Sex-specific retinal pigmentation results in sexually dimorphic long-wavelength-sensitive photoreceptors in the eastern pale clouded yellow butterfly, *Colias erate*

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**SUMMARY**

The compound eyes of the eastern pale clouded yellow butterfly, *Colias erate*, contain three types of ommatidia (I, II and III), identifiable by the differing arrangements of pigment clusters around the rhabdons. The pigment color is red in all ommatidial types except for type II ommatidia of females, where the pigment is orange. Intracellular recordings demonstrated that the spectral sensitivities of the proximal photoreceptors (R5–8) of all ommatidia in both sexes are strongly tuned by the perirhabdomal pigments. These pigments act as long-pass filters, shifting the peak sensitivities into the wavelength range above 600 nm. Due to the sex-specific pigments in type II ommatidia, the spectral sensitivities of the R5–8 photoreceptors of females peaked at 620 nm while those in males peaked at 660 nm. The measured spectral sensitivities could be well reproduced by an optical model assuming a long-wavelength-absorbing visual pigment with peak absorbance at 565 nm. Whereas the sexual dimorphism was unequivocally demonstrated for the ventral eye region, dimorphism in the dorsal region was not found. Presumably the ventral region is adapted for sexual behaviors such as courtship and oviposition.

Key words: color vision, insect, ommatidium, opsin, spectral sensitivity, visual pigment.

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**INTRODUCTION**

The eyes of butterflies are composed of thousands of ommatidia, each containing nine photoreceptor cells, R1–9. The visual pigment-containing organelles of these photoreceptors, the rhabdomerses, are closely apposed and thus form a fused rhabdom. In many butterflies, specifically pierids and papilionids, the rhabdom is tiered. The distal tier is made up of the rhabdomeric microvilli of four distal photoreceptors (R1–4), and the proximal tier consists of the microvilli of four proximal photoreceptors (R5–8). At the base of the rhabdom, the basal photoreceptor, R9, contributes a few additional microvilli (Fig. 1).

The spectral sensitivities of the photoreceptors are principally determined by their visual pigments, but may be modified by additional optical effects. Notably, clusters of pigment granules that are concentrated near the light-guiding rhabdons act as long-pass filters and thus can shift the spectral sensitivity of the photoreceptors. Detailed studies on the small white butterfly, *Pieris rapae* (Pieridae), identified clusters of pale red and deep red pigments located inside the photoreceptors, adjacent to the rhabdons. These pigments cause a pronounced shift towards red in the spectral sensitivity of the proximal photoreceptors. Whereas all proximal photoreceptors express a visual pigment with an absorption spectrum peaking in the green range at around 560 nm, photoreceptors in ommatidia with pale-red pigment have a spectral sensitivity peaking at 620 nm, and those in ommatidia with deep-red pigment have a peak sensitivity of 640 nm (Qiu and Arikawa, 2003; Wakakuwa et al., 2004). The receptors are accordingly termed red (R) and deep-red (dR) receptors.

When observed with an epi-illumination microscope, the eyes of most butterflies (with the exception of all papilionids and some pierids) exhibit a striking eyeshine, due to the presence of a tracheal tapetum at the bottom of the rhabdom (Fig. 1) (Arikawa and Stavenga, 1997; Bernard and Miller, 1970; Takemura et al., 2007). The tapetum reflects light that has escaped absorption while propagating through the rhabdom. Some of the reflected light travels back through the rhabdom and leaves the eye, and is visible as the eyeshine. The eyeshine spectrum (that is, the reflectance spectrum of the individual ommatidium) depends on the absorption by the visual pigments as well as the spectral filtering by the pigment clusters surrounding the rhabdons, and thus the eyeshine color is a useful indicator of the type of ommatidium. In *P. rapae*, the individual ommatidia have eyeshine peaking at 635 or 675 nm. By a combination of histological, optical and electrophysiological methods, it could be demonstrated that ommatidia with reflectance spectra peaking at 635 nm contained 620 nm-peaking R-receptors and ommatidia with eyeshine peaking at 675 nm contained 640 nm-peaking dR-receptors (Qiu and Arikawa, 2003).

In the pale clouded yellow butterfly, *Colias erate* (Pieridae), we have recently found that the eyeshine spectrum peaks near the infrared, at 730 nm, in a subset of ommatidia, suggesting that their photoreceptors may have a spectral sensitivity peaking at an even longer wavelength than those of *P. rapae*. We therefore decided to perform an extensive study of the *C. erate* eye, involving anatomical (Arikawa et al., 2009), molecular biological (Awata et al., 2009) and physiological (Pirih et al., 2010) analyses. We previously investigated the distal photoreceptors and found a clear sexual dimorphism due to sex-specific pigmentation of the ommatidia (Ogawa et al., 2012). Here we report a subsequent study on the properties of the proximal photoreceptors, with particular reference to the sexual dimorphism in their spectral sensitivities. We...
discovered that the long-wavelength receptors peaking in the wavelength range longer than 600 nm, which are generically called ‘red’ receptors, are much more variable in females than in males, implying that females have an improved wavelength discrimination ability in the long wavelength region of the spectrum.

**MATERIALS AND METHODS**

**Animals**
Adults of the eastern pale clouded yellow butterfly, *Colias erate* (Esper 1805), were obtained from a laboratory culture derived from eggs laid by females captured around the Sokendai-Hayama campus, Kanagawa, Japan. Hatched larvae were reared under natural light conditions on fresh clover leaves.

**Electrophysiology and dye injection**
Electrophysiological methods were as described previously (Ogawa et al., 2012). Briefly, photoreceptor spectral sensitivities were determined by intracellular recording of responses to monochromatic stimuli delivered by a 500 W Xenon arc lamp via a series of narrow-band interference filters ranging from 300 to 740 nm. The light beam was focused on the tip of an optical fiber, the other end of which was attached to a Cardan-arm perimeter device in a Faraday cage, where it provided a point light source (1 deg in diameter). The quantum flux of each monochromatic stimulus was measured using a radiometer (Model-470D, Sanso, Tokyo, Japan) and adjusted to a standard number of photons using an optical wedge. For each experiment, a butterfly was mounted on a plastic stage in the Faraday cage, with its dorsal side up. A silver wire inserted into the head served as the reference electrode. A glass microelectrode filled with 10 mmol l\(^{-1}\) Alexa Fluor 568 (peak excitation/emission at 576/599 nm) in 200 mmol l\(^{-1}\) KCl (A10441, Molecular Probes, Invitrogen, Carlsbad, CA, USA), with a resistance of approximately 100 MQ, was inserted into the retina through a small hole made in the cornea. Membrane potentials were recorded through a preamplifier (MEZ-7200; Nihon Kohden, Tokyo, Japan) connected to a computer via an AD converter (MP-150, BIOPAC Systems, Goleta, CA, USA).

After penetrating a photoreceptor, the optical fiber was adjusted so as to yield maximal responses. First, the spectral type of the impaled photoreceptor was determined using a series of monochromatic flashes of duration 30 ms, spaced 1 s apart. The response–stimulus intensity (\(V–\log I\)) function was recorded over a 4 log unit intensity range at the photoreceptor’s peak wavelength. The photoreceptor was subjected to further analyses only if the maximal response amplitude exceeded 30 mV. At the end of the recording, Alexa Fluor 568 was injected into the photoreceptor by applying a 2 nA hyperpolarizing DC current for ~5 min.

**Anatomy**
Immediately after the electrophysiological experiment, we observed the eyes with a fluorescence microscope (Olympus, BX50, Tokyo, Japan) applying 550 nm excitation light, and thus localized and photographed the ommatidium containing the dye-filled photoreceptor. We fixed the eyes in 4% paraformaldehyde in 0.1 mol sodium cacodylate buffer (pH 7.4) at room temperature for 30 min, then embedded the eyes in Quetol 812 (Nisshin EM, Tokyo, Japan) and made a series of 10-μm-thick transverse sections. Subsequently we identified the type of the ommatidium containing the dye-filled photoreceptor based on the pigmentation pattern (as revealed by normal transmitted light microscopy) combined with the fluorescence and eyeshine pattern of the intact eye. To determine the distribution of the perirhabdomal pigment along the rhabdom, we fixed intact eyes as described above and cut 5-μm-thick serial sections from the distal to the proximal end of the rhabdom.

**Microspectrophotometry**
The absorbance spectra of the perirhabdomal pigments were measured from pigment clusters in transverse light-microscopic sections using a microspectrophotometer consisting of a Leitz Ortholux microscope with an Olympus 20× objective (NA 0.46), connected to an Avaspec 2048-2 CCD detector array spectrometer (Avantes, Eerbeek, The Netherlands).

**Model calculation**
To quantitatively estimate the spectral sensitivity of the proximal photoreceptors of *C. erate*, we constructed an optical simulation model based on the anatomical details of the three types of ommatidia (I, II and III) present in the eye of *C. erate* (Arikawa et al., 2009). Based on the anatomical study of Arikawa et al. (Arikawa et al., 2009), we used the following values in the model calculations. The total length of the rhabdom was 500 μm (Fig. 2). The depth of the upper tier of the rhabdom, with rhabdomeres of photoreceptors R1–4, was 250 μm in type I ommatidia, and 200 μm in type II and III ommatidia. The depth of the second tier (containing the rhabdomeres of photoreceptors R5–8) was the remaining part of the rhabdom, except for the basal 10 μm, which was assumed to be fully
occupied by the rhabdomere of R9. A fluorescent pigment, whose absorption spectrum was assumed to be identical to that of the fluorescent pigment in P. rapae (Arikawa et al., 2005), was present in the most distal 80μm of the rhabdoms in type I ommatidia in male eyes and type II ommatidia of female eyes (Ogawa et al., 2012). The visual pigment expressed in each photoreceptor (Awata et al., 2009) and the occupancy ratio of the rhabdomere of R9. a rhabdomere in the entire rhabdom (Arikawa et al., 2009) are shown in Table 1. Visual pigment absorbance spectra were calculated using the Govardovskii template (Govardovskii et al., 2000). We applied the simple model approach of Stavenga and Arikawa (Stavenga and Govardovskii template (Govardovskii et al., 2000). We applied

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\[ \Delta \]

in Table 1. Parameters used in the model

<table>
<thead>
<tr>
<th>Type</th>
<th>( \kappa_{S,\text{max}} )</th>
<th>Visual pigment absorption peak (nm)/rhabdomere occupancy ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \kappa_{S,\text{max}} )</td>
<td>R1</td>
</tr>
<tr>
<td>Type I</td>
<td>3.0</td>
<td>2.5</td>
</tr>
<tr>
<td>Type II</td>
<td>2.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Type III</td>
<td>3.0</td>
<td>4.0</td>
</tr>
</tbody>
</table>

\( \kappa_{S,\text{max}} \): peak absorbance coefficient of red pigment.
The present model did not reproduce the secondary sensitivity bands in the short wavelength region, showing that the model is incomplete, as will be discussed below.

**DISCUSSION**

**Red receptors in *Colias erate***

We characterized the spectral sensitivities of the proximal photoreceptors in the compound eye of *C. erate* by intracellular recording and optical modeling. All photoreceptors were maximally sensitive in the red wavelength region, but their peak wavelengths differed strikingly between males and females. According to the actual recordings and model calculations, we conclude that the proximal photoreceptors of males peak at 660 nm in all ommatidial types, and those in females peak at 610, 650 or 660 nm depending on the ommatidial type. To the best of our knowledge, 660 nm is the longest peak wavelength of any red receptor reported in an insect.
This is also the first example of a distinct sexual dimorphism of red receptors in any animal.

The simple model we applied could well reproduce the principal sensitivity peaks of the red receptors. Two mechanisms appeared to account for the differences in the calculated sensitivity spectra. First, the different absorbance spectra of the red and orange pigment clusters mean that different spectral filters act on the proximal photoreceptors (Figs 2, 3). Second, the orange pigment in the female type II ommatidia extends over just 150 μm (Fig. 2), which is much shorter than in the other ommatidia, resulting in a lower density of the pigment filter. Accordingly, the proximal photoreceptors in female type II ommatidia have the shortest peak wavelength (620 nm). Furthermore, the extent of red pigment in type I ommatidia is longer in males (265 μm) than in females (230 μm). Consequently, the spectral sensitivity of the proximal photoreceptors in type I ommatidia peaks at 660 nm in the male and at 640 nm in the female.

The modeled spectra deviate markedly from the experimental spectra in the short- and middle-wavelength range, where the calculations predict a low sensitivity but the measured spectra show distinct sensitivity bands (Fig. 5). The same problem was encountered in our study of the small white butterfly, *P. rapae*. In that instance, calculations with a similar simple model also yielded low sensitivities in the short and middle wavelength range. An elaborate wave-optical model of the *P. rapae* rhabdom produced substantial sensitivity bands in the shorter wavelength.
range (Stavenga and Arikawa, 2011). Our attempts to construct a wave-optical model similar to that of P. rapae have remained somewhat unsatisfactory, due to the considerably more complicated structure of the rhabdoms of C. erate; whereas the rhabdoms of P. rapae approximate a homogeneously tapering cylinder, the rhabdoms of C. erate have a pronounced constriction in the middle (Arikawa et al., 2009). At the constriction, the rhabdomeric microvilli are locally replaced by concentrated red pigment. This makes wave-optical modeling difficult, because the rhabdoms’ waveguide properties strongly depend on both the rhabdom diameter and the refractive indices of the medium within and surrounding the rhabdom, which is unknown in the case of the red pigment. Nevertheless, preliminary modeling attempts yielded clear sensitivity bands in the short and middle wavelength range. From this observation, together with the exemplary case of P. rapae, it seems reasonable to hypothesize that the ultraviolet and blue sensitivity bands of the proximal photoreceptors of C. erate (Fig. 5) are due to waveguide effects.

Note that the Rml and Rfl are similar in the principal peaks in red wavelength region, but the secondary sensitivity band in the UV–V wavelength region is smaller in Rml than in Rfl (Fig. 5A,D). In type II red receptors, the relationship is reverse. This sexual difference is attributable, at least in part, to the sexually dimorphic distribution of the fluorescence pigment (Fig. 3C): the male type I and the female type II ommatidia contain the fluorescence pigment (Ogawa et al., 2012).

### Function of the multiple red receptors

Remarkably, the expression pattern of visual pigments is identical in both sexes of C. erate (Awata et al., 2009; Ogawa et al., 2012), but the spectral sensitivities of their photoreceptor sets differ. Sexual dimorphism in photoreceptor spectral sensitivities was already encountered in the distal photoreceptors of Colias (Ogawa et al., 2012). A fluorescent pigment that absorbs maximally at around 420 nm, concentrated near the distal tip of the rhabdom in male type I ommatidia and female type II ommatidia, produces shouldered-blue receptors (sB) in males and narrow-blue receptors (nB) in females (Fig. 6) (see Ogawa et al., 2012). Together with the present study, this shows that the sexual dimorphism of C. erate eyes is rather pronounced (Fig. 6, Table 2). It is therefore most likely that males and females view the colored world quite differently. The eyes’ set of spectral photoreceptors provides an animal with the ability to see color and/or to discriminate light sources that differ in wavelength. Extensive studies of wavelength discrimination in the honeybee Apis mellifera (von Helversen, 1972) demonstrated that the threshold for wavelength discrimination (that is, the minimal discriminable wavelength difference) is the lowest at wavelengths in between the sensitivity peaks of the trichromatic set of photoreceptors (Kelber et al., 2003). The eye of the Japanese yellow swallowtail butterfly, Papilio xuthus, has eight classes of photoreceptor, distributed in three types of ommatidia. Behavioral tests revealed that the wavelength discrimination acuity of P. xuthus is maximal at 420, 480 and 560 nm. Assuming a spectral opponency
mechanism between the spectral receptors through the interphotoreceptor connections in the lamina (Takemura and Arikawa, 2006), we applied the receptor noise-limited color opponent model (Vorobyev and Osorio, 1998) to identify the photoreceptors that were involved in the wavelength discrimination behavior in P. zuthus. We thus found that only four spectral classes of photoreceptors participate in wavelength discrimination, i.e. P. zuthus is tetrachromatic (Koshitaka et al., 2008).

The sexual dimorphism of the spectral photoreceptors of C. erate implies that wavelength discrimination differs between the two sexes. To investigate this wavelength discrimination ability, we have applied the receptor noise-limited color opponent model (Vorobyev and Osorio, 1998) to the C. erate visual system (Fig. 7A). For comparison, we performed the same analysis for the visual system of P. rapae (Fig. 7B). We assumed that all spectral receptors of an eye participate in the wavelength discrimination system. Because in C. erate males the sensitivities of all red receptors peak at 660 nm, wavelengths above approximately 650 nm cannot be distinguished (Fig. 7A, blue line). In contrast, C. erate females have a set of three red receptors, which extends their discriminable range to much longer wavelengths up to approximately 700 nm (Fig. 7A, red line). Both male and female P. rapae have the same two types of red receptor, R (620 nm) and dR (640 nm) (Qiu and Arikawa, 2003). In both sexes wavelength discrimination in the red wavelength range is similar, extending to about 670 nm (Fig. 7B). Clearly, C. erate females should have the best spectral resolution in the longer wavelength range.

### Table 2. Characteristics of the three types of ommatidia of the Colias erate ventral eye

<table>
<thead>
<tr>
<th>Type</th>
<th>Pigment</th>
<th>Fluorescence</th>
<th>R1</th>
<th>R2</th>
<th>R3, 4</th>
<th>R5–8</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>♂</td>
<td>Red</td>
<td>+</td>
<td>UV</td>
<td>sB</td>
<td>bG</td>
</tr>
<tr>
<td></td>
<td>♀</td>
<td>Red</td>
<td>-</td>
<td>UV</td>
<td>V</td>
<td>G</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CeUV</td>
<td>CeV1+V2</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>♂</td>
<td>Red</td>
<td>-</td>
<td>bB</td>
<td></td>
<td>G</td>
</tr>
<tr>
<td></td>
<td>♀</td>
<td>Orange</td>
<td>+</td>
<td>nB</td>
<td></td>
<td>GfII</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CeV1+V2+B</td>
<td></td>
<td>CeL</td>
</tr>
<tr>
<td>III</td>
<td>♂</td>
<td>Red</td>
<td>-</td>
<td>UV</td>
<td></td>
<td>G</td>
</tr>
<tr>
<td></td>
<td>♀</td>
<td>Red</td>
<td>-</td>
<td>UV</td>
<td></td>
<td>G</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CeUV</td>
<td></td>
<td>CeL</td>
</tr>
</tbody>
</table>

Photoreceptor nomenclature: UV, ultraviolet; V, violet; sB, shouldered-blue; bB, broad-blue; G, green; bG, broad-green; GfII, green in female type II ommatidia; Rm(f)I(II,III), red of male (female) type I (II, III) ommatidia. See Fig. 6 for the spectral sensitivity curves. For opsins, see Awata et al. (Awata et al., 2009) and Ogawa et al. (Ogawa et al., 2012).
Red receptors are presumably crucial for female lycaenids to identify larval food sources (Bernard and Remington, 1991). Similarly, the ovipositing Australian orchid butterfly, *Papilio aegeus*, uses a red receptor peaking at 610 nm to select young green leaves as being the best food for larvae (Kelber, 1999a). These examples suggest the hypothesis that the three red receptors of *Colias* females are highly beneficial for detecting suitable leaves for oviposition. The light reflected by leaves is highest in the green and red ranges, but weaker additional reflections occur at shorter wavelengths. The sensitivity bands of the photoreceptors in the short- and middle-wavelength ranges (Fig. 5) will hence create a non-negligible background signal, but the high red sensitivity – especially the strongly red-shifted spectral sensitivity of the proximal photoreceptors in female type III ommatidia – will enable the discrimination of subtle differences in leaf coloration. Some of the spectral receptors of *C. erate* have high polarization sensitivity as well (Pirih et al., 2010), which might enhance the spectral discrimination as in the case of *Papilio* (Kelber, 1999b). Of course, not all spectral receptors necessarily contribute to the wavelength discrimination process, as is exemplified by *P. xuthus* (Koshitaka et al., 2008). Elucidation of the actual wavelength discrimination ability of *Colias* will require behavioral experiments.

Regionalization and sexual dimorphism of the *Colias erate* retina

The eye of *C. erate* has a clear discontinuity between the dorsal third and the ventral two-thirds (Awata et al., 2009). The dorsal region is characterized by its light pigmentation (Arikawa et al., 2009) and the absence of the blue-absorbing visual pigment CeB (Ogawa et al., 2012). We have performed some preliminary studies to investigate whether the dorsal photoreceptors are also sexually dimorphic. However, intracellular recordings did not reveal any sexual dimorphism in the distal nor proximal tiers of the dorsal retina. The proximal photoreceptors in the dorsal region of both sexes were maximally sensitive at 600–620 nm, and the spectral sensitivity curves had no secondary peak sensitivities in the shorter wavelength range (cf. Fig. 5E). This is presumably due to the low density of perirhabdomal pigments, resulting in only moderate filtering. The experimental results indicated that the dorsal eyes of both males and females have the same, rather simple set of UV, V, G and R receptors. Possibly, the visual system of the dorsal retina mediates general visual activities, such as flight control and evading predators. The ventral retina may have evolved to mediate sexual behaviors including courtship and oviposition.

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AUTHOR CONTRIBUTIONS

Y.O. designed the study, performed all the experiments and wrote the paper. M.K. contributed to the design of the study and performed electrophysiological and histological experiments in part. D.G.S. measured the spectra shown in Fig. 3C, and contributed to the modeling of photoreceptor spectral sensitivities and the writing of the paper. K.A. contributed to the design of the study, performed histology in part and contributed to the writing of the paper. This work is a part of the PhD thesis of Y.O.

COMPETING INTERESTS

No competing interests declared.

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