J Exp Biol Advance Online Articles. First posted online on 31 May 2016 as doi:10.1242/jeb.136523
Access the most recent version at http://jeb.biologists.org/lookup/doi/10.1242/jeb.136523

Title: Sexual dimorphism in the compound eye of *Heliconius erato*: a nymphalid butterfly with at least five spectral classes of photoreceptor

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Key Words: rhodopsin, opsin, electrophysiology, *Heliconius*, insect, color vision

Summary Statement

Female *Heliconius erato* butterflies have two UV-sensitive photoreceptors, but males only have one, and both sexes have a red-sensitive photoreceptor.

Abstract

Most butterfly families expand the number of spectrally-distinct photoreceptors in their compound eye by opsin gene duplications together with lateral filter pigments, however most nymphalid genera have limited diversity, with only three or four spectral types of photoreceptor. Here we examine the spatial pattern of opsin expression and photoreceptor spectral sensitivities in *Heliconius erato,* a nymphalid with duplicate ultraviolet opsin genes, *UVRh1* and *UVRh2*. We find that the *H. erato* compound eye is sexually dimorphic. Females express the two UV opsin proteins in separate photoreceptors, but males do not express UVRh1. Intracellular recordings confirmed that females have three short wavelength-sensitive photoreceptors (λ_{max} = 356 nm, ~390 nm and 470 nm), while males have two (λ_{max} = 390 nm and ~470 nm). We also found two long wavelength-sensitive photoreceptors (green, λ_{max} ~555 nm, and red, λ_{max} ~600 nm), which express the same LW opsin. The red cell's shifted sensitivity is probably due to perirhabdomal filtering pigments. Sexual dimorphism of the UV-absorbing rhodopsins may reflect the females' need to discriminate conspecifics from co-mimics. Red-green color vision may be used to detect differences in red coloration on *Heliconius* wings, or for host-plant identification. Among nymphalids so far investigated, only *H. erato* is known to possess five spectral classes of photoreceptor; sexual dimorphism of the eye via suppression of one class of opsin (here UVRh1 in males) has not--to our knowledge--been reported in any animal.

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Introduction

As vision incurs energetic costs (Niven et al., 2007; Niven and Laughlin, 2008; Moran et al., 2015), selective pressure for efficiency leads us to expect that the number of spectral receptor types, their tuning, and the way in which they are distributed across the retinal mosaic will reflect the evolutionary significance of color to an animal. Accordingly, sex differences in color vision might be expected where the sexes forage differently for food, and especially where one sex (normally females) chooses mates by their coloration. It is therefore surprising that within some taxonomic groups for which color is ecologically important, such as old-world primates, birds, and bees, there is little variation in photoreceptor spectral sensitivities between species within a given clade (Osorio and Vorobyev, 2005; Bloch, 2015), or between sexes. Aquatic taxa including teleost fish (Carleton and Kocher, 2001; Bowmaker and Hunt, 2006) and stomatopods (Cronin and Marshall, 1989; Porter et al., 2009) do have substantial spectral diversity of photoreceptors between related species, which can often be related to the spectral variation in ambient illumination in water. Among terrestrial animals dragonflies (Futahashi et al., 2015) and butterflies (Briscoe, 2008) are known for the diversity of their photoreceptor spectral sensitivities, but the evolutionary causes and physiological significance of these differences remain unclear, and there is limited evidence for sexual dimorphism in photoreceptor spectral sensitivities (but see below).

Ancestral holometabolous insects probably had compound eyes with three spectral types of photoreceptor, each containing a unique type of opsin (Briscoe and Chittka, 2001; Henze and Oakley, 2015). The butterfly eye ground plan seems to have retained the ancestral form with three opsins, UV, B, and LW, having sensitivity maxima (λ_{max}) at about 360 nm, 470 nm, and 560 nm respectively (Briscoe and Chittka, 2001; Briscoe, 2008). Butterfly ommatidia contain nine photoreceptor cells R1-R9, whose photosensitive membranes form a fused rhabdom (Fig. 1A-C); (Wernet et al., 2015). In the nomenclature used for butterflies, R1 and R2 cells are long visual fiber (LVF) photoreceptors, which lie on opposite sides of the rhabdom, and typically express either *UVRh* or *BRh* mRNAs (encoding SW opsins) (Fig. 1C). R3-R8 cells are short visual fiber (SVF) photoreceptors, which express *LWRh* mRNAs (Fig. 1C), and the R9 cell is a tiny LVF, which contributes a few microvilli to the proximal tip of the rhabdom (Briscoe, 2008).

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Butterflies are known for the diversity in their photoreceptor spectral sensitivities (Arikawa et al., 1987; Arikawa et al., 2005; Sison-Mangus et al., 2006; Briscoe, 2008; Ogawa et al., 2012). In several butterfly families, this diversity has been achieved by independent increases in the number of spectrally distinct photoreceptors in the adult compound eye, via three mechanisms. First, opsin gene duplication followed by subfunctionalization and spectral tuning produces divergent sensitivities of the resulting photoreceptor cells. Second, photostable lateral filtering pigments typically absorb short wavelengths, narrowing the shape and shifting the peak of a cell's spectral sensitivity toward longer wavelengths without any change in the endogenous opsin sequence or expression level. Finally, two opsins may be expressed together in the same cell to broaden spectral sensitivity.

The swallowtails (Papilionidae) have the greatest known number of photoreceptor spectral sensitivities among butterflies. *Papilio xuthus* uses the three processes above to produce at least eight spectrally-distinct types of photoreceptors (Kitamoto et al., 1998; Arikawa et al., 1999b; Arikawa et al., 1999a; Kitamoto et al., 2000; Arikawa et al., 2003; Stavenga and Arikawa, 2006). *Papilio glaucus* has eight visual opsins, six of which result from LW opsin duplications (Briscoe, 2000; Cong et al., 2015), while another papilionid, the birdwing *Troides aeaca* has nine spectral classes of photoreceptor (Chen et al., 2013). Duplicated LW opsin genes are found in several species including a representative from the basal lineage of the family, *Parnassius glacialis*, suggesting the LW opsin duplication may have been present in the ancestral papilionid (Matsushita et al., 2012).

In other butterfly families, similar mechanisms have resulted in expansions of photoreceptor classes. At least one species in the Riodinidae has duplicated a LW opsin (Frentiu et al., 2007). B opsin gene duplications have resulted in spectrally distinct receptors in the range 435 nm – 500 nm in the family Lycaenidae (Sison-Mangus et al., 2006; Sison-Mangus et al., 2008) and independently in Pieridae. In addition, both *Pieris* and *Colias* (Pieridae) have complex patterns of lateral filtering pigments, which in *Colias erate*, results in nine spectral classes of photoreceptor, including multiple red-sensitive cells (Qiu et al., 2002; Qiu and Arikawa, 2003; Arikawa et al., 2005; Awata et al., 2009; Ogawa et al., 2012; Ogawa et al., 2013).

In contrast to other investigated butterfly families, the photoreceptor spectral sensitivities in the largest butterfly family, Nymphalidae, have not diverged from the ancestral holometablous

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insect form (Briscoe et al., 2003; Sauman et al., 2005; Stalleicken et al., 2006). Nymphalids with three known spectral types of photoreceptor include iconic and cosmopolitan species such as the monarch, *Danaus plexippus,* and the painted lady, *Vanessa cardui*. The satyrine butterfly *Hermeuptychia hermes,* is an exception with a single *LWRh* duplication (Frentiu et al., 2007). Although perirhabdomal filtering pigments are common to many insects, including butterflies (Stavenga, 2002a, b), they seem to be absent from some nymphalid eyes, thus eliminating one way to generate additional photoreceptor spectral sensitivities (Briscoe and Bernard, 2005; Frentiu et al., 2007). The narrower spectral variability in this speciose and colorful group of butterflies highlights the general problem of relating color vision to visual ecology (Osorio and Vorobyev, 2008), where the underlying ecological and evolutionary processes responsible for the observed patterns of visual traits are difficult to identify.

In this context the genus *Heliconius* is of particular interest owing to the presence of two evolutionary innovations (synapomorphies), namely a duplicated UV opsin gene that evolved under positive selection (Briscoe et al. 2010), and the use of the UV-yellow-reflecting molecule, 3-hydroxykynurenine (3-OHK), for wing pigmentation (Brown, 1967; Briscoe et al., 2010; Bybee et al., 2012). Color space modeling predicts that the presence of two UV receptors would be beneficial for discriminating *Heliconius* 3-OHK wing pigmentation from co-mimics belonging to other genera that use a different non-UV reflecting wing pigment (Bybee et al., 2012). Thus, the novel wing pigment and enhanced UV color vision may be important in the success of *Heliconius*, allowing the genus to benefit from defensive mimicry without losing the ability to recognize conspecifics and select mates by their color (Bates, 1862; Brown et al., 1974; Hines et al., 2011; *Heliconius* Genome Consortium, 2012; Merrill et al., 2015)

If *Heliconius* use 3-OHK UV-yellows as a private channel of communication to identify conspecifics while maintaining mimicry in the eyes of predators (Bybee et al., 2012), we would expect that *Heliconius* do indeed have two types of UV receptors with different spectral sensitivities. In attempt to answer this question using *in situ* hybridization, a previous experiment found that *H. erato UVRh1* and *UVRh2* transcripts co-localized to the same R1 and R2 photoreceptor cells (Zaccardi et al., 2006a). We could not, however, rule out the possibility of cross-hybridization of the riboprobes due to the high nucleotide sequence similarity between the duplicated genes. We next attempted to estimate UVRh1 and UVRh2 visual pigment (opsin + chromophore) absorbance by epi-microspectrophotometric densitometry on *H. erato in vivo*,

which yields peak absorbances through reflectance measurements of a group of (~20) ommatidia (Briscoe et al., 2010). This method identified separate peak absorbances at 355 nm and 398 nm, as expected for an eye with two functional UV opsins, but strong evidence linking specific UV opsins to specific photoreceptors with divergent sensitivities was still missing.

Due to their significance for understanding the co-evolution of color vision and communication signals, we aimed to characterize the photoreceptor subtypes and ommatidial classes in *H. erato* compound eyes. We predicted that the duplicate UV opsins are expressed in distinct R1 and R2 photoreceptor subtypes with different spectral sensitivities, consistent with sub- and neo-functionalization after gene duplication. Among nymphalids, *H. erato* is notable for the ability to discriminate color in the red range (Zaccardi et al. 2006b), and as only one LW opsin gene is found in gDNA (Hsu, 2001) or cDNA synthesized from head tissue (Zaccardi et al., 2006b), we predicted a red-sensitive photoreceptor cell is present in the eye of *H. erato,* probably due to filter pigments.

This study uses immunohistochemistry to determine the pattern of opsin expression in photoreceptor cells and intracellular recordings to measure photoreceptor spectral sensitivities. We confirm that the UV opsins are expressed in separate R1 and R2 photoreceptor subtypes, and we provide evidence for the presence of a red-sensitive photoreceptor. Unexpectedly, *H. erato's* compound eye is sexually dimorphic, with the male lacking UVRh1 expression. This is the first instance, to our knowledge, of a nymphalid using both an opsin duplication and filtering pigments to increase the number of spectral receptor types, and the first case of a sexually-dimorphic eye in the family Nymphalidae.

Materials and Methods

Animals

We obtained *H. erato petiverana* pupae from The Butterfly Farm - Costa Rica Entomological Supply. After eclosion, butterflies were housed for at least a day in a humidified chamber, and were fed a diluted honey solution daily before recording. Animals were sacrificed by rapidly severing the head and crushing the thorax.

Cryosectioning and Immunohistochemistry

Freshly severed butterfly heads were cut in two to separate the eyes, and immediately fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in 0.1 M phosphate buffered saline (PBS) for 30 minutes at room temperature. Eyes were then sucrose-protected in successive concentrations of 10%, 20%, and 30% sucrose in PBS, either for one hour at room temperature or overnight at 4°C. Excess cuticle around each eye was cut away before it was placed on a bed of Tissue Tek O.C.T. compound (VWR, Radnor, PA, USA) and frozen at -20°C. Frozen eyes were sectioned at 14 µm thickness on a Microm HM 500 OM microtome cryostat (Fisher Scientific, Pittsburgh, PA, USA), and placed on slides to dry overnight at room temperature.

An antibody against the peptide DGLDSVDLAVIPEH in the N-terminal domain of *Heliconius erato* UVRh1 was generated in guinea pigs and immunoaffinity purified (Open Biosystems, Inc., Huntsville, AL, USA). An anti-blue opsin antibody was generated in rats against the *H. erato* peptide RYRAELQKRLPWMGVREAD, and also immunoaffinity purified (Life Technologies, Grand Island, NY, USA). The rabbit anti-pan-UV antibody was generated against a *Papilio glaucus* peptide CISHPKYRQELQKRMP (Lampel et al., 2005), which has a sequence similar to *H. erato* UVRh1 and UVRh2. In *H. erato*, this antibody strongly labels R1 and R2 cells that do not stain for anti-UVRh1 or anti-blue antibodies. The long-wavelength opsin antibody was generated in rabbits against the *Limenitis astyanax* sequence KYRAALYARFPALACAPEPQD (Quality Controlled Biochemicals, Hopkinton, MA, USA). After labeling, dry slides were placed in 100% ice-cold acetone for 5 minutes, then washed 3 x 10 minutes in 0.1 M PBS. Slides were then placed in 0.5% sodium dodecyl sulfate in 0.1 M PBS for 5 minutes. Each slide was blocked for one hour at room temperature using 8% (v/v) normal donkey serum and normal goat serum, and 0.3% Triton X-100 in 0.1 M PBS. Slides were

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incubated with 2:75 rabbit anti-pan-UV, 1:15 rat anti-blue antibody (pooled from two animals), and 1:15 guinea pig anti-UVRh1 antibody or 1:15 rabbit anti-LWRh antibody in blocking solution overnight at 4°C. Slides were washed 3 x 10 minutes in 0.1 M PBS and then incubated with 1:1000 goat anti-rat Alexafluor 488 and 1:500 donkey anti-rabbit Cy3 or Alexafluor 555, and 1:250 goat-anti-guinea pig Alexafluor 633 (Life Technologies, Grand Island, NY, USA) in blocking solution for two hours at room temperature. Slides were washed again 3 x 10 minutes in 0.1 M PBS. Slides were stored for imaging by coverslipping with Aqua Poly/Mount (Polysciences, Inc. Warrington, PA, USA). Slides were viewed using a Zeiss Axioskop 2 under a 20x lens. Images were taken using a Zeiss Axiocam HRc and associated Axiovision software. For some images, a Leica confocal SP700 microscope was used in the UC Irvine Optical Core Facility. Stains were pseudocolored, and contrast and brightness were adjusted for clarity using Adobe Photoshop CS4 and Fiji (Schindelin et al., 2012).

Cell and Ommatidial Counts

Ommatidia were counted when images contained more than 100 ommatidia, the tissue was not sheared or folded, and cell bodies were clearly labeled without a high level of background. Images were viewed at full resolution in Adobe Illustrator and whenever possible, ommatidia were individually marked according to their R1/R2 cell staining. Background autofluorescence was retained, to reveal any unstained ommatidia. Ommatidia were not counted if the staining was unclear or the sectioned tissue was of poor quality (e.g. folded). Total ommatidia were counted over as much area as possible for a single high quality section per individual and the percentages of each class of ommatidia were calculated due to differences in the area of different sections or partial sections. From these ommatidial classes we derived the total number of individual R1 and R2 photoreceptor subtypes in each section. The numbers of photoreceptors in each subtype were also converted to percentages. Ommatidial and photoreceptor counts of all animals were pooled by class and sex, converted to proportions, and each proportion was compared between the sexes using a two tailed Z-test. The data were tested for normality using a Shapiro-Wilk test, and a nonparametric Mann-Whitney-Wilcoxon test was performed to test for sex differences when the null hypothesis of normality was rejected.

Intracellular Recording

Before beginning an experiment the sex was determined. For *in vivo* recordings, an individual was affixed inside a small plastic tube using hot wax. The abdomen was held down with a dry piece of wax and the tube was humidified by placing a wet tissue inside. The entire tube was mounted on a stage and an indifferent silver electrode of 0.125 mm diameter inserted into the head via the mouthparts. A small hole (~10-20 ommatidia in diameter) was cut in the left cornea using a thin razorblade chip and sealed with Vaseline to prevent desiccation.

The recording setup and procedure are described in detail elsewhere (McCulloch et al., 2016). Briefly, we used an Oriel Xenon Arc lamp (Irvine, CA, USA) as a light source, aiming the light through a condenser lens assembly (Newport, Irvine, CA, USA, Model 60006), a convex silica lens (Newport, SPX055), a neutral density (ND) filter wheel (from 0 to 3.5 optical density), 10 nm bandwith spectral interference filters (Edmund Optics, Barrington, NJ, USA) a concave silica lens (Newport SPC034), a shutter with drive unit (Uniblitz, Rochester NY, USA, 100-2B), a collimating beam probe (Newport 77644), and finally into an attached UV transmitting 600 µm diameter fiber optic cable (Oriel 78367), all held by an optical rail. Photoreceptors were recorded intracellularly with borosilicate capillary electrodes filled with 3M KCL (~100 MΩ resistance).

Once the recording was stable, i.e. little to no change in resting potential, low background noise, and consistently large depolarizing responses (at least 10:1 signal to noise ratio, at least ~50 mV response amplitude), recording began. Responses were recorded to narrow-band spectral flashes of 50 ms, presented at 0.5 s time-intervals and covering the spectrum from 300 nm to 700 nm in steps of 10 nm. Intensity response curves were recorded from 3.5 to 0 optical density before and after an experiment when possible. During an experiment, the ND filter wheel was left in place at an optical density that elicited a strong response from the peak interference filter wavelength but one that did not approach the maximum response with white light. intensities adjusted as appropriate using quartz neutral density filters (McCulloch et al., 2016). When possible, wavelengths near the peak spectral response were recorded more than once.

After a recording, the spectral sensitivity of each cell was derived from the recorded spectral responses. The amplitudes of responses to white light at each ND filter step were used to create a response-log Intensity (VlogI) curve. The VlogI data was used to estimate parameters for the Naka-Rushton equation: $V/V_{max} = I^n / (I^n + K^n)$, where V is the amplitude of a given response; V_{max} is the maximum response amplitude; I is the intensity of the stimulus for the given response,

V; K is the intensity of the stimulus that elicits ½ of V_{max}; n is the exponential slope of the function (Fig. S1) (Naka and Rushton, 1966; Aylward, 1989). Due to differences in total photon flux for each interference filter, correction factors were calculated to approximate constant photon flux over all filters from 300 to 700 nm, and multiplied by raw intensities. Corrected intensities were divided by the maximum for each cell to calculate relative spectral sensitivity. Photoreceptors were classified by peak sensitivity and shape of the spectral sensitivity curve. Averages were taken of the same spectral class of photoreceptor for each sex, and standard error (SE) bars were applied. Each cell recording came from a different individual. To estimate peak sensitivities, we used least-squares regression to fit rhodopsin templates to our data (Stavenga, 2010).

Eyeshine and Tapetal Reflectances

Eyeshine images were taken with a 4x objective on a Zeiss Axioskop2 plus microscope using white light epi-illumination. Live butterflies were affixed to a glass slide on their side using wax, and positioned so that the ommatidia directly faced the objective, and then were allowed to dark adapt for a few minutes. To rule out the possibility of heterogeneous tapetal reflectances, we removed the photoreceptor layer from 4% paraformaldehyde-fixed eyes and visualized the tapeta under 10x magnification on a Zeiss Axioskop2.

Results

We labeled UVRh1, UVRh2, BRh, and LWRh by immunohistochemistry, and observed the distribution of labeling across the main retina (excluding the dorsal rim area) of *H. erato* (Fig. 1). All R3-R8 cells express LWRh (Fig. 1D,E). Each R1 and R2 photoreceptor expresses only one opsin: either UVRh1, UVRh2, or BRh, confirming and extending previous *in situ* hybridizations that could not distinguish between the two *UVRh* opsin mRNAs (Zaccardi et al., 2006b). We identified ommatidial classes by their staining for UVRh1, UVRh2, and BRh (Fig. 1F,G), and examined the sexes separately (see below). Additionally, because previous experiments and our own data show that BRh is expressed in the R1 and R2 cells that do not express UV opsin, we included double UV stains in our dataset, treating an unstained cell in either the R1 or R2 position as a blue cell. We pooled data from ommatidial classes if the same

opsins were expressed in either the R1 or the R2 cells. Thus an ommatidium with BRh in R1 and UVRh in R2 would be the same as one with UVRh in R1 and BRh in R2.

Comparing sexes we found that females (n=6) express both UVRh1 and UVRh2, while males (n=8) express only UVRh2 (Fig. 1F,G) in the main retina. Male ommatidia have all three possible combinations of R1-R2 cells given the loss of UVRh1 (B-UV2, UV2-UV2, and B-B), while females have three of their six possible combinations (B-UV1, UV2-UV2, or B-B). Females lack the male B-UV2 ommatidial class. There were no exceptions to these expression patterns in 24 individuals visualized under the microscope, and from 4784 ommatidia counted in high quality sections from 14 individuals. Differences in eye morphology and function along the dorso-ventral axis of the eye are common in insects (e.g., the ventral stripe in the cricket retina; (Henze et al., 2012)) so we also stained longitudinal sections of the eye (n=6), but did not find any signs of regionalization (Fig. 2).

To further characterize photoreceptor cell and ommatidial classes, we counted 299-730 ommatidia in the six females and 181-428 ommatidia in the eight males for which we had high quality tissue. Within the same sex, variation in the percentages of both cell and ommatidial classes is low (see boxplots in Fig. 3, Table 1). Approximately half the ommatidia (46.9-55.2 %) in females are UV1-B ommatidia, and the other half are split into similar proportions of UV2-UV2 (20.3-27.5 %) and B-B ommatidia (20.7-27.5 %). Based on ommatidial counts, female R1 and R2 cells express roughly half blue cells (48.3-53.6 %) and half UV cells (46.3-51.7 %), split between either UV1 (23.5-27.6 %) or UV2 (20.4-27.5 %) opsin. Males, rather than mirroring female expression, have a much higher proportion of B-B ommatidia than UV2-UV2 ommatidia. Male R1 and R2 cells comprise between 53.8-66.9 % blue photoreceptors and 33.1-46.2 % UV2 photoreceptors. In males 29.8-49.5 % of ommatidia are B-B, 13.2-22.2 % are UV2-UV2, and 36.5-48.0 % are UV2-B ommatidia. The proportions for the same ommatidial type differ significantly between males and females (Z-test, $p < 0.001$ for all cases), as do the relative abundances for the different cell types (Z-test, $p < 0.01$ for all cases). Using a Shapiro-Wilk test, the null hypothesis of normality could not be rejected for all cell and ommatidial counts ($p > 0.05$) except for the male UV2/B ommatidial type ($p = 0.029$). Females do not express this ommatidial subtype, so we were still confident that the difference in expression was real. A nonparametric Mann-Whitney-Wilcoxon test was performed, which showed that the sex difference in the number of the UV2/B ommatidial type was significant ($p = 0.00159$).

Ommatidia are expressed spatially in a stochastic manner, but the proportion of each type of ommatidium is consistent within each sex, as in other insects (Wernet et al., 2015).

Intracellular recordings of *H. erato* photoreceptor cells between 300 and 700 nm are reported from 42 cells. As expected from opsin expression, we found that females have two UV and one blue spectral receptor type, with λ_{max} at 356 ± 1.5 nm (n = 3) (SE after fitting a rhodopsin template to our data, see Methods), 389 ± 1.6 nm (n = 3), and 470 ± 2.0 nm (n = 7), respectively (Fig. 4A). Males have one UV and one blue receptor type with λ_{max} at 390 ± 1.2 nm (n = 4) and 469 ± 1.8 nm (n = 4) (Fig. 4B). Based on sexually dimorphic opsin labeling together with sexually dimorphic intracellular recordings, we infer that the UVRh1 is present in the female-specific 356 nm-sensitive photoreceptors, while the UVRh2 is found in the ~390 nm-sensitive photoreceptors found in both sexes. Both sexes have typical butterfly LW receptors with λ_{max} = 555 ± 1.0 nm for females (n = 12) and λ_{max} = 556 ± 2.2 nm for males (n = 7). We also found single examples of red sensitive cells in each sex, with λ_{max} ~ 600 nm (Fig. 5). The spectral sensitivity of the red receptor is narrowed in a manner that is consistent with tuning by a red filter pigment associated with LW opsin expression. Qualitatively, the spectral curve dips at the peak wavelengths of the green-sensitive photoreceptor, from ~540 nm to 570 nm, and then rises to a sharp, narrow-band peak sensitivity at ~600 nm (Fig. 5). Red pigment that may be responsible for heterogeneous eyeshine reflectance *in vivo* (Fig. 6), and that could produce such an effect on sensitivity, is visible in plastic sectioned eyes of *H. erato*. (Zaccardi et al., 2006b)

Discussion

Heliconius erato ommatidia resemble those of other insects, with the long visual fiber (LVF) receptors R1 and R2, containing SW opsins, while the short visual fibers R3-8, contain a single opsin, LWRh (Wernet and Desplan, 2004; Hadjieconomou et al., 2011; Wernet et al., 2015). *H. erato* compound eyes have at least five spectral types of photoreceptor and are sexually dimorphic, despite monomorphic wing patterns. We confirm the existence of R1 and R2 cells with sensitivity maxima (λ_{max}) ~355 nm, 390 nm and 470 nm, based on UVRh1, UVRh2, and BRh expression, respectively, however males lack UVRh1. Despite expression of only one long

wavelength opsin, both sexes have green- (λ_{max} ~555 nm) and red-sensitive photoreceptors (600 nm), with the longer wavelength peak attributable to the presence of lateral filtering.

UV photoreceptors and sexual dimorphism

Sexual size dimorphism is well documented in insect compound eyes (Meyer-Rochow and Reid, 1994; Lau et al., 2007; Meyer-Rochow and Lau, 2008), especially in Diptera, where males in several groups have larger eyes than females, probably due to the need for males to find females (Wehrhahn, 1979; Straw et al., 2006). In the butterfly *Bicyclus anynana*, the relative eye sizes of males and females are sexually dimorphic and differ according to the time of year together with opsin expression levels (Everett et al., 2012; Macias-Munoz et al., 2015). These differences can be interpreted as reflecting selection for optimal eye size, which is dependent on the behavioral requirements of the different sexes. In several dipteran species, males aerially chase and catch females for mating and the male-specific "love spot," is specifically adapted to this task (Land and Collett, 1974; Wehrhahn et al., 1982). In ommatidia found in the male love spot only, facet lenses are larger and an atypical R7 cell is present. Unlike other R7 cells in either sex, these love spot R7 cells resemble the outer R1-R6 cells; they express the same visual pigment and project their axons to the same region in the optic lobe as the R1-R6 cells (Franceschini et al., 1981). Neuronal circuitry in the optic lobe is also sexually dimorphic, maximizing the male's spatial resolution and motion detection for tracking females while sacrificing color vision (Hornstein et al., 2000). In honeybees (Hymenoptera), the male drone similarly has a dorsal acute zone with larger facet diameter and unique opsin expression for tracking females aerially, while the male ventral eye and the female worker bee eye are similar in opsin expression and morphology (Menzel et al., 1991; Velarde et al., 2005). Most known examples of sex differences in opsin expression are from flies, bees, and butterflies (see below), where both sexes express the same opsins, and only the domain of expression (or filtering) changes.

There are few examples of sex differences in photoreceptor spectral sensitivities, and by implication color vision. Among vertebrates, in most New-World primate species, a proportion of females can be trichromats, whereas all males are dichromats (Mollon et al., 1984). Males and females of the beetle *Rhagophthalmus ohbai* have different ERG response peaks, but opsin

expression in the eyes is unknown (Lau et al., 2007). Several butterflies have sexually dimorphic spectral sensitivities due to lateral filtering pigments, which may be related to wing pattern dichromatism. For instance in the butterfly *Pieris rapae crucivora* (Pieridae), photoreceptors that express violet-absorbing visual pigments are tuned in males by filtering pigments to a narrow blue sensitivity to detect sexually dichroic wings displayed by females (Obara, 1970; Arikawa et al., 2005). In the European subspecies *P. r. rapae* wing colors are monomorphic (Obara and Majerus, 2000; Stavenga et al., 2004), and photoreceptor sensitivities lack this sex-specific filtering (D. Stavenga, unpublished observation); (Arikawa et al., 2005). Similarly, sexually dimorphic expression of filtering pigments in the clouded yellow butterfly, *Colias erate* (Pieridae), leads to differences in ommatidial classes (Ogawa et al., 2013). In the small copper butterfly, *Lycaena rubidus* (Lycaenidae), sexual dimorphism in the eye again coincides with wing dichromatism, and is based on differences in the spatial pattern of blue opsin expressing cells, as well as opsin co-expression in a subset of photoreceptors (Bernard and Remington, 1991; Sison-Mangus et al., 2006).

Thus despite the widespread occurrence of sexual dimorphism in insect compound eyes, *H. erato* is unusual among insects so far investigated in that the males lack protein expression of one of the visual opsins (UVRh1). Sexually dimorphic expression of opsin mRNA levels has been found in insects like the fig wasp, *Ceratosolen solmsi,* but protein spatial expression and spectral sensitivity data are missing (Wang et al., 2013). Furthermore, other known examples of sexually dimorphic eyes in butterflies are accompanied by sexually dimorphic wings. *H. erato* wing patterns are sexually monomorphic. Other butterflies modify sex-specific color receptor differences with filtering pigments, however in *H. erato* filtering pigments do not contribute to sexual dimorphism. Lastly, we provide the first quantitative evidence in a butterfly that the relative abundance of shared photoreceptor and ommatidial classes are different between the sexes.

Given the new findings of this study, it is worthwhile to reconsider the hypothesis of Briscoe et al. (2010) who proposed that when the genus *Heliconius* arose, both sexes used both UV photoreceptors to facilitate detection of a UV-yellow wing signal that arose at the same time**.** However we find *H. erato* males lack one of the photoreceptors that should have given them an advantage in identifying conspecific females via UV color discrimination. This unexpected result raises the question of how selection may be acting on the visual systems of male and female *H.*

erato. It may be less costly for males to mistake female co-mimics of another species because their investment in reproduction is small and they mate multiple times in their lifetime. Thus males may have lost the circuitry required for UV discrimination because it is metabolically costly and/or because of trade-offs with other uses of color vision. Females may benefit in discriminating the correct male colors, due to a much larger investment in egg production and the ability to mate with only a few males.

Sexual dimorphism exists in *H. erato* among blue photoreceptors as well, where males have 20% more BRh-expressing R1 and R2 photoreceptor cells compared to females (Figs. 3, 7A). This small but non-trivial sex difference might reflect differences in the costs and benefits for the chromatic and perhaps achromatic signals that can be derived from the different types of compound eye. The reflectance spectrum of the *H. erato* 3-OHK yellow wing pigment has a step-like function peaking at about 470-480 nm, in the same range as the peak sensitivity of the blue photoreceptor (blue highlight, Fig. 7B). Males and females might use the blue-sensitive cell to detect differences between *Heliconius* and non-*Heliconius* yellows (yellow vs. green spectra, Fig. 7B) via either a chromatic or an achromatic channel. Males that lack UV discrimination could potentially benefit from more blue cells in the compound eyes than females.

Red receptors

Although the ancestral pterygote eye had only three opsin-based receptors: UV, B, and LW, red-sensitivity is widespread in insect compound eyes. Species in Odonata (Meinertzhagen et al., 1983), Hymenoptera (Peitsch et al., 1992), and Lepidoptera have receptors with $\lambda_{max} > 570$ nm (Briscoe and Chittka, 2001). Earlier physiological studies (Bernhard et al., 1970) and behavioural tests (Zaccardi et al., 2006b) have provided evidence for *Heliconius* red receptors, but to our knowledge these are the first intracellular recordings of red receptors in the retina of *H. erato*, and possibly any other nymphalid butterfly. Swihart (Swihart, 1972) predicted *H. erato* photoreceptors with peaks at 440 nm, 490 nm, and 600 nm based on single unit recordings from visual interneurons in the protocerebrum. Although the 440 nm estimate has not been replicated in subsequent experiments (Figs. 4,5, (Briscoe et al., 2010)), the 490 nm and 600 nm estimates match ours. Only one other nymphalid, *Polygonia c-album* ($\lambda_{max} = 580$ nm), has been found with a sensitivity $\lambda_{max} > 570$ nm, from electroretinograms (ERGs) (Eguchi et al., 1982).

For photoreceptors expressing non-A2 pigments including those found in insects, sensitivity maxima exceeding 580 nm are nearly always achieved by filters associated with an rhodopsin of λ_{max} <580 nm; the exception being the riodinid *Apodemia mormo,* which has a rhodopsin with λ_{max}=600 nm (Frentiu et al., 2007). Thus pierid and papilionid butterfly red receptors rely on photostable filters (Arikawa et al., 1999a; Qiu and Arikawa, 2003; Ogawa et al., 2013). Consistent with this pattern our recordings of red receptors from a male and female *H. erato* indicate a peak sensitivity of 600 nm, which is significantly narrowed when compared to the predicted rhodopsin absorbance spectrum at a peak of 600 nm. *H. erato* ommatidia have red and yellow eyeshine, and it is theoretically possible that the red-shifted sensitivity is partly due to tuning of the tapetum, but tapetal reflectance is in fact uniform (Fig. S2). Thus it is more likely that red eyeshine is due to the heterogeneous distribution of the red filter pigment between ommatidia. *H. erato* has a candidate photostable filter (possibly an ommine) that together with filtering by overlaying green cells, absorbs light at about 550 nm, near the peak sensitivity of the green cell (Langer and Struwe, 1972), which could explain the depression between 540 nm and 570 nm in the red cells' spectral sensitivities.

We did not label the red-sensitive cell, therefore we do not know which of the R3-9 cells correspond to the red-sensitive cells. However the rarity of our recordings (2 of 42) might not be reflective of a rarity of cells, as the abundance of dark red ommatidia in eyeshine reflectance suggests this cell type might be common (Fig. 6). In plastic sections the red pigment was found adjacent to the rhabdom starting at approximately 320 µm below the cornea and extending to 480 µm (Zaccardi et al., 2006b). The location of the pigment suggests that if the *H. erato* retina is tiered or partially tiered, the red-sensitive cells might be the more proximal R5-R9 cells. However in the absence of transmission electron microscopy data we cannot rule out the possibility that some R3/R4 cells might also be red-sensitive (Fig. 1B). Heterogeneity of ommatidial classes as well as the location of the cell bodies in the proximal retina may make recording from these photoreceptors difficult. Although we did not examine R9 cells of *H. erato* specifically, previous *in situ* hybridization experiments of individual ommatidia in the compound eyes of *Papilio glaucus* (Briscoe, 2008) and *Vanessa cardui* (Briscoe et al., 2003), indicate butterfly R9 cells express *LWRh* mRNAs.

H. erato uses color vision to discriminate between wavelengths from 590-640 nm (Zaccardi et al. 2006b). We show here that the *H. erato* retina has two spectrally distinct LW receptors (Fig. 6) forming the physiological basis of red-green color discrimination using only one LW opsin. Color vision has been demonstrated in *Papilio,* where tetrachromatic vision involves three typical insect receptors (UV, B, LW) plus an additional red receptor (Kelber and Pfaff, 1999; Koshitaka et al., 2008). Other studies further demonstrated that pierids and papilionids with red receptors use color discrimination to choose green leaves for oviposition (Kolb and Scherer, 1982; Kelber, 1999), as opposed to insects that lack a red receptor and use a monochromatic signal to choose yellow leaves for oviposition (Prokopy and Owens, 1983). Lythgoe proposed that using photoreceptors with λ_{max} above 580 nm would be suited to discriminating leaf colors, whose spectra tend to vary more in the red range from the reflectance maximum of chlorophyll (> 555 nm) (Lythgoe, 1979). Discriminating green leaves among many different plants would probably be difficult without chemosensory cues, but using the red receptor for color discrimination may allow females to oviposit on leaves of an individual plant that are most suitable for offspring growth - typically younger leaves (Kelber, 1999).

As social and sexual signals, red wing colors of hetero- or conspecifics may be better discriminated by *Heliconius* using both green- and red-sensitive photoreceptors. Crane (Crane, 1955) showed behaviorally that the red colored band on the *H. erato* forewing is important for courtship and approach in both sexes. In *H. melpomene,* the gene responsible for the red color pattern is genetically linked to the preference for that same pattern (Merrill et al., 2011). It has been previously proposed that overlap in color receptor sensitivities should match steep slopes in the spectra of salient signals to better discriminate more color differences between similar signals (Chittka and Menzel, 1992). It is likely that *H. erato* are using the green and red receptors for color vision in the context of mate choice because the reflectance spectra of the red color patches on wings correspond to a region that quickly rises from low to very high in this range (~550-590) of the spectrum (Fig. 7B) (Briscoe et al., 2010).

Here we show spectrally distinct UV photoreceptors due to a UV opsin duplication in the compound eye of *Heliconius erato.* We identify a new mechanism of sexual dimorphism among butterflies: namely, complete repression of expression of one UV opsin in males, together with a

concomitant increase in the abundance of blue photoreceptors. Lastly, we physiologically characterize both green and red receptors that are likely to be responsible for color vision in the red range due to filtering of a LW rhodopsin. An open question remains as to how these sexual dimorphisms affect color and motion vision, and whether the 'unit of discrimination' is at the ommatidial level, the sum of all individual photoreceptors found in the eye regardless of ommatidium structure, or some combination in between. The evolutionary and ecological consequences of this sexual dimorphism for *H. erato* behavior and life history have yet to be elucidated. We are only now able to frame these new questions in this long-studied system because of the novel work we present here.

Acknowledgements

We thank Kimberly Jameson, Matthew McHenry, and Raju Metherate for generously loaning equipment for the electorphysiology rig, Willi Ribi for technical advice on visualizing tapeta, Timothy Bradley, Almut Kelber and Kentaro Arikawa for advice and encouragement, and two anonymous reviewers for their useful feedback. We thank Charlie and Neil Podvin for assistance with eyeshine reflectance imaging, and Paola Vargas for sending us pupae. This work was made possible, in part, through access to the confocal facility of the optical biology shared resource of the Cancer Center support grant (CA-62203) at the University of California, Irvine.

Competing Interests

The authors declare they have no competing financial interests.

Author contributions

K.J.M., D.O., and A.D.B. conceived of the study and set up the recording equipment. K.J.M. and A.D.B. made new reagents. K.J.M. performed antibody staining, imaging, cell counts, intracellular recordings, and analysis. A.D.B. and D.O. assisted with spectral sensitivity analysis. K.J.M. and A.D.B. wrote the manuscript and D.O. provided input.

Funding

This work was funded by a National Science Foundation (NSF) Graduate Research Fellowship to KJM and NSF IOS-1257627 to ADB.

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Figures

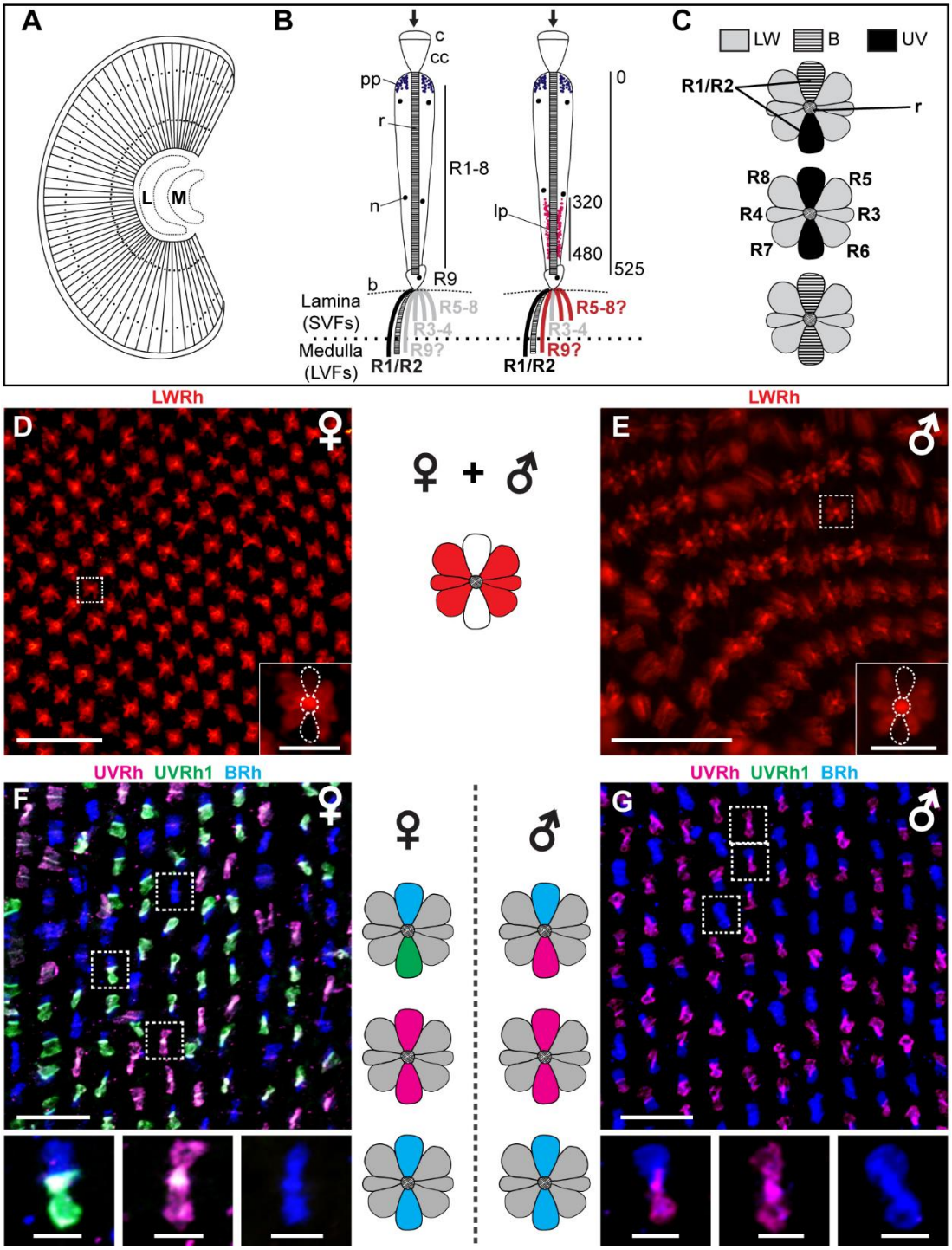

Fig. 1 Butterfly compound eye anatomy and *H. erato* eye sections stained for opsins (n = 24). **A.** Schematic of a longitudinal section of ommatidia and optic lobe immediately proximal to the retina. **B.** Longitudinal view of two *H. erato* ommatidia. Arrow indicates direction of light

entering the eye. Numbers indicate length of rhabdom from most distal (0) to most proximal point (525) in µm. Perirhabdomal filtering pigment extends from about 320 to 480 µm. **C.** Transverse sections of individual ommatidia, showing R1-R8 cells. In butterflies, R1/R2 cells express SW opsins, either BRh (light gray) or UVRh (dark gray). R3-R8 cells (and possibly R9) express LWRh (white). **D,E.** Ommatidia stained with anti-LW opsin are shown in red. Scale bar = 50 µm. Inset shows a single ommatidium at higher magnification, where six cells, R3-R8 are labeled. Dotted lines indicate R1 and R2 cells, which are not labeled. Scale bar = 10 µm. **F,G.** Triple stains of pan-UVRh (magenta), UVRh1 (green) and BRh (blue) opsins. Males (n = 8) and females (n = 6) are sexually dimorphic, with males lacking UVRh1 expression. Cartoon ommatidia show differences in classes of ommatidia found in males and females. Scale bar = 50 µm. Smaller boxes correspond to individual classes of ommatidia under higher magnification found in males and females. Scale bar = 10 µm. **L,** lamina; **M,** medulla; **c**, cornea; **cc**, crystalline cone; **pp**, pupillary pigment; **r**, rhabdom; **n**, cell nucleus; **R1-9** photoreceptor cells; **b**, basement membrane through which axons from cells R1-9 pass to reach the lamina and medulla; **SVF**s, short visual fibers; **LVF**s, long visual fibers; **lp**, lateral filtering pigment.

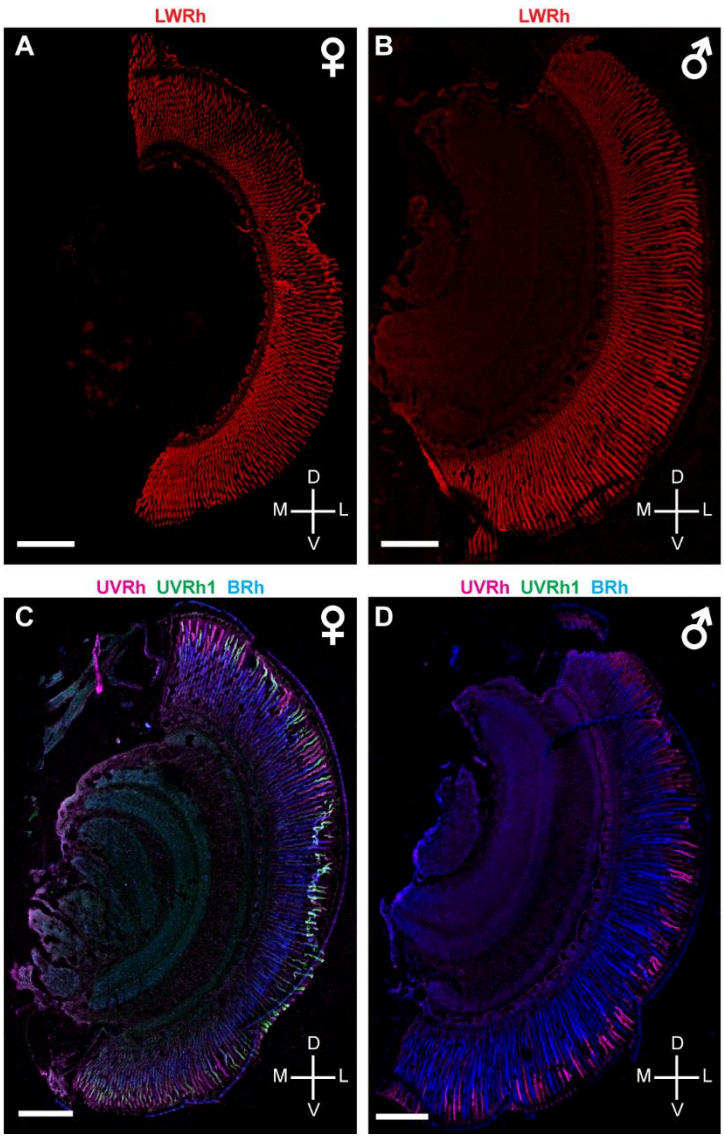

Fig. 2 Longitudinal sections of the adult compound eye stained for opsins in both males (n = 3) and females (n = 3). **A,B.** LW opsin (red) is present throughout the dorsal-ventral axis of the eye in both males and females. **C,D.** Triple stains show no obvious regionalization with respect to SW opsins with UVRh1 (green), UVRh2 (magenta) and BRh (blue) cells found across the dorsal and ventral compound eye. Sexual dimorphism is evident with the male eye lacking UVRh1 expression. Crosses show orientation along the dorsal (D)/ventral (V) axis and the lateral (L)/medial (M) axis. Scale bars = 250 μm.

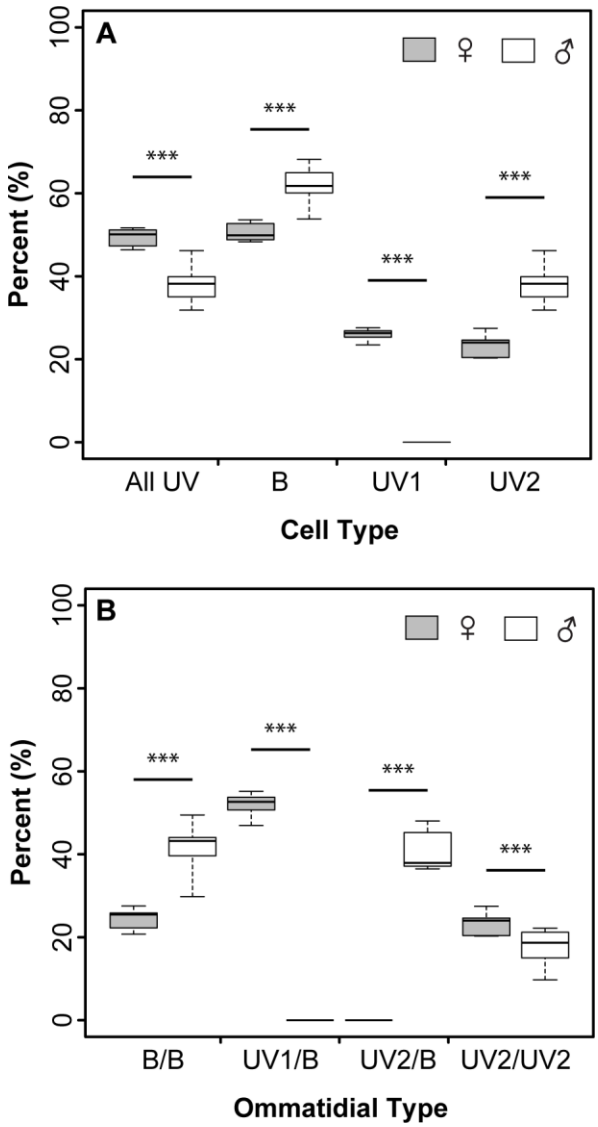

Fig. 3 Boxplots of female and male photoreceptor and ommatidial classes. **A.** Percent of R1 and R2 photoreceptors in the compound eye of males (white boxes, n=8) and females (gray boxes, n=6) expressing a particular opsin. Males lack any UVRh1 expression. Percent of UV1 and UV2 cells were summed and are indicated by *All UV*. **B.** Percent of each ommatidial class in the compound eye based on specific R1 and R2 opsin expression. Female eyes do not have UV2/B ommatidia, while male eyes do not have UV1/B ommatidia. A Z-test was performed to compare pooled male and female proportions for each photoreceptor and ommatidial class. For the Z-test, the number of photoreceptors sampled was 4712 for males and 4856 for females, and the number of ommatidia sampled was n = 2356 for males and 2428 for females. (***) indicates $p < 0.001$ between the two groups.

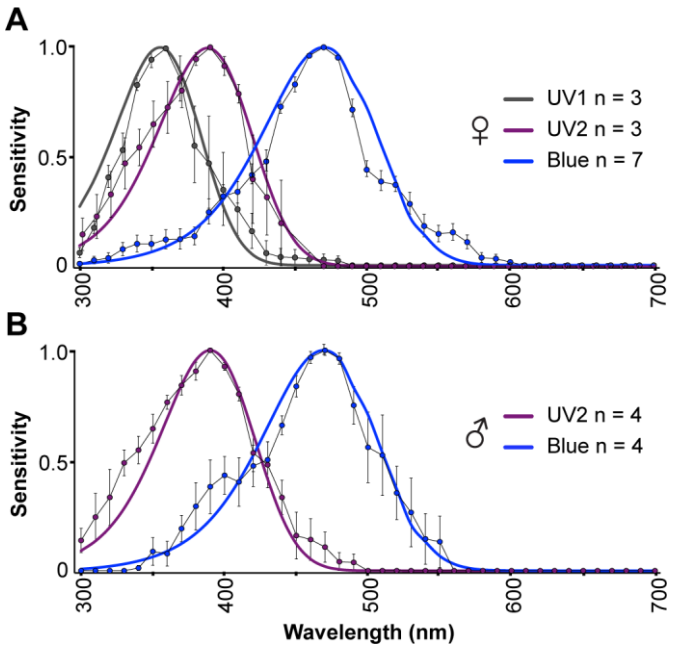

Fig. 4 Short-wavelength photoreceptor cell spectral sensitivities in *H. erato.* **A.** Females and **B.** males with standard error bars. Sensitivity is normalized to one. Females have a UVRh1 photoreceptor cell with a $\lambda_{max} = 356 \pm 1.5$ nm, which males do not possess. UVRh2 cells in females have a $\lambda_{max} = 389 \pm 1.6$ nm and in males a $\lambda_{max} = 390 \pm 1.2$ nm, while BRh cells in females have a $\lambda_{max} = 470 \pm 2.0$ nm and in males a $\lambda_{max} = 469 \pm 1.8$ nm.

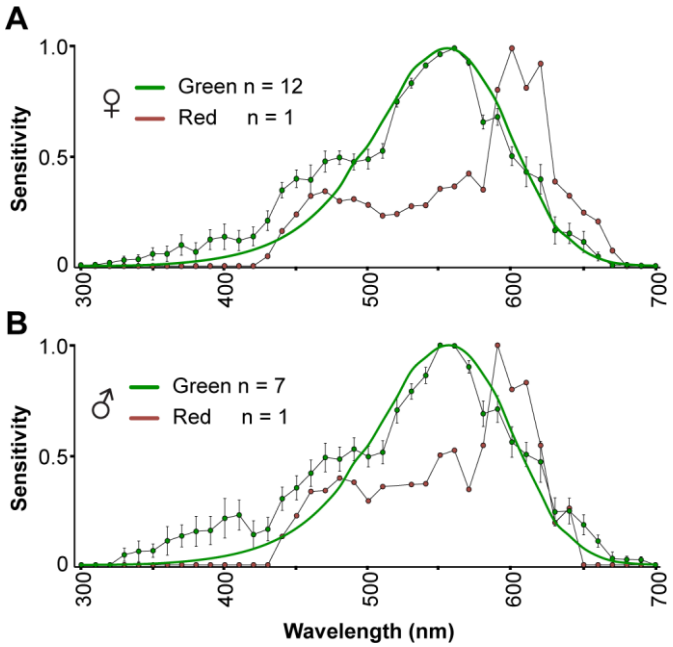

Fig. 5 Long-wavelength photoreceptor cell spectral sensitivities in *H. erato.* **A.** Females and **B.** males with standard error bars. Sensitivity is normalized to one. Both sexes possess green- and red-sensitive spectral types of photoreceptor cell. Green-sensitive photoreceptor cells have a λ_{max} = 555 ± 1.0 nm in females and a λ_{max} = 556 ± 2.2 nm in males. The red-sensitive photoreceptors have a depression in their sensitivity from 540 to 570 nm in both females and males, then show a narrow peak with λ_{max} ~ 600 nm.

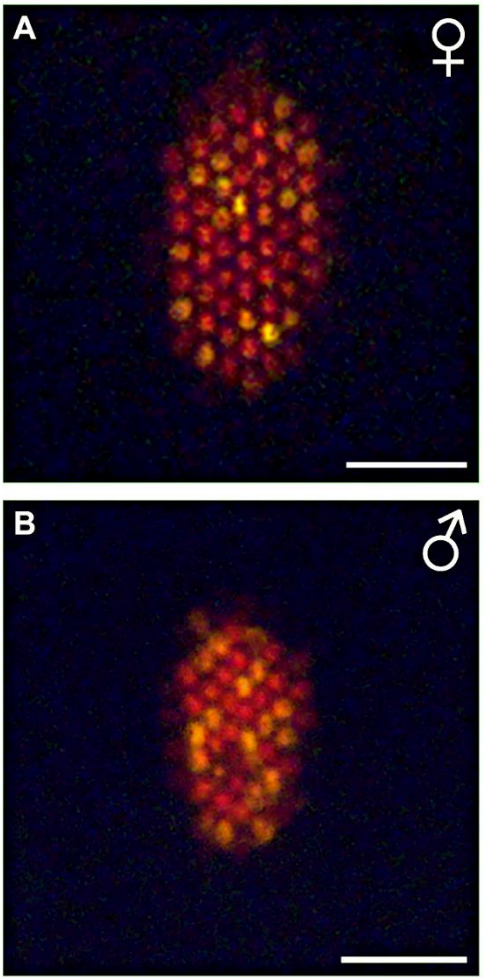

Fig. 6 *H. erato* eyeshine reflectance *in vivo*. **A.** Female. **B.** Male. Red and yellow ommatidia are inferred to be the result of heterogeneity of filtering pigments in the compound eye. Scale bars = 50 μm.

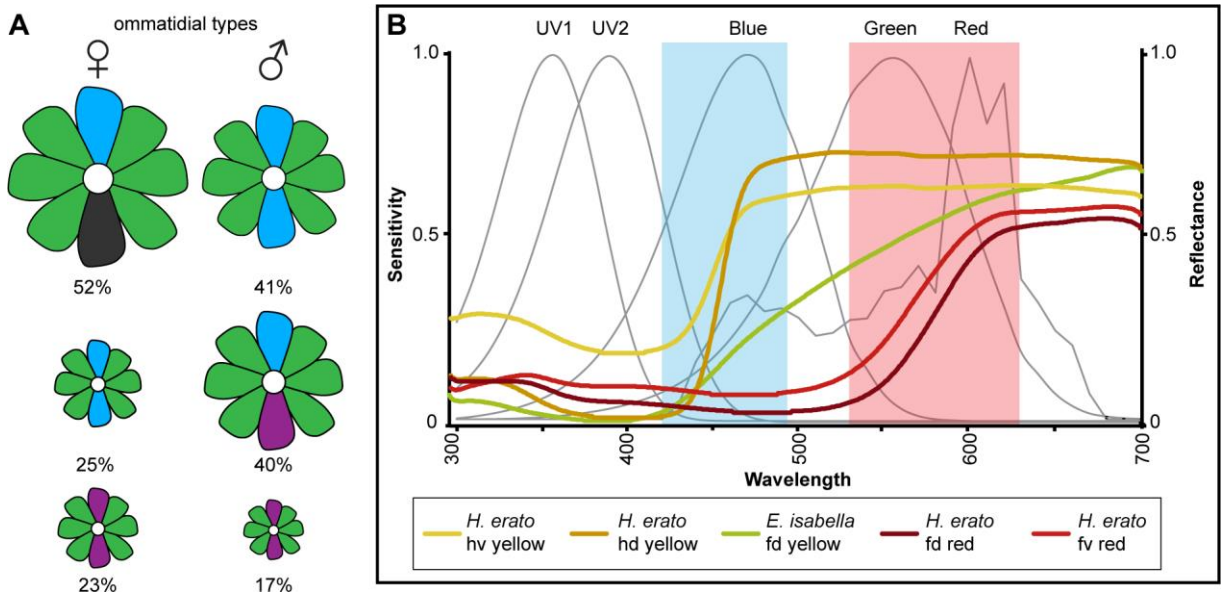

Fig. 7 Summary of *H. erato* retinal mosaic, photoreceptor physiology and wing reflectance spectra. **A,** Ommatidial classes found in the sexually dimorphic eyes of *H. erato.* Size of the ommatidium is proportional to the average abundance of that class found in the eye of either females or males. Both differential regulation of opsin expression levels and the abundance of these ommatidial classes may be playing a role in color vision. **B,** The five known spectral classes of photoreceptor in female *H. erato* overlayed with yellow and red wing reflectance spectra from *H. erato,* compared with the yellow wing reflectance spectrum of a *Heliconius* co-mimic, *Eueides isabella.* Blue shaded area indicates the overlap of the yellow reflectance spectra with the idealized sensitivity peak of the blue receptor, while the pink shaded area indicates the overlap of red wing reflectance spectra with the idealized sensitivities of the green and red receptors. Brightness differences between yellow wing colors may be detected by the *H. erato* blue receptor. Wing reflectance spectra data are from (Briscoe et al., 2010; Bybee et al., 2012). **d**, dorsal; **v**, ventral; **f**, forewing **h**, hindwing.

Table 1. Ommatidial counts in adult compound eyes of male and female *H. erato*.

		Total Ommatidial Counts (N)							Percentages (%)					
Specimen	**Sex**	**UV2/UV2**	**UV2/B**	**B/B**	**UV1/UV1**	**UV1/UV2**	**UV1/B**	**Total**	**UV2/UV2**	**UV2/B**	**B/B**	**UV1/UV1**	**UV1/UV2**	**UV1/B**
1	female	175	0	185	0	0	370	730	23.97	0	25.34	0	0	50.68
2	female	98	0	124	0	0	258	480	20.41	0	25.83	0	0	53.75
3	female	72	0	62	0	0	165	299	24.08	0	20.73	0	0	55.18
4	female	83	0	75	0	0	179	337	24.62	0	22.25	0	0	53.11
5	female	42	0	57	0	0	108	207	20.28	0	27.53	0	0	52.17
6	female	103	0	96	0	0	176	375	27.46	0	25.6	0	0	46.93
7	male	77	136	142	0	0	0	355	21.69	38.3	40	0	0	0
8	male	25	71	94	0	0	0	190	13.15	37.36	49.47	0	0	0
9	male	82	158	188	0	0	0	428	19.15	36.91	43.92	0	0	0
10	male	33	68	80	0	0	0	181	18.23	37.56	44.19	0	0	0
11	male	59	104	122	0	0	0	285	20.7	36.49	42.8	0	0	0
12	male	19	91	85	0	0	0	195	9.74	46.66	43.58	0	0	0
13	male	67	145	90	0	0	0	302	22.18	48.01	29.8	0	0	0
14	male	71	184	165	0	0	0	420	16.9	43.8	39.28	0	0	0

Total numbers of ommatidia were counted for each sample, excluding unclear staining or poor tissue quality. Mean percentages for each sex were calculated and used in Fig. 3. Total cell counts used in Fig. 3 were derived from ommatidial counts.