light microscope sections of the *P. xuthus* retina. We discovered in serial sections that both R3 and R4 and R5–R8 photoreceptors in any one ommatidium contain either yellow or red pigment near the rhabdom (Figs 1, 2A). Further electron microscopy demonstrated that the yellow and red pigmentation is made up of pigment granules, residing in the cell body 1–1.5 µm from the edge of the rhabdom (Fig. 2B). R1 and R2 of all ommatidia have a purple pigmentation positioned at the distal tip of the ommatidia. The anatomical results are summarized schematically in Fig. 1.

The rhabdom diameter is approximately 2.0 µm (Fig. 2B) and it therefore functions as an optical waveguide (for a review, see van Hateren, 1989). Consequently, the light-absorbing pigment surrounding the rhabdom will act as a colour filter (Stavenga, 1989; Marshall *et al.* 1991). This effect

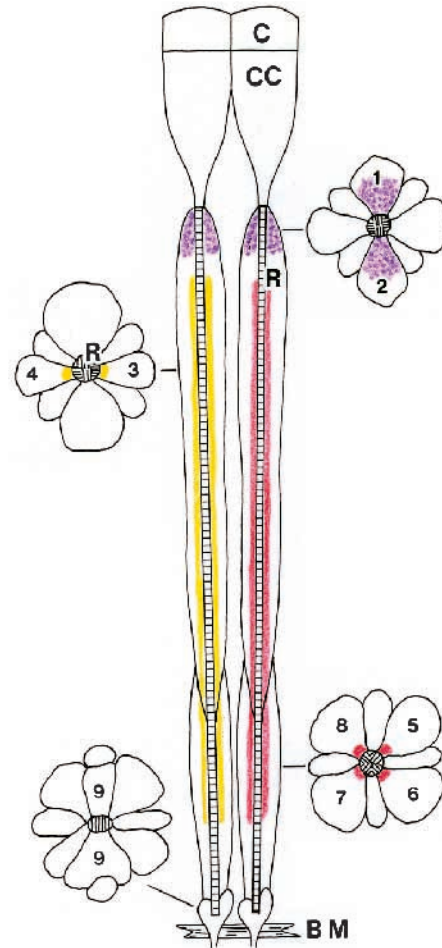


Fig. 1. Schematic representation of the ommatidia of *Papilio xuthus*. The ommatidium is of the apposition type with a fused and tiered rhabdom (R) consisting of nine photoreceptors (R1–R9). The distal two-thirds of the rhabdom (total length approximately 500 µm) is made up of the rhabdomeral microvilli of four distal cells (R1–R4), whereas the proximal photoreceptors, R5–R8, bear microvilli in the proximal one-third of the rhabdom, except for the very basal part near the basement membrane (BM), where the bilobed R9 photoreceptor contributes microvilli to the rhabdom. R1 and R2 of all ommatidia contain purple pigmentation at the region immediately proximal to the crystalline cone (CC). R3–R8 have yellow or red pigmentation along the rhabdom except for the distal and proximal parts. R9 is pigment-free. Numbers represent photoreceptor number. C, cornea.

can be directly visualized by cutting a fresh compound eye with a vibrating microtome at the level of the proximal photoreceptors. In transmitted light, the ommatidia then appear either yellow or (more or less saturated) red (Fig. 3A). The purple pigment is shared by R1 and R2 in all ommatidia, so that the observed difference in coloration must be attributed to the yellow or red pigment in R3–R8 (Fig. 2A).

The ratio of yellow to red ommatidia is approximately 1:3. For 653 ommatidia in 10 transverse sections collected from four individuals, the ratio was 166:487; there was no significant variation among individuals. We further investigated whether the array of differently coloured

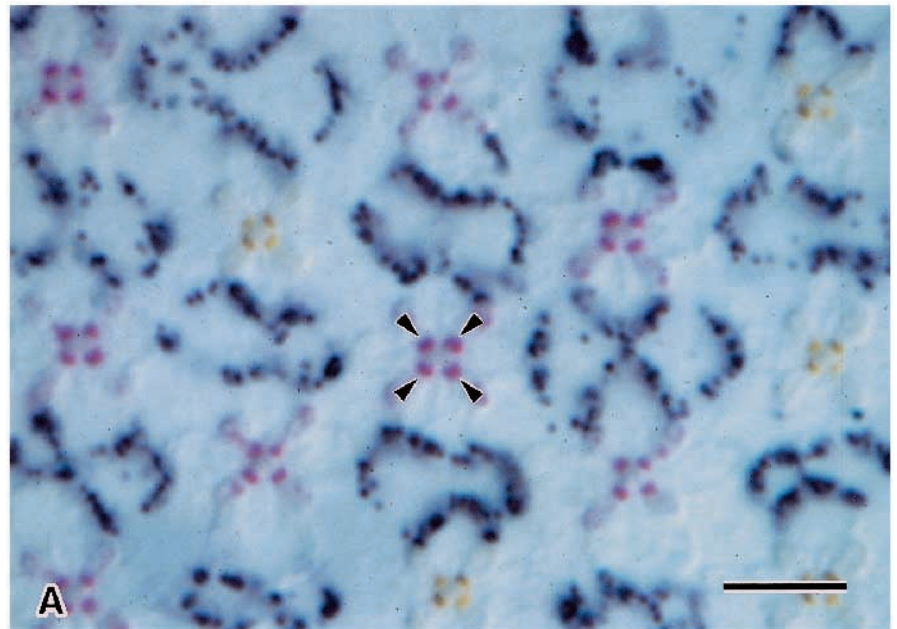
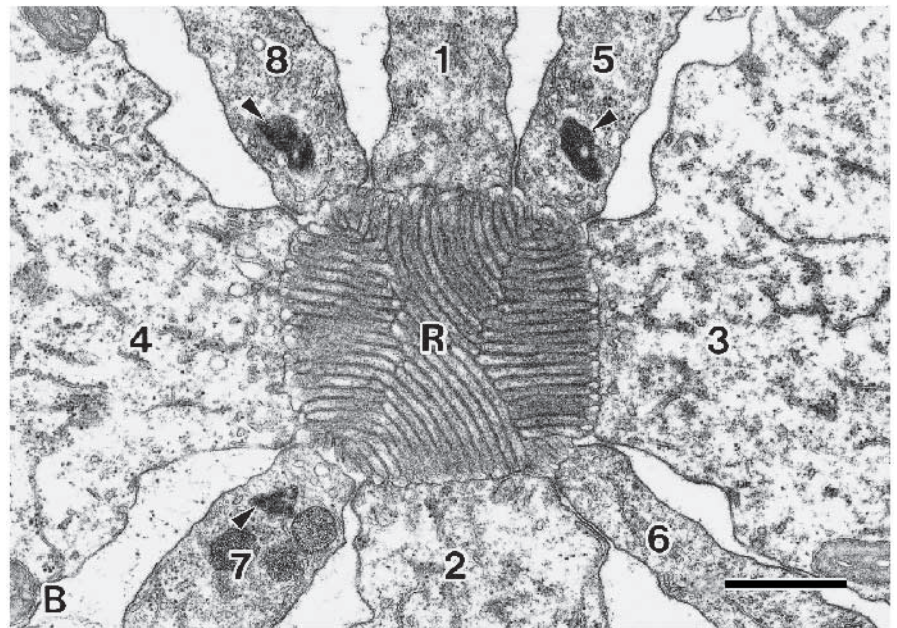


Fig. 2. Pigmentation in the ommatidia of *Papilio xuthus*. (A) Unstained Epon-embedded section through the proximal tier of the ommatidium, where four proximal photoreceptors, R5–R8, contribute their microvilli to the rhabdom. The four coloured patches (arrowheads) are pigment clusters in R5–R8. Red-pigment-containing and yellow-pigment-containing ommatidia are distributed randomly throughout the retina. Comparisons of frozen sections with Epon-embedded sections show that the colours of the pigments are virtually unchanged by the short fixation time (30 min) without osmication used in preparing these sections for light microscopy. Scale bar, 10 μm . (B) Electron micrograph of a red-pigment-containing ommatidium. The pigment granules (arrowheads) reside in the cell body area 1–1.5 μm from the edge of the rhabdom (R). Numbers indicate photoreceptor number. Scale bar, 1 μm .



ommatidia has any regularity. To do this, we counted the frequency of transition, for example from yellow to red or from red to red, along the three axes of the hexagonal lattice. It appeared that the transition frequency is independent of the frequency of the type of its neighbours and only reflects the absolute probability of the ommatidial type (10 micrographs yielded $\chi^2=2.25$, d.f.=4).

To investigate any possible correlation between the spectral receptor types and the pigmentation, we recorded spectral sensitivities from single photoreceptors and marked the cells by injecting Lucifer Yellow. Subsequently, we identified the pigmentation of the ommatidia to which the penetrated photoreceptors belonged by light microscope histology. We found that the proximal R5–R8 cells in the yellow-pigment-

containing ommatidia, without exception, appear to be green-sensitive receptors. The R5–R8 cells in the red-pigment-containing ommatidia are always red-sensitive receptors.

Of course, it is virtually impossible to prove conclusively using electrophysiology that cells R5–R8 in a single ommatidium are always identical. A powerful alternative is offered by histological *in situ* hybridization to localize different mRNAs encoding visual pigment opsins. Our preliminary results indicate that photoreceptors R5–R8 in a single ommatidium always contain identical opsin mRNA (Kitamoto *et al.* 1996); that is, it is most likely that R5–R8 in the red-pigment-containing and the yellow-pigment-containing ommatidia are all red- and green-sensitive receptors, respectively.

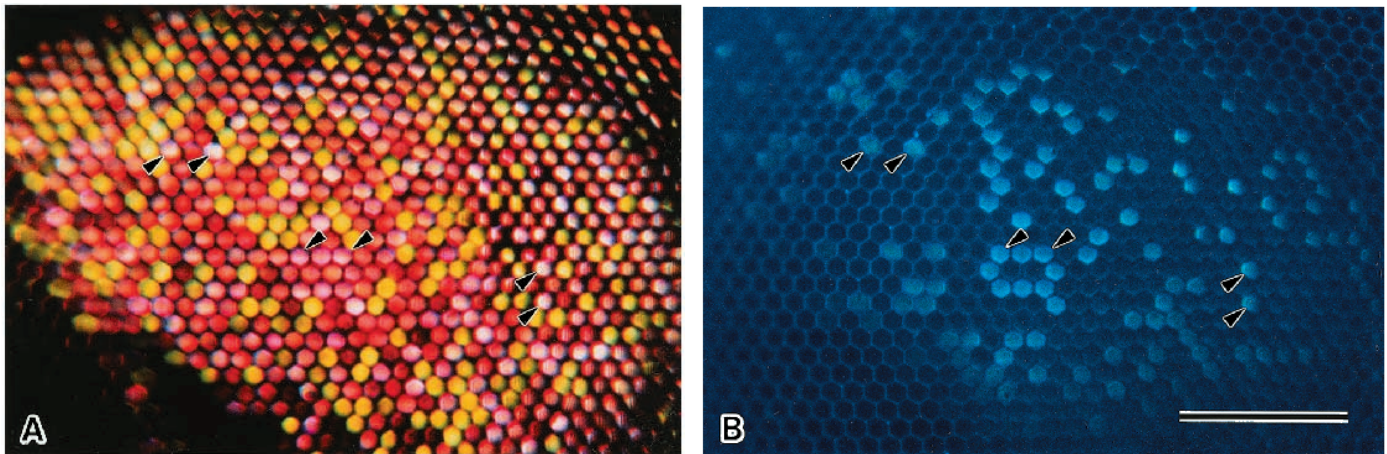


Fig. 3. (A) Ommatidial pigmentation seen in a slice of fresh eye observed in transmitted light, i.e. under antidromic illumination. (B) Ommatidial fluorescence under ultraviolet epi-illumination of the same preparation as in A. Arrowheads indicate identical ommatidia. Fluorescing ommatidia in B appear very pale or pink in A. Scale bar, 200 μm .

Fluorescing ommatidia

We also investigated the intact eye of *P. xuthus* using fluorescence microscopy and found that a further aspect of retinal heterogeneity can be directly observed *in vivo*. Ultraviolet epi-illumination of the *P. xuthus* eye excites a distinct whitish emission in approximately one-quarter of the

ommatidia in the ventral half of the eye (Fig. 3B). In six micrographs, 28% of the ommatidia fluoresced. Again, their distribution appears to be random. Upon focusing at levels proximal to the cornea, waveguide mode patterns appear, indicating that the fluorescence originates from the rhabdom (Franceschini *et al.* 1981; Nilsson *et al.* 1988; van Hateren,

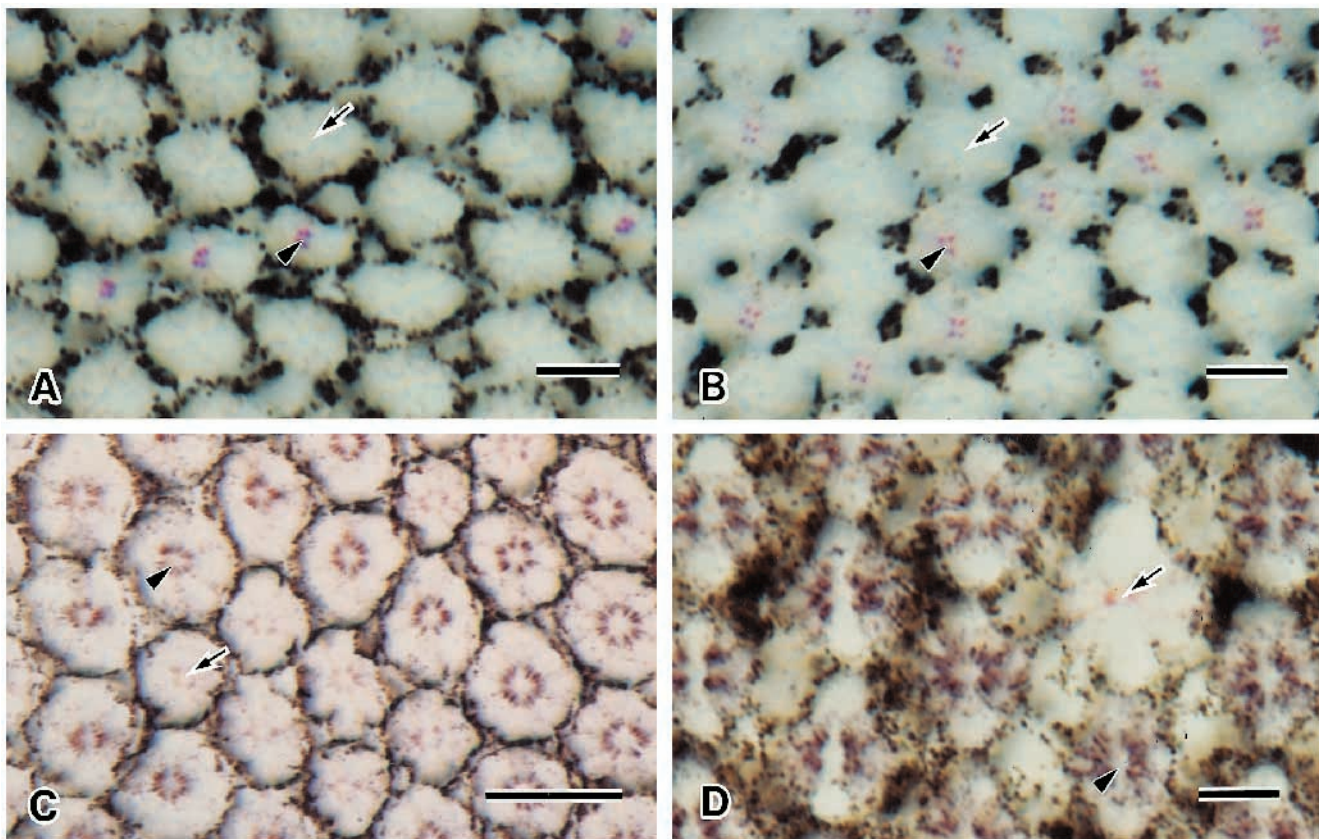


Fig. 4. Photoreceptor pigmentation in lycaenid and satyrid butterflies. Transverse sections of ommatidia were observed without staining. Pigmented ommatidia (arrowheads) and non-pigmented ommatidia (arrows) are evident. (A) *Zizeeria maha* (Lycaenidae), (B) *Lycaena phlaeas* (Lycaenidae), (C) *Neope goschkevitschii* (Satyridae), (D) *Ypthima argus* (Satyridae). Scale bars, 10 μm (A, B, D) and 25 μm (C).

1989). Preliminary photochemical and biochemical experiments suggest that the fluorescing pigment is a retinoid, but conclusive results have yet to be obtained. The ultraviolet-fluorescing ommatidia are not seen in the dorsal half of the eye. Their presence, if they are there, is obscured by a distinct corneal fluorescence.

Combined fluorescence and transmission microscopy of eye slices revealed that those ommatidia that fluoresce under ultraviolet excitation appear a less saturated red, i.e. very pale to pink, in transmitted light (Fig. 3A,B). Further electrophysiological experiments, combined with Lucifer Yellow staining and fluorescence microscopy, demonstrated that violet receptors only occur in the fluorescing ommatidia. Ultraviolet and blue receptors were found exclusively in the non-fluorescing ommatidia.

The sensitivity spectrum of the violet receptor is very sharp-peaked (Arikawa *et al.* 1987). This suggests the challenging hypothesis that the fluorescing pigment might act as a selective ultraviolet absorbance filter. The red receptor also has a narrower spectral sensitivity spectrum than predicted from a rhodopsin template (Arikawa *et al.* 1987). This is probably a direct consequence of the strong high-pass filtering by the red photoreceptor pigmentation (Arikawa *et al.* 1996).

Comparative aspects

Our studies on *P. xuthus* show that its retina is a mesh of at least three types of optically distinguishable ommatidia: one containing yellow pigment and two containing red pigment, one type of which contains an ultraviolet-fluorescing pigment. Together with the visual pigments of the photoreceptors, these pigments are crucial in shaping the spectral sensitivity of the five receptor types. How *P. xuthus* connects these receptors to achieve colour discrimination, which might even be pentachromatic, remains an intriguing problem. Behavioural experiments detailing the colour discrimination capabilities of *P. xuthus* are now in progress.

A mesh of different classes of ommatidia has been found previously in the retina of flies (Franceschini *et al.* 1981; Hardie, 1986; Chou *et al.* 1996), where the two central photoreceptors determine colour vision (Troje, 1993). The observed randomness of the retinal organization in *P. xuthus* is also shared with primates: a random distribution of M and L cones has, for instance, been demonstrated in talapoin monkeys (*Cercopithecus talapoin*) (Mollon and Bowmaker, 1992). In contrast, several fish species have a very regular retina (Lythgoe, 1979).

In a survey of the compound eyes of other butterflies (Fig. 4), we found a marked variation in pigmentation around the rhabdom, not only in the species belonging to the family Papilionidae (*Papilio bianor*, *Papilio protenor*, *Papilio polytes*, *Graphium sarpedon*, *Parnassius glacialis*) but also in Lycaenidae (*Zizeeria maha*, *Lycaena phlaeas*) and Satyridae (*Neope goschkevitchii*, *Ypthima argus*). These results are in agreement with previous optical studies on intact butterfly eyes using epi-illumination microscopy (Bernard and Miller, 1970; Miller, 1979). These studies revealed a multicoloured

reflection from a tapetal mirror proximal to the rhabdom that exists in the eye of all butterflies except for the Papilionidae. Although no direct evidence is available so far, the difference in pigmentation and coloration of the tapetal reflection strongly indicates that the ommatidia are different in terms of the spectral receptor types they contain.

Compound eyes often exhibit considerable regionalization, because different parts of the eye are devoted to specific tasks, such as prey or mate recognition or polarization vision (Stavenga, 1992). Here, we have shown that, within restricted regions of the butterfly eye, ommatidial characteristics can differ considerably. Clearly, the concept that compound eyes, at least locally, consist of identical building blocks, the ommatidia, does not hold. The random organization of the spectral receptors in the retina of butterflies seems to be a universal feature, presumably because a diverse set of spectral receptors is essential for a highly developed colour vision system (Bernard, 1979; Bernard and Remington, 1991; Goldsmith, 1990).

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