Opponent processing in the retinal mosaic of nymphalid butterflies

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1,2,3,4,5,6 The eyes of nymphalid butterflies, investigated with incident illumination, show colourful facet reflection patterns—the eye shine—which is uniform or heterogeneous, dependent on the species. Facet colours suggest that the ommatidia contain different sets of photoreceptors and screening pigments, but how the colours and the cell characteristics are associated has not been clearly established. Here, we analyse the retinae of two nymphalids, Apatura ilia, which has a uniform eyeshine, and Charaxes jasius, a species with a heterogeneous eye shine, using single-cell recordings, spectroscopy and optical pupillometry. Apatura has UV-, blue- and green-sensitive photoreceptors, allocated into three ommatidial types. The UV- and blue-sensitive cells are long visual fibres (LVFs), receiving opponent input from the green-sensitive short visual fibres (SVFs). Charaxes has an expanded set of photoreceptors, allocated into three additional, red-reflecting ommatidial types. All red ommatidia contain green-sensitive LVFs, receiving opponent input from red receptors. In both species, the SVFs do not receive any opponent input. The simple retina of Apatura with three ommatidial types and two colour-opponent channels can support trichromatic vision. Charaxes has six ommatidial types and three colour-opponent channels. Its expanded receptor set can support tetrachromatic vision.

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1. Introduction

Colour vision is based upon the analysis of the spectral composition of the visual scene. The neural signals originating from photoreceptors with different spectral sensitivities are compared in a process of colour opponency. The spectral properties of colour opponent visual neurons match the statistics of natural scenes, maximizing the signal variance and allowing for optimal information processing in the visual pathway [1,2]. The optimal transformation for trichromatic vision is achieved through decomposing the retinal signals into an achromatic channel and two colour-opponent channels [3]. In flies and butterflies, the first stage of colour processing is performed already by the photoreceptors that directly inhibit each other through inter-photoreceptor synapses with histaminergic chloride channels [4–8]. Colour processing is continued downstream in the optical ganglia, the lamina and the medulla [5,8,9]. Here, we study colour opponency in the retinal mosaic of brush-footed butterflies (Nymphalidae).

Compound eyes are built from discrete optical units, the ommatidia. In all butterfly families with afocal apposition eyes studied thus far (families Papilionidae, Pieridae, Lycaenidae and Nymphalidae), each ommatidium contains eight large photoreceptors R1–8 and a small basal R9. The photoreceptors contribute their light-sensing microvilli to the common light guide, the rhabdom. The microvilli of R1,2 are oriented vertically (i.e. parallel to the dorsoventral eye axis), horizontally in R3,4 and diagonally in R5,7 and R6,8. The horizontal and diagonal photoreceptors R3,4 and R5–8 are the short visual fibres (SVF)
that are presynaptic to the large monopolar neurons of the lamina [10,11], which in turn condition the signals for processing of achromatic contrasts and motion. In most butterflies studied so far, the SVFs are expressing a long-wavelength (LV) rhodopsin, peaking in the green range (520–560 nm), but exceptions do exist: in the dorsal eye of the lycaenid butterfly *Lycaneta rubidus*, blue opsin mRNA is co-expressed with LV opsin mRNA in the females and exclusively expressed in the males [12].

The vertical photoreceptors R1,2 and the basal R9 are the long visual fibres (LVF) that project axons directly to the medulla and contribute to the processing of colour and polarization content of the visual scene. In most studied butterflies, receptors R1,2 express the short-wavelength (SW), ultraviolet (U, 340–370 nm) or blue-peaking (B, 420–460 nm) opsins [13,14]. The U and B receptors allocated to R1,2 give rise to three ommatidial types [UU, UB, BB] that are randomly distributed across the retina with species-specific fractions [15]. The random expression of the various visual pigments is driven by the spinless mechanism [16]. The basal cells R9 likely express the same LW opsin as R3–8 [13].

The retinai of papilionid and pieriid butterflies are fully tiered [17]. The rhabdomeres of R1–4 occupy the distal tier of the rhabdom and the rhabdomeres R5–8 constitute the proximal tier. The SW part of the downwelling light is filtered by the visual and screening pigments in the distal tier, resulting in red-shifted sensitivities of the proximal photoreceptors R5–8 [18,19]. In papilionids, the basal LVF R9 can be either green- or red-sensitive [20] and expresses the LW opsin mRNA [21]. The red-sensitive basal LVF photoreceptor in pieriids [11] likely also expresses a LW opsin. The inter-photoreceptor opponent synapses are located both downstream in the optic ganglia—the lamina and the medulla—and in the retina, along the photoreceptor axons, rendering the opponent signals detectable by intracellular recordings from the photoreceptors [5–7]. In the Japanese swallowtail, *Papilio xuthus*, opponent signals were detected in all photoreceptors R1–8 [6].

In brush-footed butterflies (nymphalids), the retina is incompletely tiered. The rhabdomeres of R3–8 are present along the entire length of the ommatidium, while the rhabdomeres of R1,2 may be present only distally [10,22] or may span along the whole rhabdom [13,23]. A tracheal basket at the base of the retina forms the reflective *tapetum lucidum* in all studied butterfly families with apposition eyes [24,25] except in the papilionids [26]. The light launched into the rhabdom is reflected from the tapetum, bringing about the eye shine. The reflections from individual ommatidia are coloured, depending on the composition of visual pigments (rhodopsin R and its isomere metarhodopsin M) and the presence of screening pigments. A red ommatidial colour is a tell-tale sign for red (i.e. blue- and green-absorbing) screening pigment being opposed to the rhabdom. Ommatidia without such a screening pigment appear yellow, green, pale or blue, depending on the tuning of the tapetum, rhodopsin composition and rhabdom length [15,25,27,28]. In species with a uniform eye shine, e.g. *Vanessa atalanta*, vertical cells R1,2 express exclusively UV- or blue-peaking rhodopsins, similarly to papilionids and pieriids [13,28].

Many nymphalids have a non-uniform eye shine with red-reflecting ommatidia [25,29], which may indicate the presence of more than three ommatidial types. For instance, in some *Heliconius* butterflies, R1,2 can express another type of UV opsin (UV2, due to opsin duplication) [14] or a green-absorbing (G; LW) opsin [30], which leads to the expansion of possible ommatidial types and a complex retinal mosaic. The LW opsin-expressing R1,2 likely reside in the red ommatidia, which furthermore contain a functional, red-sensitive R9 that inhibits the green-sensitive vertical cells [7]. The functional significance of R9 in the non-red ommatidia is currently unknown, but their spectral sensitivity is likely less red-shifted [15,28], similarly to *Papilio* [20].

Here, we study the inter-photoreceptor opponent mechanisms in brush-footed butterflies (family Nymphalidae). We focus our study on the lesser purple emperor *Apatura ilia*, and the two-tailed pasha, *Charaxes jasius*, the former with a simple and the latter with a complex eye mosaic. We show that the emperor is equipped with the basic trichromatic photoreceptor set, while the pasha has five additional photoreceptor types. We show that the expanded set of receptors in the pasha (green-sensitive R1,2, yellow-sensitive R3–9 and red-sensitive R9) is allocated to the red ommatidia. We identify two and three colour opponent channels in the emperor and the pasha, respectively. Taken together, the eyes of brush-footed butterflies can have either a simple retinal mosaic with three ommatidial types and two colour opponency channels, or a complex retinal mosaic with six ommatidial types and three opponency channels that are presumably used as the substrate for tetrachromatic vision.

2. Results

(a) Eye shine and rhodopsin photochemistry

The eye shine mosaic in *C. jasius* is predominantly green in the dorsal part and yellow-green in the central and ventral parts, speckled with red ommatidia whose fraction is higher ventrally (figure 1a). The red ommatidia stand out prominently in the hyperspectral image of the dark-adapted eye of *Charaxes* (figure 1f), while in the eye of *Apatura*, mosaic regionalization is not observed (figure 1g).

When the eye of *Charaxes* was left in the dark for longer than 10 min, the dorsal non-red ommatidia were blueish, turning green after a few seconds of illumination (figure 1b,c) before the pupil activation would extinguish the eyeshine. The reflectance change had two phases: the first phase was due to rhodopsin-metarhodopsin (R–M) photoisomerization [31]. Photoisomerization caused a pronounced reduction of reflectance below the isosbestic point at approximately 530 nm (figure 1d). The reflectance increase in the wavelength range (550–650 nm) is consistent with the observed eyeshine colour change (figure 1c). The second phase, due to the pupil closure [32], started about 3 s after the light onset and completed about 15 s afterwards (figure 1e).

The measured reflectance spectra were low-pass filtered, background-corrected and log-transformed. The difference between the first and any later log10(R) spectrum gave a series of absorbance-difference spectra ΔA with a peak trough shape (figure 1h). The first 50 log10(R) spectra were processed using singular value decomposition (SVD) of the (wavelength × time) matrix. The principal temporal component η(t) was decaying exponentially, as expected for a photoisomerization process. A fit of a template absorbance-difference model to the principal spectral component η(t) yielded the estimate for *Charaxes* LW rhodopsin and metarhodopsin, peaking at 543 nm and 502 nm, respectively (R543/
M502. The estimated R&M templates, the steady state metarhodopsin fraction \( f_{502}(\lambda) \) and the normalized isomerization time constant \( \tau(\lambda) \) for isosbestic monochromatic illumination are shown in figure 1i. The estimated LW opsin of *Apatura* is R527/M496 (electronic supplementary material, figure S1).

(b) Intracellular recordings

Photoreceptor identities were revealed by intracellular recordings, using rapid spectral sequence stimulation provided by a fast narrow-band LED source [33]. In both species, we found photoreceptors that depolarized maximally upon ultraviolet or blue stimulation, respectively, and hyperpolarized upon green stimulation (*Charaxes*: figure 2a,b; *Apatura*: electronic supplementary material, figure S2). We termed the two classes U+G− and B+G−; the letters signify the human colours of the unit spectral maxima, [UBGYR] for ultraviolet, blue, green, yellow and red, respectively. In *Charaxes*, we additionally found B+Y− photoreceptors and G+R− photoreceptors (figure 2c,d). The responses of the depolarizing units U+ and B+ could be isolated by saturating the hyperpolarizing units G− and Y− using appropriate LW adapting illumination (green or orange triangle, middle trace, figure 2a–c). Isolation of the hyperpolarizing units was less successful due to the sensitivity overlap with the depolarizing units in the SW range (purple or blue triangle, bottom trace, figure 2a–c). Both units of the G+R− opponent pair could be isolated by selective adaptation of the opponent unit (figure 2d), confirming that the sensitivity overlap between the G+ and R− units is minimal [7]. Hyperpolarization could be enhanced, suppressed or reversed by current injection (figure 2f). Both depolarizing and hyperpolarizing responses were graded along the approximately 3 log stimulus intensity range. The aperture of the light stimulus had a minor effect on the hyperpolarization in the green (electronic supplementary material, figure S2). The most frequently encountered cells belonged to the broad-sensitive LW photoreceptor class G and were without a detectable inhibitory input at the retinal level. In these cells, monochromatic adaptation light (of any wavelength) caused a wavelength-independent suppression of the
light response (525 nm or 625 nm adapting light used in green and red traces in figure 2e).

The ancestral set of insect spectral photoreceptors (U, B, G) was found both in Aputura and Charaxes (figure 3a, b; electronic supplementary material, figure S2). The U and B receptors were maximally sensitive to vertically (parallel to the dorsoventral body axis) polarized light, consistent with allocation to photoreceptors R1,2. The cells of the main LW receptor class G were maximally sensitive either to horizontally or diagonally polarized light, consistent with the allocation to R3,4 and R5–8, respectively (figure 3d, e). In both species, the measured spectral sensitivity of G cells was broader and red shifted with respect to the corresponding opsin templates with peak sensitivity parameters (λ_max) determined spectrophotometrically (Aputura: λ_max = 527 → 534 nm; Charaxes: λ_max = 543 → 548 nm), probably due to self-screening in long photoreceptors [7,15,34]. In Charaxes, the additional, yellow-peaking LW receptor class Y (figure 3c) was similarly consistent with allocation to R3–8 (figure 3f). The G+R− receptors, allocated to R1,2, are maximally sensitive to green light and likely receive opponent input from the red-sensitive, basal R9 that could not be directly impaled [7].

In Charaxes, the B cells with vertical microvilli were maximally hyperpolarized by their opponent units either around approximately 550 nm (figure 3c) or approximately 600 nm (figure 3f). The latter is best explained with the opponent of Y units to B cells, yielding the class B+Y− additional to the class B+G−. A similar, albeit more modest distinction could also be made between U+G− and U+Y− classes (figure 3b, c; electronic supplementary material, figure S3). The receptive fields of the main and the opponent units always overlapped, indicating that the opponency at the level of the retina does not involve pooling from neighbouring ommatidia (figure 3h, k).

Allocation of the main and the opponent units to the receptor positions was further studied by measuring the polarization sensitivity (PS) of the hyperpolarizing responses. In most UV+G− and some B+G− cells, PS in the green spectral range was low, suggesting that several green-sensitive cells converge onto a single UV or B cell, so their PS cancels out (figure 3f; electronic supplementary material, figure S3). In a subclass of B+G− and B+Y− cells, the opponent PS was modest (2–3) and had a horizontal angular maximum, while the main PS was modest to high (2–4): these opponent cells are good candidates for being the retinal substrate for polarization vision (figure 3e; electronic supplementary material, figure S3).

The onset of depolarizing responses was lagging the stimulus onset for 6–9 ms at the highest stimulus intensity and for 10–15 ms when being stimulated with light attenuated by 1.5 log (figure 3g, j). The shortest delay was, as expected, in the class G SVF photoreceptors and class Y photoreceptors (not shown); the fastest LVF was the class B. Response latency of hyperpolarizing units is a sum of the phototransduction and synaptic latency. In B+Y− cells, where the sensitivities of the main and opponent unit are about equal (electronic supplementary material, figure S4), the opponent response was delayed relative to the depolarizing response for approximately 1 ms, which is our estimate for the synaptic latency. This latency is consistent with the situation in U+G− class, where the latency of hyperpolarizing units G− was approximately 1 ms longer than that of SVF G cells (figure 3g, j). In this class, the opponent response was even slightly faster than the depolarizing response, possibly due to the slower phototransduction of the U+ unit. The most striking response delay difference was found in G+R− cells, where the opponent response was lagging the depolarizing response by 4–10 ms, likely due to the slow transduction in the minute, light-starved R9 cells (figure 3f; electronic supplementary material, figure S5).

(c) Optical retinography

The electrophysiological results in Aputura suggested a simple retinal mosaic with two types of LVFs, both receiving opponent signals from G receptors. Physiological evidence suggests that the classes U+G− and B+G− are possibly
allocated in pairs to form three types of ommatidia, [UU, UB, BB], similarly to the genus Vanessa [15]. In the red-eyed Charaxes, however, the expanded retinal mosaic contains an additional distal LVF class G+R–, which could, in combination with U and B classes, form three additional ommatidial types, [GG, GU, GB]. The proposed allocation nevertheless awaits molecular validation in both species.

We checked the proposed allocation of six ommatidial types into the eye mosaic of Charaxes with optical retinography (ORG) [15], an optical method that reports the compound pupillary sensitivity of the photoreceptors in each ommatidium. We expected that the spectral sensitivity of the pupillory responses, evoked with isosquantal pulses, would resemble the weighted sum of opsins templates in LVFs and SVFs, with relative transduction gains as weights. For each ommatidium and each wavelength, we acquired bouts of linearly polarized light were delivered to the eye, causing a partial closure of the pupil. The polarizer was rotated for 37.25° between stimulations, completing three revolutions of linearly polarized light were delivered to the eye, causing a partial closure of the pupil. The polarizer was rotated for 37.25° between stimulations, completing three revolutions in 29 steps. For each ommatidium and each wavelength (λ) bout, we used a linear model to estimate the constant part of pupillary response bDC(λ) and its modulation part bPS(λ), which is due to the compound polarization sensitivity of photoreceptors. The constant (DC) and modulation (PS) parameters of the pupillary response were estimated using a linear model. The two parameters were normalized to the pupil working range, determined from the images taken in the central eye region of a single eye. The pupil sensitivity was measured with an isosquantal spectral sequence (red to UV; UV to red). The sequence was repeated at full, half and quarter intensity. At each wavelength, we acquired bouts of 30 images: the first image was of the dark-adapted eye (λ), the remaining were taken after 15 s adapting stimuli (λ). (g/j) Response latency (mean ± s.d., number of analysed cells in brackets) of main cells (full circles), opponent units (empty circles) and non-opponent G cells (black circles) as a function of graded stimulus intensity. (h,k) Receptive fields of single main cells (top), their opponent units (middle) and overlap between main and opponent units (bottom). (λmax and PS of all spectral classes given in table 1.)
Table 1. Spectral sensitivity maxima, polarization sensitivity and angular maxima of photoreceptor classes encountered in Charaxes jasius and A. ilia.

| species       | class | $\lambda_{\text{max}}$ | $\lambda_{\text{max}}$ opp | PS  | N   | PS opp | N   |
|---------------|-------|------------------------|----------------------------|-----|-----|--------|-----|
| Charaxes jasius | U+G   | 337                    | 500–590                     | 1.7 ± 0.4 | 17 | 1.3 ± 0.5 | 6   |
|               | U+Y   | 335                    | 590                         | 2.3  | 1  | 1.2 ± 0.1 | 3   |
|               | B+G   | 430                    | 560                         | 1.7 ± 0.5 | 18 | 1.2 ± 0.1 | 7   |
|               | B+Y   | 430                    | 600                         | 1.9 ± 0.5 | 6  | 1.3 ± 0.3 | 6   |
|               | G+R   | 533                    | 633                         | 1.2 ± 0.1 | 6  | 1.3 ± 0.1 | 5   |
|               | G     | 548                    | —                           | 1.8 ± 0.4 | 22 | —        | —   |
|               | Y     | 580                    | —                           | 2.0 ± 0.4 | 12 | —        | —   |
|               | R     | 615                    | —                           | 1.3 ± 0.1 | 5  | —        | —   |
| Apatura ilia  | U+G   | 340                    | 480–540                      | 2.2 ± 0.6 | 20 | 1.3 ± 0.3 | 9   |
|               | B+G   | 435                    | 575                         | 2.2 ± 0.6 | 44 | 1.2 ± 0.2 | 11  |
|               | G     | 534                    | —                           | 2.2 ± 0.6 | 58 | —        | —   |
The constant (DC) pupil responses of the ommatidial clusters are shown as median and shaded inter-quartile range; the median of PS modulation is depicted with bars (figure 4a–c, e–g). The clusters {BB, UB} had pronounced PS in the UV-blue spectral part (figure 4a, b). The cluster UU had a high PS across the whole spectrum (figure 4c). The remaining three clusters had generally lower PS (figure 4e–g). The distribution of the six clusters in the ommatidial lattice seems to be random (figure 4d, h). The boosted colours measured from the hyperspectral image of the eyeshine (figure 4k) were mapped to the six clusters. The two most numerous clusters had generally lower PS (figure 4e–g). The distribution of the six clusters in the ommatidial lattice seems to be random (figure 4d, h). The boosted colours measured from the hyperspectral image of the eyeshine (figure 4k) were mapped to the six clusters. The two most numerous clusters had generally lower PS (figure 4e–g). The distribution of the six clusters in the ommatidial lattice seems to be random (figure 4d, h). The boosted colours measured from the hyperspectral image of the eyeshine (figure 4k) were mapped to the six clusters. The two most numerous clusters had generally lower PS (figure 4e–g). The distribution of the six clusters in the ommatidial lattice seems to be random (figure 4d, h).

3. Discussion
We have shown that in Apatura ilia, a nymphalid butterfly with a basic set of spectral photoreceptors (U, B, G), the retina is likely built from three ommatidial types without red pigments that form a simple mosaic. In Charaxes jasius, a nymphalid with an expanded set of LVF photoreceptor classes (G+R−, R) and a SVF photoreceptor subclass Y, the retinal mosaic is complex, having three additional ommatidial types, allocated to the red ommatidia (figure 5).

We found similar expanded spectral sets of photoreceptors in other species with red ommatidia and with a complex mosaic eye shine, e.g. the monarch (Danaus plexippus), blue morpho (Morpho peleides) and prepona (Archaeoprepona demophon) (electronic supplementary material, figure S6). Interestingly, these butterflies likely have only three opsin genes {U, B, G = LW} [35,36], suggesting that the expanded set of receptors {G+R−, Y and R} is implemented on the basis of optical filtering of a single LW opsin. The simple retinal mosaic can support trichromatic vision in the ultraviolet to green range [28,37]. Colour discrimination in the red wavelength range and the putative tetrachromatic vision are only supported in the eyes with a complex retinal mosaic, confirmed behaviourally in Danaus and Heliconius [28,38], but not yet in Charaxes. The retinal complexity is likely costly and seems to be evolutionarily switched on or off, depending on the visual ecology of the species. Extension of colour vision into the red wavelength range in brush-footed butterflies is associated with the simultaneous occurrence of multiple features of the visual system: the green-sensitive R1,2, the red-screening
pigment, and a functional R9 with direct retinal opponency to the class G+R− LVF. These features are likely controlled by an additional stochastic genetic switch similar to the known spinoless transcription factor [16].

Quite possibly, the expanded retinal mosaic is also associated with a red-shifted LW opsin. Nymphalid LW opsin is subjected to extensive evolutionary tuning, with $\lambda_{\text{max}}$ varying between 515 nm and 585 nm [35], but the physiological relevance of this tuning is unknown. In nymphalids with a uniform eyeshine, the LW opsin tends to peak below 530 nm [27], whereas in the red-eyed butterflies, the LW opsin’s peak tends to shift above 540 nm. A similar shift has been implicated in the evolution of red colour vision in lycaenid butterflies [39].

We note that a basal photoreceptor, receiving light filtered by red-screening pigments, can have high sensitivity and a high signal-to-noise ratio only by expressing a red-shifted LW opsin. Assuming narrow-band red light (620 nm), R9 would be approximately twice and five times more sensitive, if R525 is red shifted for 10 and 25 nm, respectively. Photoconversion of metarhodopsin seems to be very ineffective in a red-sensitive R9 (figure 1f), so the cell probably relies on enzymatic pigment conversion to maintain high sensitivity [40].

The simple nymphalid retina contains two colour-opponent channels (here U+G− and B+G−) and an achromatic channel (here S+M−), following the design principles for optimal information transfer in trichromatic vision [3]. In the expanded retina, a new opponent channel (G+R−) and a Y subclass achromatic channel are added, likely on the basis of the same LW opsin as the green-sensitive R3–8. The channel expansion is implemented through a red-screening pigment that tunes the basal red receptor R9. Three interesting functional features can be elucidated. (i) LVF R1,2 can receive opponent inputs from either LVF or SVF (U&B from R3–8, G from R9). (ii) Opponency seems to be unidirectional, SVFs (R3–8) do not receive opponent inputs from LVFs. (iii) Opponent cells (G SVFs and R9 LVF) have a red-shifted sensitivity, compared to their postsynaptic partners R1,2. (We note that due to the absence of direct recordings, we cannot exclude the possibility that R9 are receiving an opponent input from another class of SVF). This implementation is quite different from Papilio, where direct opponency is present among all kinds of visual fibres, including LVFs being opponent to both LVFs and SVFs, and SW-peaking classes being opponent to LW classes [5], or from Drosophila, where direct opponency is only present between the LVFs [4].

The SW photoreceptors U and B have a higher phototransduction gain (effectively more millivolts of depolarization per absorbed quanta) than the LW photoreceptors (electronic supplementary material, figure S4). Direct opponency from U&B LVF would cause strong inhibition of G&Y SVF. This would be detrimental for the signal-to-noise ratio in the achromatic visual pathway relayed via the lamina monopolar cells. The opponent signalling from SW to LW is likely implemented at a later stage in the visual pathway, probably via interneurons in the medulla [8,9]. In the complex nymphalid retina with red ommatidia, the tiny, light-starved, high-gain R9 cells can use a novel, private opponent channel in the form of green-sensitive LVF R1,2, thereby avoiding sending an opponent signal into the R3–8 achromatic pathway.

The LVFs in our study exhibited various levels of hyperpolarization due to the opponent cells; generally, the opponency in Apatura was much less pronounced than in Charaxes (figure 2a–c). The sensitivity of the receptors in opponent pairs must be tuned so that the degree of inhibition is just right. The inhibition should not be too weak, or else spectral discrimination would not work, nor too strong, to the detriment of signal generation and propagation. We hypothesize that the inhibition gain may be adjustable by yet unknown mechanisms that depend on light adaptation and internal state-dependent efferent inputs from the central nervous system. Interestingly, many LVFs from all spectral classes exhibited slow spikes (figure 2f; electronic supplementary material, figure S2a) that were very loosely correlated with light stimulation and depolarization. These spikes may have been initiated by the adjacent cells that may be modulating the physiological properties of their impaled neighbour. Further comparative research is needed to assess how the opponent pairs respond to the changes in spectral composition of ambient light across different time scales, and what are the optimal spectral opponent combinations for the particular photonic environment.

4. Experimental methods

(a) Animals

The butterflies were collected near Zadar and Mali Lošinj, Croatia (C. jasius) or bred and shipped from the UK (A. ilia) by Mr Mark Youles as a part of ongoing collaboration. Adult butterflies were kept at 27°C and 80% relative humidity and regularly fed sucrose solution.

(b) Rhodopsin isomerization spectroscopy

The template spectra of the LW rhodopsin and metarhodopsin can be estimated using a spectroscopic method employing a broadband light source both for isomerization and the measurement [40]. The animal was immobilized in a pipette tip attached to a manual goniometer and placed under a Leitz Orthoplan microscope with a custom epi-illumination attachment with a 50% beamsplitter. The light source was a modified violet-chip-based white LED (Sora MR16-50-B03) driven with a constant current source. A long working distance objective (either Nikon Plan ELWD 20 × NA0.40 1.2/160, part 120152, or an Olympus MPlan 10 × NA0.25 0/∞) was focussed on the eye’s curvature centre. A Bertrand eyepiece was used to finely position the animal and set the illuminator’s aperture and field iris so that the whole objective back-focal plane was filled with ommatidial reflections. The eye’s luminous deep pseudopupil was imaged onto the iris plane of a custom-made microspectrometer head and relayed to a rosette-to-line fibre bundle attached to a spectrometer (Ocean Optics USB2000) controlled from GNU Octave. Eyeshine images were taken with a Raspberry Pi4, equipped with a Pi HQ Camera (RGB CMOS, 20MP).

Before each measurement, the animal was left to dark adapt for 10–60 min, so that the visual pigment would be predominantly in the rhodopsin isof orm. The shutter was opened for 10–15 s, and several hundred spectra with integration time 15–25 ms were taken. Electronic (thermal) noise and the animal or objective background were subtracted from the spectral series. Data processing was implemented in GNU Octave with Signal and Optim toolboxes, using SVD, where a matrix with a temporal series of spectra M was decomposed.
into \( n \) spectral (U) and temporal (V) components, \( M_{\{k\times l\}} \rightarrow U_{\{k\times n\}} D_{\{n\times n\}} V_{\{n\times l\}}^T \).

The raw data matrix \( M_k \) with count spectra (2048 pixels \( \times \) 500 acquisitions) was decomposed into spectral and temporal components that were separately low-pass filtered using a zero-phase filter (function \text{filtfilt}). A filtered matrix \( M_k \) was recomposed from the first few signal-bearing components and then log-transformed, \( M_2 = \log_{10}(M_0) \). The first 50 spectra of \( M_2 \) were again decomposed. The fundamental component \( \{u_0, v_0\} \) was approximately constant in time and could be discarded, while the next temporal component vector \( v_t \) followed an exponential relaxation course, indicative of a photoisomerization process. The corresponding spectral component vector \( u_t \), analogous to the absorbance-difference spectrum, was fitted with a template absorbance-difference model based on Govardovskii et al. [41] templates \( \Gamma_\lambda = a (\epsilon \Gamma_M - \Gamma_R) + ab \), where \( c \) is \( M/R \) peak absorbance ratio, \( b \) is (relative) baseline correction and \( a \) is a technical scaling parameter. We used nested models where the parameters could be constrained to \( b = 0 \) or \( c = 1.25 \), the latter being a biblical value in the fully dark-adapted state. A model fit yielded \( \lambda_R \), \( \lambda_M \) and optionally \( c \). In the case of \text{Charaxes}, the value \( c = 1.25 \) seems to be correct, while the experimental data for \text{Apatura}, due to close-lying R&M peaks, does not allow for a reliable estimation of \( c \). The data points below 430 nm were excluded from the fit due to a systematic deviation (see also electronic supplementary material, figure S1).

(c) Electrophysiological recordings

Intracellular recordings were performed as described previously [7]. Briefly, immobilized animals were placed with the head in the centre of rotation into a goniometer that also carried the micromanipulator with sharp electrodes (Sensapex, Oulu, Finland). The recordings were performed with an amplifier (SEC-10LX, NPI, Tamm, Germany) in bridge mode or discontinuous clamp mode at 20 kHz and 0.25 duty cycle. The electrodes were pulled from borosilicate glass and had resistance in the range 80–120 MΩ when filled with 3 M KCl. Light stimulation was provided by a 75 W XBO lamp (Cairn Research, Kent, UK), filtered through a motorized monochromator (B&M Optik, Limburg, Germany), a computer-controlled neutral density wedge filter (Thorlabs, Dachau, Germany) and, for PS measurements, a UV-capable polarizer (OUV2500, Knight Optical, UK). The second source was a ‘LED Synth’ [33] with narrow-band LEDs between 365 and 685 nm in 15 nm intervals. Both sources were combined with a beam splitter (Thorlabs, Dachau, Germany) and isosquantized with an irradiance-calibrated spectrophotometer (Flame, Ocean Optics, USA) to yield equal photon (isosquantal) flux density at all wavelengths (max. 1.5 \( \times \) 10^7 photons cm\(^{-2}\) s\(^{-1}\)). The aperture of the coaxial stimulating beam was adjusted to between 1.5° and 20° by an iris. Receptive fields of the photoreceptors were mapped with an RGB DLP projector (LightCrafter 4500, Texas Instruments, USA) that projected to a back-projection screen (ST-Pro-X, Screen-Tech e.K., Hohenaspe, Germany) at a refresh rate of 220 Hz using software package PsychoPy.

(d) Eyeshine

Eyeshine was observed with a custom epi-illumination microscope built from Thorlabs, Edmund Optics and Linos parts as described elsewhere [15,29]. The relaying lenses were near-UV achromatic doublets (Edmund Optics). The main objective lens was a Zeiss LD-Epiplan 20 \( \times \) NA0.40 objective (part 442840). Images were taken with monochrome or RGB CMOS cameras (1.6, 2.3, 20.0 MP, BFS-U3-1652, BFLY-U3-2356, BFS-U3-20056, all FLIR/PointGray).

The illumination for RGB images was provided by a white LED (colour temperature approx. 3000 K), filtered by a purplish filter (Lee Filters) that brought the three colour channel gains close to unity. Hyperspectral images were taken with a monochrome camera, using the LED synh [33] as the light source. Exposure and gain were optimized for each image. The instrumental background, due to reflections from the objective lenses, was acquired separately, averaged and subtracted from the image series. Images were processed in ImageJ/Fiji [42]. Background correction was performed by subtracting an averaged image of lens reflections without the animal. The stacks were aligned using StackReg [43]. Substacks covering spectral ranges where a similar eyeshine pattern was observed (e.g. 380–440 nm, 480–540 nm and 600–660 nm) were averaged and joined into a pseudo-coloured image (figure 1f and figure 4f).

(e) Optical retinography

ORG is an optical method reporting the compound sensitivity of the photoreceptors in each ommatidium [15]. We measured the pupillary action spectra of ommatidia in the central eye region of \text{Charaxes}. The experiments were performed in the epi-illumination microscope equipped with a monochrome CMOS camera as described in §4d. The light source for test images was a white LED, filtered with an orange- or red-coloured glass long-pass filter. The adapting light source was the LED synh [33] with 20 LEDs covering the range 365–660 nm. The combined beam was depolarized using a liquid crystal depolarizer (DPP25-A, Thorlabs) before being focussed to a 1 mm diameter, NA 0.40 polymer fibre. The fibre output was collimated with an aspheric lens and sent through a rotatable, UV-capable polarizer.

The adaptation experiment was conducted three times, at full, half and quarter intensity of the isoquantal adapting light source. In each of the experiments, we acquired \( m = 1200 \) images, divided into 40 bouts. In each bout, we recorded a dark-adapted image and 29 images where monochrome, isoquantal, linearly polarized adapting light was switched on for 15 s prior to taking the test image. The polarizer was rotated for 37.25° between the images, so the 30th image completed the third rotation in the same angular position as the second image. Each bout was followed by a 1 min dark adaptation period. The bouts #1 and #21 were dark-adapted control; the bouts #20 and #40 were control with saturating adaptation light. The bouts [2# … #19] and [2#2 … #39] were sequences with spectral adaptation going from red to UV and from UV to red, respectively. We reversed the bouts [#2 … #19], yielding six experimental runs with adaptation wavelength going from UV to red. An example of median responses of GU ommatidia from bouts 21–40 of the full intensity run is shown in figure 4g.

The ROI of ommatidia were found using ImageJ [42] functions ‘Find Maxima’ and ‘Analyse Particles’. The adaptation states of \( n = 666 \) ommatidia in \( m = 3600 \) images (6 experimental runs \( \times \) 20 bouts \( \times \) 30 images) were exported as a table of average ROI grey values \( g_{n \times m} \). The pupil range of each ommatidium was obtained by finding the maximal grey value in the fully dark-adapted state \( g_{n \times DA} \) and the minimal grey in the fully light-adapted state \( g_{n \times LA} \). The relative pupil
activation for the ommatidium \( n \) in image \( m \) was calculated as
\[
P_{nm} = 1 - \frac{(g_{n,m} - g_{n,LA})}{(g_{n,DA} - g_{n,LA})}.
\]
The 30 pupil responses in each bout, \( P_{m} = [P_{0,m} \cdots P_{30,m}]^{T} \) were fitted with a linear model \( p = Ab \). The matrix \( A_{30 \times 3} \) was constructed from column vectors containing a constant (DC) component, sine and cosine modulation, linear trend and exponential decay with a predetermined time constant. Solving the matrix equation \( b = A^{{-1}}p \) for each bout yielded a parameter vector containing the constant pupillary response parameter \( b_{DC} \) and the oscillating parameters \( b_{\cos}, b_{\sin} \). From the latter two, PS modulation amplitude was calculated as \( PS = 2 |(b_{\cos} + ib_{\sin})| \); we did not analyse the PS phase. The parameters \( b_{DC} \) and \( b_{PS} \) obtained at each wavelength in the six experimental runs were placed into six \( b_{DC} \) and six \( b_{PS} \) matrices of size \([666 \text{ ommatidia} \times 18 \text{ wavelengths}] \). Each of these 12 matrices were separately normalized and decomposed using SVD, yielding score and spectral matrices. The first three score vectors \([666 \times 3]\) from each decomposition were concatenated, yielding a matrix of size \([666 \text{ ommatidia} \times 36 \text{ scores}] \), coming from two parameters \([b_{DC}^{{\text{opt}}}, b_{PS}^{{\text{opt}}}] \times 6 \) experimental runs \( 3 \) score vectors. This matrix was again decomposed, giving the final score matrix with \( 3 \)–\( 5 \) signal-bearing score vectors that were used for \( k \)-means clustering. The first two-score components are shown as the scatter in figure 4i inset.

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Data accessibility. Data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.cnpbhjqk [44]. The data are provided in the electronic supplementary material [45].

Authors’ contributions. P.P.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, software, supervision, validation, visualization, writing—original draft and writing—review and editing; M.I.: formal analysis, investigation, methodology, software, validation, visualization and writing—original draft; A.M.: investigation, methodology, validation, visualization and writing—original draft; G.B.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, software, supervision, validation, visualization—writing—original draft and writing—review and editing.

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