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Published in:

Journal of comparative physiology a-Neuroethology sensory neural and behavioral physiology

DOI:

[10.1007/s00359-019-01330-8](https://doi.org/10.1007/s00359-019-01330-8)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version

Publisher's PDF, also known as Version of record

Publication date:

2019

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Blake, A. J., Pirih, P., Qiu, X., Arikawa, K., & Gries, G. (2019). Compound eyes of the small white butterfly *Pieris rapae* have three distinct classes of red photoreceptors. *Journal of comparative physiology a-Neuroethology sensory neural and behavioral physiology*, 205(4), 553-565. <https://doi.org/10.1007/s00359-019-01330-8>

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Compound eyes of the small white butterfly *Pieris rapae* have three distinct classes of red photoreceptors

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Received: 29 October 2018 / Revised: 25 February 2019 / Accepted: 18 March 2019 / Published online: 24 May 2019
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Abstract

The two subspecies of the small white butterfly, the European *Pieris rapae rapae* and the Asian *P. r. crucivora*, differ in wing colouration. Under ultraviolet light, the wings of both male and female *P. r. rapae* appear dark, whereas the wings of male *P. r. crucivora* are dark and those of females are bright. It has been hypothesized that these sexually dimorphic wing reflections in *P. r. crucivora* may have induced the evolution of a fluorescing-screening pigment in the violet-opsin-expressing photoreceptors of males, thus facilitating greater wavelength discrimination near 400 nm. Comparing the compound eyes of the two subspecies using genetic, microscopical, spectrographic, and histological methods revealed no differences that would meaningfully affect photoreceptor sensitivity, suggesting that the fluorescing-screening pigment did not evolve in response to sexually dimorphic wing reflections. Our investigation further revealed that (i) the peri-rhabdomal reddish-screening pigments differ among the three ommatidial types; (ii) each of the ommatidial types exhibits a unique class of red photoreceptor with a distinct spectral peak; and (iii) the blue, green, and red photoreceptors of *P. rapae* exhibit a polarization sensitivity > 2, with red photoreceptors allowing for a two-channel opponency form of polarization sensitivity.

Keywords Spectral sensitivity · Polarization sensitivity · Fluorescence · Pigments · Phylogeny

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00359-019-01330-8>) contains supplementary material, which is available to authorized users.

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Introduction

The compound eyes of many insects are composed of thousands of ommatidia. These ommatidia are often not uniform across the eye and differ in the expression of visual pigments in a stochastic mosaic governed by a single transcription factor (Perry et al. 2016). The ventral compound eye of the Asian subspecies of the small white butterfly, *Pieris rapae crucivora* Boisduval 1836 (Pieridae), exhibits a mosaic of three ommatidial types (Qiu et al. 2002) which are most readily distinguished by the trapezoidal (I), square (II), or rectangular (III) arrangement of pigment clusters surrounding the rhabdom. As is typical of pierid and papilionid butterflies, the distal, proximal, and basal parts of the fused rhabdom are composed of the photoreceptors R1–4, R5–8, and R9, respectively (Fig. 1; Shimohigashi and Tominaga 1991). The R1–4 photoreceptors express four different rhodopsins (PrUV, PrV, PrB, PrL) which absorb maximally in the ultraviolet (UV), violet, blue, and green-yellow wavelength ranges, respectively; all R5–8 photoreceptors (and presumably also R9) express PrL (Table 1; Wakakuwa et al. 2004; Arikawa et al. 2005). Remarkably, however, intracellular recordings have revealed at least eight spectral classes

Fig. 1 Anatomy of the ommatidia in the compound eye (fronto-ventral part) of *Pieris rapae rapae*. **a** Diagram of the tiered ommatidium showing the position of the nine photoreceptors. The length of the corneal lens and crystalline cones is ~100 μm , with the rhabdom extending between 200 and 600 μm depending on the specimen's sex and the position in the compound eye. The rhabdom is composed of microvilli from photoreceptors R1–4 in the distal half of the ommatidium and from photoreceptors R5–8 in the proximal half of the ommatidium. Only in the very basal part of the ommatidium does photoreceptor R9 contribute microvilli. Clusters of peri-rhabdomal pigment exist in the somata in the upper two-thirds of the proximal photoreceptors R5–8. The horizontal bold line indicates the depth level of the histological sections shown on the right. **b**, **c** Sections of the compound eyes of a female and a male *P. r. rapae*, respectively. The pigment colour and the arrangement of pigment clusters differ among ommatidial types. Pale-orange (solid circle), deep-pinkish (dotted circle), and pale-red pigments (dashed circle) are arranged in trapezoidal, square, and rectangle clusters in type I, II, and III ommatidia, respectively. Scale bar: 10 μm

of photoreceptors (Qiu and Arikawa 2003a, b; Arikawa et al. 2005).

As the rhodopsin molecules of a photoreceptor reside in its rhabdomere, the spectral sensitivity of the photoreceptor is determined by how much of the light flux propagating in the rhabdom its rhodopsin molecules absorb at each wavelength (Snyder 1979; Stavenga 2006). The rhabdom acts

as an optical waveguide, and therefore the light flux does not fully propagate inside the rhabdom boundary. The part outside the boundary encounters the rhabdom-surrounding pigment clusters that thus act as spectral filters and consequently affect the spectral sensitivity of the photoreceptors. This holds specifically for those photoreceptors (R5–9) that contribute microvilli in the proximal and basal parts of the rhabdom (Qiu et al. 2002; Arikawa et al. 2005; Stavenga and Arikawa 2011).

Microspectrophotometry on histological sections identified two peri-rhabdomal pigment types. Pale-red pigment clusters were found in ommatidial types I and III, and deep-red pigment in ommatidial type II (Stavenga and Arikawa 2011). This finding agreed with observations of the eyeshine observable in live butterflies studied under epi-illumination light microscopy (Qiu et al. 2002). Eyeshine in butterfly eyes is created by a strongly folded tracheole, the tapetum, that exists below each rhabdom and that acts as an interference reflector. Incident light that has propagated down to the basal end of the rhabdom, without having been absorbed by either the rhodopsins in the rhabdom or the surrounding pigment clusters, is reflected by the tapetum and then travels back through the rhabdom, eventually exiting the ommatidium through its facet lens. The eyeshine colour has been characterized as either pale-red or deep-red, corresponding well to the colour of the pigment clusters that surrounds the rhabdoms (Qiu et al. 2002; Stavenga and Arikawa 2011).

Fluorescence microscopy with a blue-violet excitation light revealed the presence, exclusively in males, of a distinctly fluorescing pigment in type II ommatidia. This pigment was shown to act as a violet-absorbing pigment on the R1 and R2 photoreceptors. These photoreceptors express the PrV rhodopsin, so that in females they are violet-sensitive photoreceptors, but the filtering effect of the pigment results in the double-peaked blue photoreceptors of males (Fig. 2b; Table 1; Arikawa et al. 2005). Similarly, in *Papilio* a fluorescing pigment, presumably 3-hydroxyretinol, narrows the sensitivity of a UV opsin-expressing photoreceptor to create a narrow-band violet receptor (Arikawa et al. 1999a). However, the identity of the pigment in *P. rapae* remains elusive as its peak absorbance of ~420 nm contrasts that of 3-hydroxyretinol (~320 nm) (Arikawa et al. 2005).

The sexual differences in the eye pigmentation of *P. r. crucivora* might be related to the marked sexual dimorphism in UV reflections from their wings (Fig. 2a). The reflectance of male and female wings in the UV wavelength range is low and high, respectively. In contrast, in the European subspecies of *P. rapae* (Linnaeus, 1758), *P. r. rapae* (Linnaeus, 1758), the UV reflectance of both male and female wings is low (Obara 1970; Obara and Majerus 2000; Giraldo and Stavenga 2007). As a behavioural “flutter response”, used by resting males to deter approaching males, is present in both subspecies, the UV reflectance of *P. r. rapae* wings is likely

Table 1 Summary of the three ommatidial types in *Pieris rapae*

Ommatidial type	I	III ^a	IIm ^a	III												
Rhabdom shape	Trapezoid	Square	Square	Rectangular												
Eye shine/ reflection $\lambda_{\max}$ (nm)	Pale-red/~640	Deep-red/~680	Deep-red/~680	Pale-red/~660												
Pigment colour ^b / absorption $\lambda_{\max}$ (nm)	Pale-orange/~487–503	Deep-pinkish-red/~494	Deep-pinkish-red/~485	Pale-red/~472–489												
Fluorescence	Weak (R1 or R2)	None	Strong (R1 and R2)	None												
Photoreceptor	$S(\lambda)/\lambda_{\max}$ (nm)	Opsin	PS	$\phi_{\max}$ (°)	$S(\lambda)/\lambda_{\max}$ (nm)	Opsin	PS	$\phi_{\max}$ (°)	$S(\lambda)/\lambda_{\max}$ (nm)	Opsin	PS	$\phi_{\max}$ (°)				
R1	UV ^c /~350	PrUV	1.1	NA	V/~400	PrV	1.2	7	dB/~460	PrV	1.3	9	UV/~350	PrUV	1.1	NA
R2	B ^c /~450	PrB	2.9	6	V/~400	PrV	1.2	7	dB/~460	PrV	1.3	9	UV/~350	PrUV	1.1	NA
R3,4	G/~560	PrL	1.9	95	G/~560	PrL	1.3 ^d	91 ^d	dG/~560	PrL	1.3	91	G/~560	PrL	1.9	95
R5,7	Ri/~610	PrL	2.2	155	Rii/~640	PrL	1.9 ^d	131 ^d	Rii/~640	PrL	1.9	131	Riii/~630	PrL	2.1	156
R6,8	Ri/~610	PrL	2.2	34	Rii/~640	PrL	1.9 ^d	52 ^d	Rii/~640	PrL	1.9	52	Riii/~630	PrL	2.1	33
R9	R?	PrL?	?	?	R?	PrL?	?	?	R?	PrL?	?	?	R?	PrL?	?	?

This table compiles published information on *P. r. crucivora* (see Table 1, Stavenga, and Arikawa 2011), new observations of *P. r. rapae*, and a re-examination of electrophysiological recordings (some previously unpublished) from *P. r. crucivora* eyes

$S(\lambda)$ photoreceptor class as derived from the wavelength range of its spectral sensitivity, UV ultraviolet, V violet, dB double-peaked blue, B blue, G green, dG blue-suppressed green (Stavenga and Arikawa 2011), Ri red with peak wavelength $\lambda_{\max}$ ~610 nm, Rii red with $\lambda_{\max}$ ~640 nm, Riii red with $\lambda_{\max}$ ~630 nm, PS polarization sensitivity, $\phi_{\max}$ angle of maximal polarization sensitivity

^aIIf—type II ommatidia of females, IIm—type II ommatidia of males

^bThe pigments were previously reported for type I and type III ommatidia as pale-red, and for type II ommatidia as deep-red (Qiu et al. 2003b)

^cThe R1 and R2 photoreceptors of type I ommatidia contain either the UV and B rhodopsins (narrow trapezoidal side up) or the B and UV rhodopsins (wide trapezoidal side up), see Fig. 1

^dPS and $\phi_{\max}$ (°) values for females were inferred from electrophysiology recordings of males

Fig. 2 **a** Diagram showing the appearance of the dorsal wing surface of male and female *Pieris rapae crucivora* and *Pieris rapae rapae* in conventional (human visible) photographs (left) and in UV photographs (right), based on photographs from Obara and Majerus (2000). **b** Comparison of the spectral sensitivity of the PrV opsin, expressing double-peaked blue and violet R1,2 photoreceptors in male and female *P. r. crucivora*

the ancestral state (Obara and Majerus 2000). It therefore has been hypothesized that the divergent spectral sensitivity of the double-peaked blue photoreceptors of *P. r. crucivora* males (Fig. 2b) is an adaptation that enables greater wavelength discrimination near 400 nm, thereby improving the males' ability to discriminate females from males based on differential UV reflectance of their wings (Arikawa et al. 2005).

To test this hypothesis, we performed a comparative study of the compound eyes of *P. r. rapae* and *P. r. crucivora*, using a combination of genetics, spectrophotometry, electron microscopy, and in vivo eyeshine observations coupled with histological localization. Most of these data were available for *P. r. crucivora* but additional spectrophotometry, histology, and microscopy measurements were necessary for *P. r. rapae*. Our findings also prompted a re-examination of previously obtained electrophysiological recordings of *P. r.*

crucivora photoreceptors that yielded spectral and polarization sensitivities.

Materials and methods

Experimental insects

Males and females of *P. r. crucivora* were taken from a laboratory colony started from eggs laid by females collected around the campus of Yokohama City University. Larvae were reared on fresh kale leaves at 19 °C under a photoperiod of 8:16 h (L:D). Pupae were stored at 4 °C for at least 3 months before adults were allowed to eclose at 25 °C. Adults were used in experiments within 4 days of eclosion.

Males and females of *P. r. rapae* were taken from a laboratory colony started from eggs procured from Carolina Biological Supply Company (Item # 144102; NC, USA). Larvae were reared on a wheat-germ casein diet. Adults were allowed to eclose from pupae that were kept at room temperature or held at 4 °C for up to 2 weeks (Webb and Shelton 1988). Both larvae and adults were held in a rearing room with a temperature and light regime of 18–24 °C and 8:16 h L:D.

P. r. rapae opsin sequences

The complete genome of *P. r. rapae* was recently sequenced and annotated (Shen et al. 2016). MEGA7 (Kumar et al. 2016) was deployed to generate a maximum likelihood phylogenetic tree of opsins from an alignment of the *P. r. rapae* sequences and all available full opsin protein sequences from the Pieridae.

Histology

To examine ommatidial heterogeneity in pigment clusters, isolated eyes were fixed at room temperature for 30 min in 4% paraformaldehyde (PA) in a 0.1 M sodium cacodylate buffer (CB) with a pH of 7.4. Eyes were then dehydrated in a graded series of acetone, infiltrated with propylene oxide, and embedded in Epon. Unstained transverse sections of 5–10 µm thickness were observed and photographed with a regular transmission light microscope.

Eyes prepared for electron microscopy were fixed with 2.5% glutaraldehyde and 2% PA in 0.1 M CB at 4 °C overnight, post-fixed in 2% OsO₄ in 0.1 M CB for 2 h at room temperature, and embedded as described above. Ultra-thin sections were prepared and stained with 1% uranyl acetate prior to observations with a transmission electron microscope (H7600 or H7650, Hitachi, Tokyo).

Eyeshine and ommatidial fluorescence

The spatial arrangement of ommatidial types, both in the ventral and dorsal eye regions, was investigated in the eyes of living butterflies, using epi-illumination with a modified telemicroscopic optical assembly (Stavenga 2002) equipped with a 50% beam splitter, a long working distance air objective (LUCplanFLN20X, NA 0.45, WD 6.4–7.6 mm, Olympus, Tokyo, JP), and a monochrome digital camera (Chameleon3, CM3-U3-31S4M-CS, Point Grey/FLIR BC, CA). The light source, a 500 W Xenon lamp, was bandpass-filtered between 500 and 710 nm (20 nm bandwidth; Asahi Spectra, Tokyo, JP). Ommatidial fluorescence was recorded by replacing the beam splitter with standard fluorescence cubes (U-MWU, U-MWBV; Olympus).

Reflectance and fluorescence measurements from four eyes of three females and five eyes of three males, with 100–450 ommatidial measurements per eye, were used to classify the ommatidia into groups using *k*-means clustering (Hartigan and Wong 1979). After the *in vivo* measurements, the eyes were fixed, sectioned, and observed under a light microscope. The histology indicated four distinct ommatidial types (dorsal; ventral types I–III), which mapped well with the ommatidial groups derived by *k*-means clustering.

Absorbance spectra of the reddish screening pigments

Absorbance spectra of the reddish peri-rhabdomal screening pigments were obtained from unstained transverse eye sections of both male and female *P. r. rapae*. Hyperspectral imaging was performed using (i) a modified Zeiss Axioskop 2 FS microscope with a fixed tube lens (f165 mm), (ii) a motorized *z*-stage and either a Zeiss Neofluar 40×0.85 dry objective or a Zeiss Ultrafluor 40×0.60 immersion objective, and (iii) a monochrome camera (Blackfly 23S6M; IMX249, pixel size 5.86 µm or Flea 32S2M; IMX036, pixel size 2.5 µm; FLIR/PointGrey). The light source was a PTI Deltascan 4000 system with a 75 W XBO lamp and a motorized monochromator. A quartz fiber (800 µm, NA 0.22) connected the light source to an achromatic condensor (NA 0.9). Hyperspectral image stacks of the sections were taken in the range of 360–730 nm. Prior to taking the stacks, the best focus position at each wavelength was determined by maximizing the variance of subimage blocks of a calibration slide. Using Fiji (Schinderlin et al. 2012), the measured stack was divided with a reference stack taken in the same microscope slide, yielding a transmittance stack, and was then registered using the StackReg plugin (Thévenaz et al. 1998). In each section, the transmission of the reddish screening pigments of 10–64 ommatidia of each type were taken and used to calculate non-normalized and normalized absorbance spectra.

Electrophysiology

Intracellular recordings of the spectral sensitivities of *P. r. crucivora* photoreceptors reported by Qiu and Arikawa (2003a, b) and Arikawa et al. (2005), together with unpublished recordings lacking histological identification, were all re-analysed using the originally recorded response voltages. Previous electrophysiological analyses were constrained to recordings with histological localization. All available recordings with maximal response amplitude > 30 mV were included, and the spectral class and (in most cases) ommatidial type of these recordings were inferred by comparing their spectral sensitivity with that of marked photoreceptors. In our analyses, red photoreceptors were assigned to three rather than two classes (Qiu et al. 2002), based on ommatidial type. The procedure for intracellular recordings has been described in detail (Qiu and Arikawa 2003a; Arikawa et al. 2005) and is only outlined below. Isoquantal monochromatic 30 ms stimuli were generated by a 500 W Xenon arc lamp directed through a series of narrow-band interference filters and a neutral density wedge. The light beam was focused on the tip of an optical fiber that was attached to the perimeter device, where it provided a point source of light (diameter 0.6°). The spectral sensitivity of the recorded photoreceptor was determined by stimulating the cell with a series of monochromatic flashes (300–700 nm). The stimulus intensity–response function was measured at the peak wavelength ($\lambda_{\max}$) over an intensity range of 5 log units. For most cells, the polarization sensitivity ratio (PS: maximal sensitivity/minimal sensitivity) at the $\lambda_{\max}$ was also measured. An ultraviolet-capable polarizer was inserted in front of the eye and rotated through 360° to produce flashes varying in axis of polarization from 0 to 180°. A sinusoidal curve was fitted to the data, yielding the PS and the axis of peak sensitivity to linearly polarized light, $\phi_{\max}$. The presented PS measurements for all receptor classes, except for the female violet class, are from male *P. r. crucivora*.

Results

Opsin sequences of *P. r. rapae* and *P. r. crucivora*

The amino acid sequences of *P. r. rapae* opsins deduced from genomic sequences (Shen et al. 2016) closely resembled those of *P. r. crucivora*, deviating by zero to three amino acids, and grouped tightly when the phylogeny of these sequences was reconstructed (Fig. 3). None of the amino acid differences occurred in regions identified as important for spectral tuning (Salcedo et al. 2003; Wakakuwa et al. 2010). Based on the similarity in opsin sequences between *P. r. rapae* and *P. r. crucivora*, the absorbance of their opsins can be inferred to be nearly identical.

Fig. 3 Phylogeny of opsin amino acid sequences in the Pieridae, as determined by maximum likelihood analysis. The numbers at nodes indicate the maximum likelihood bootstrap values. Accession numbers, opsin type [UV, Violet (V, V1, V2), Blue (B), Long (L)], and absorption peak wavelength, $\lambda_{\max}$ (where available), are listed with species names

Ommatidial heterogeneity in *P. r. rapae*

Light- and electron-microscopic examination of the ommatidial structures of *P. r. rapae* and *P. r. crucivora* did not reveal any differences between the subspecies in the arrangement of microvilli or pigment clusters that would significantly affect photoreceptor sensitivity (Figs. 1, 4; Qiu et al. 2002). As in *P. r. crucivora* (Arikawa et al.

2005), ommatidial structures of *P. r. rapae* were found not to be sexually dimorphic (Fig. 4, female ommatidia; Fig. S1, male ommatidia).

The clusters of pigment surrounding the rhabdoms of *P. r. crucivora* have previously been divided into two pigment classes: pale-red and deep-red (Qiu et al. 2002). However, careful observation of unstained light microscopic sections of *P. r. rapae* and *P. r. crucivora* compound eyes revealed that both subspecies appear to have three pigment classes, pale-orange, deep-pinkish-red, and pale-red, located in ommatidial types I, II, and III, respectively (Fig. 1).

Eyeshine in *P. r. rapae*

The spectral composition of the light reflected from the ommatidia is affected both by the perirhabdomal screening pigments and by the tuning of the tapetum. In both males (Fig. 5) and females (Fig. S2), there were distinct differences in the eyeshine of the three ventral ommatidial types. Under monochromatic illumination, type I ommatidia appeared bright under 620 nm light, type II ommatidia under 670 nm light, and type III ommatidia appeared bright both under 670 nm and 690 nm light. The reflectance $\lambda_{\max}$ of ommatidial types I, II, and III was ~640 nm, 680 nm, and 660 nm, respectively (Fig. 6). Variation in peak ommatidial reflectance among eyes was small relative to variation among ommatidia in a single eye. However, these measurements were taken from a single laboratory colony and greater variation among or within wild populations is possible. We did not compare the reflectance between the two subspecies or between the reared and wild animals.

The dorsal area of *P. rapae* eyes also has a prominent eyeshine but its colour varies between green and red (see Fig. 2c in Stavenga 2002). The mean reflectance spectrum of the dorsal eye area peaks at ~610 nm (Fig. 6). It is likely that the low reflectance below 610 nm is chiefly determined by the absorption of visual pigments, as the dorsal ommatidia are devoid of the red screening pigments (Qiu and Arikawa 2003a).

Absorbance spectra of reddish screening pigments

The absorbance spectra of reddish screening pigments of male and female *P. r. rapae* differed among ommatidial types, with type II ommatidia having the greatest absorbance followed by type III and I (Fig. 7a, b). Additionally, type II pigments had greater absorbance in the red wavelength range of the spectrum (Fig. 7c, d), with only minor differences between the normalized spectra of type I and III ommatidia.

Fig. 4 Electron micrographs of transverse sections of female *Pieris rapae rapae* rhabdoms. **a, d, g** Ommatidial type (I) **b, e, h** Ommatidial type (II) **c, f, i** Ommatidial type (III). **a–c** Depth from corneal surface: ~165 μm . **d–f** Depth ~210 μm . **g–i** Depth ~250 μm . **j** Depth ~335 μm . Numbers in **b** and **j** refer to the photoreceptor number. **b** R—rhabdom. Scale bar (all panels): 1 μm

Ommatidial fluorescence

Like eyeshine observations, fluorescence microscopy offers a powerful means to distinguish ommatidial types. Type II ommatidia of both male *P. r. rapae* and *P. r. crucivora* exhibited a strong blue-violet and ultraviolet light-induced fluorescence (Fig. 5). The fluorescence in ommatidial types I and III of males was weak, resembling that of all ommatidial types of females (Figs. 5d, e, S2d, e).

Re-examination of spectral sensitivity in *P. r. crucivora*

Our adjusted view that the three ommatidial types differ in fluorescence characteristics and the composition of

screening pigments prompted us to re-analyse the previously reported photoreceptor spectral sensitivities (Qiu and Arikawa 2003a, b; Arikawa et al. 2005). We concluded that the spectral sensitivities of the red photoreceptors were distinct in each of the ommatidial types (Fig. 8), in line with the eyeshine and pigment absorption measurements (Figs. 6, 7). The red photoreceptors in the three ommatidial types were determined to have sensitivity peaks (λ_{max}) at around 610 nm (Ri; type I), 640 nm (Rii; type II), and 630 nm (Riii; type III). Furthermore, compared to Ri, the Riii photoreceptors have a greater sensitivity in the 400–500 nm range, and the Rii photoreceptors of males have a sensitivity trough near 420 nm, due to the distally located, fluorescing, and violet-absorbing pigment, which acts as a violet spectral filter (Qiu et al. 2002; Stavenga and Arikawa 2011).

Fig. 5 Eyeshine, fluorescence, and histology of the same set of ommatidia (encircled by the light blue polygons) of a male *Pieris rapae rapae*. **a–c** Local eyeshine elicited by epi-illumination with 620 nm, 670 nm, and 690 nm light, respectively. **d, e** Fluorescence induced by blue-violet and ultraviolet light, respectively. **f** Diagram of the arrangement of different ommatidial types: orange—type I, pink—type II, red—type III; the black circles indicate ommatidia dark in both eyeshine and fluorescence. **g** Transverse section of local ommatidia showing pigment clusters. Scale bars: 20 μm

Polarization sensitivity in *P. r. crucivora*

The photoreceptors of *P. r. crucivora* have different spectral and polarization sensitivities, as summarized in Fig. 9 and Table 1. The UV-sensitive photoreceptors (R1,2) in ommatidial types I and III have a similar ($t=2.19$, $df=6.95$, $p=0.07$) but very low PS of ~ 1 . In contrast, the blue-sensitive photoreceptors (R1,2) in type I ommatidia have a high

Fig. 6 Reflectance spectra (normalized) of *Pieris rapae rapae* ommatidia. **a, b** Mean $\pm$ the standard error of normalized reflectance spectra for ommatidial types I–III in the ventral eye region and of ommatidia in the dorsal eye region of *P. r. rapae* females and males, respectively

PS of ~ 3 , with an approximately vertical axis of maximal polarisation sensitivity ($\phi_{\text{max}} \sim 0^\circ$), i.e., about parallel to the body's dorso-ventral plane. The green-sensitive photoreceptors (R3,4) in type I and III ommatidia have a similar PS ($t=1.87$, $df=3.34$, $p=0.15$) of ~ 2 with mostly a horizontal ϕ_{max} ($\sim 90^\circ$). The blue-suppressed green photoreceptors (R3,4) in type II ommatidia have a PS of ~ 1.3 which is lower than that of green photoreceptors (R3,4) in ommatidial type I and III. Red-sensitive photoreceptors (R5–8) show a PS of ~ 2 in all three ommatidial types, but the mostly oblique ϕ_{max} differs between type II and types I and III, with the axes of polarization shifted towards vertical in types I and III (Fig. 9; Table 1).

Discussion

Comparison of the compound eyes of *P. r. crucivora* and *P. r. rapae*

Our data support the conclusion that the compound eyes of *P. r. rapae* and *P. r. crucivora* are (nearly) identical. Between the two subspecies, there are only minimal differences in (a) opsin sequences (implying functionally identical visual pigments), (b) eyeshine, (c) distributions of blue-violet fluorescing pigment among ommatidial types and sexes, and (d) microvillar arrangements (implying similar polarization sensitivities). In particular, observations of fluorescence

Fig. 7 Absorbance spectra of red screening pigments in the ommatidia of *Pieris rapae rapae*. The measurements for types I–III are from a transverse section of the compound eye at a position along the rhabdom where all three ommatidial types express screening pigments. The mean non-normalized absorbance for each ommatidial

type from a male (a) and female (c) is shown on the left with error bars showing quartiles. The mean normalized absorbance along with peak wavelength ($\lambda_{\max}$) is shown on the right for both male (b) and female (d)

strongly suggest both *P. r. rapae* and *P. r. crucivora* are sexually dimorphic in the spectral sensitivity of their type II R1,2 photoreceptors, implying that this dimorphism is an ancestral trait in both subspecies rather than an adaptation in *P. r. crucivora* to enhance discrimination between males and females. We did not perform intracellular recordings on *P. r. rapae* eyes, but given the similarity in both visual and screening pigments as well as in microvillar arrangement, photoreceptor classes in both subspecies can be expected to have similar spectral and polarization sensitivities.

Ommatidial heterogeneity of red-screening pigments

Spectrophotometry on the photoreceptor screening pigments revealed distinctly different absorbance spectra for the three ommatidial types of *P. r. rapae*. The absorbance of pigments in *P. r. rapae* differs from that previously reported for *P. r. crucivora*. The only subtle differences in pigment appearance and eyeshine reflectance between the two subspecies are likely attributable to diverging methods used to determine absorbance in each subspecies. We used colony-derived *P. r. rapae* adults measuring their

eyes with an enhanced imaging microspectrophotometry (MSP) method (Arikawa et al. 2009), whereas previous studies used wild-caught *P. r. crucivora*, and non-imaging MSP methods. These measurement also did not distinguish between type I and III ommatidia (Qiu et al. 2002; Stavenga and Arikawa 2011).

The MSP of histological sections from the compound eyes of *Papilio xuthus* and female *Colias erate* demonstrated the presence of two classes of peri-rhabdomal screening pigments (Arikawa et al. 1999b; Ogawa et al. 2013). *Pieris rapae* seems to be the first documented example of a butterfly with differences among screening pigments in all three ommatidial types (at least in terms of pigment density), although imaging MSP of male *C. erate* ommatidia have shown some small differences in pigment density among ommatidial types. Females of *C. erate* possess three classes of red photoreceptors with different $\lambda_{\max}$ but optical models suggest only two screening pigments (Ogawa et al. 2013). Possible differences in pigment density between ommatidial types may play a role in spectral tuning not only in *P. rapae* but also in *C. erate*.

Butterflies use red photoreceptors to help discern green and yellow leaves, the latter often senescing and

Fig. 8 Differences in spectral sensitivities of photoreceptors among ommatidial types in *Pieris rapae crucivora*. Lines indicate the mean spectral sensitivity among recordings $\pm$ the standard error. **a** Type I ommatidia. **b** Type II ommatidia in females. **c** Type III ommatidia. **d** Type II ommatidia in males. No electrophysiological recordings were available for female type II green or red photoreceptors; shown instead (**b** dotted lines) are predicted spectral sensitivities from a wave-optical model of visual and screening pigment absorbance within the *Pieris rapae* ommatidium (Stavenga and Arikawa 2011)

suboptimal for oviposition and larval development (Kelber 1999, 2001). As optimal wavelength discrimination is achieved by overlapping sensitivity between photoreceptor classes (Kelber et al. 2003), the additional overlap in sensitivity between three, rather than two, classes of red photoreceptors could further enhance wavelength discrimination in the > 600 nm range, thereby improving perception of subtle differences in leaf colour. Wavelength discrimination has been predicted for *P. rapae* and *C. erate* with receptor noise-limited colour opponent models (Vorobyev and Osorio 1998) that assumed all photoreceptor classes contribute to discrimination (Ogawa et al. 2013). The selection pressure for discerning wavelengths > 600 nm should be greatest for gravid females seeking host plants. Yet, only females of *C. erate*, but not *P. rapae*, possess more red photoreceptor classes than males and thus are likely to have a greater discriminatory ability than their male counterparts for wavelengths > 600 nm. The selective forces that led to multiple classes of red photoreceptor in male *P. rapae* but not in male *C. erate* are not yet known.

Polarization sensitivities of photoreceptors

Visual pigments in insect photoreceptors are bound to membranes of the microvilli composing the rhabdom (Horváth and Varjú 2004). Consequently, light polarized in a direction

parallel to the microvilli is more easily absorbed by these pigments resulting in polarization sensitivity. The alignment of a photoreceptor's microvilli affects its sensitivity to polarized light, with curving of the microvilli serving to degrade this sensitivity.

Electron micrographs of the upper part of the rhabdom of *P. r. rapae* and *P. r. crucivora* ommatidia seem to reveal a greater degree of curving in the microvilli of R3,4 photoreceptors in type II ommatidia than in type I and III ommatidia. This microvillar arrangement would explain the high PS of the green photoreceptors (R3,4) in type I and III ommatidia relative to type II ommatidia. The PS remains high in type I and III green photoreceptors despite the curving of microvilli in the middle part of the rhabdom.

The higher PS (~ 2) of the red photoreceptors (R5–8) is expected given their parallel microvilli (Qiu et al. 2002); however, the difference in $\phi_{\max}$ of red photoreceptors among ommatidial types was surprising considering the ommatidial heterogeneity in the microvillar arrangement of photoreceptors R5–8. Type II and III red photoreceptors have $\phi_{\max}$ values in line with microvillar orientations, near $45/135^\circ$ for the square type II and closer to vertical for the rectangular type III. In contrast, the type I red photoreceptors despite their trapezoidal arrangement also have $\phi_{\max}$ values shifted to the vertical. While the arrangement in all ommatidial types is similar below a depth of $420 \mu\text{m}$, one would expect a substantial portion of light to be absorbed by the photoreceptor

Fig. 9 Polarization sensitivity of photoreceptors of the different spectral classes in *Pieris rapae crucivora* as represented by a series of semi-transparent solid lines. The length of these lines indicates the polarization sensitivity (PS), whereas the line's rotation indicates

the axis of polarization where the sensitivity is greatest ($\phi_{\max}$). The labeled dashed circles and lines show the mean PS and $\phi_{\max} \pm$ the standard error, respectively

above this point in the rhabdom (Stavenga and Arikawa 2011) where microvillar arrangement differs.

Further optical modeling of the *P. rapae* compound eye is necessary to discover possible polarizing filter effects of the microvilli of the distal photoreceptors (Snyder 1973). The near-orthogonal arrangement of red photoreceptors within a single class should allow for dipolar polarization opponency (How and Marshall 2014) in *P. rapae*. This opponency mechanism could allow for the discrimination, although with

considerable ambiguity, among objects differing in polarization. If receptor input from type II ommatidia is combined with the input from type I + III ommatidia to a full tripolar mechanism, the polarization ambiguities might be reduced at the expense of introducing some colour ambiguity. Clearly, further studies are needed to determine whether and how *P. rapae* senses, processes, and uses the combined information conveyed in colour and polarization of light.

Acknowledgements We thank Atsuko Matsushita for assistance and instruction with microscopy and histology, and the UBC Bioimaging Facility for assistance with electron microscopy. We also thank Doekele G. Stavenga and two anonymous reviewers who provided most helpful and constructive comments and advice on earlier drafts of this paper.

Compliance with ethical standards

Conflict of interest The NSERC-Industrial Research Chair to GG was supported by Scotts Canada Ltd. as the industrial partner.

Funding This study was supported by an Alexander Graham Bell Canadian Graduate Scholarship to AB, by the KAKENHI of the Japan Society for the Promotion of Science (JSPS; #26251036, 18H05273 to KA; #2604764 to PP), and by an NSERC-Industrial Research Chair to GG.

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