Supplementary Materials and Methods

**EPI-MICROSPECTROPHOTOMETRY**

A living butterfly, completely intact, is mounted in a slotted plastic tube. The head, thorax, and base of wings are stabilized with SIRA (BDH Chemicals 33097) adhesive wax of low melting temperature (Stavenga et al., 1977).

Our apparatus is a computer-controlled, double-beam, incident-light microspectrophotometer (epi-MSP, see Fig. 1 of Briscoe and Bernard, 2005). The tube is fixed to the goniometric stage of the epi-MSP and adjusted for the proper eye-region viewed by the butterfly looking into the MSP objective. The illuminator aperture stop is adjusted to create eyeshine from approximately twenty ommatidia. The objective is focused inward to collapse all eyeshine to a small spot, and the field stop adjusted to mask that spot and exclude the scattered light surrounding it.

The butterfly is left resting in the dark sufficiently long that photochemical changes caused by setup illumination have recovered (metarhodopsins decayed, and rhodopsins regenerated). A measurement baseline is created with 5 nm bandwidth flashes at 10 nm increments over the spectral range 380 nm to 750 nm, the intensity of each flash adjusted so that all wavelengths create the same criterion response from the MSP photomultiplier. Several spectral runs are taken successively in forward and reverse directions, to ensure that complete spectral runs are sufficiently dim to cause no measurable photochemical changes or pupillary responses. These spectral runs are averaged to create a reference spectrum for the eye’s dark-adapted state. At the end of all experiments, the butterfly is removed and fed, and then spectral runs with a NIST-
traceable spectral reflectance standard are measured and averaged to enable calculation of absolute reflectance spectra for all spectral runs.

All known butterfly long-wavelength-absorbing (L) rhodopsins shift their absorption spectrum to shorter wavelengths upon photo-isomerization to metarhodopsin (for example, R530 to M490 for *Vanessa cardui*). Thus, a strategy to determine the spectral properties of the L rhodopsin in an eye is to flash the eye using each of a graded series of sharp-cutoff, long-pass color filters (Schott RG715, Hoya R70, Schott RG695, Schott RG665, Hoya R66, Schott RG645, Hoya R64, Schott RG630, Hoya R62, Schott RG610, Hoya R60, Schott OG590, Hoya O58, Schott OG570, Hoya O56), working from long-wavelength cutoff to short, measuring a reflectance spectrum after each delivery. This procedure is halted when a flash causes a measurable difference spectrum.

A partial bleach of L rhodopsin is created by changing to the next filter in the series, and delivering repeated flashes (e.g., 3 sec every 60 sec) for 5 or 10 minutes, then monitoring dark-recovery by occasionally measuring reflectance spectra, and determining when metarhodopsin no longer contributes to the difference spectrum. At that time, rhodopsin content has not yet recovered fully. Thus, the rhabdoms are in a partially bleached state, and the difference spectrum is a direct measure of the alpha-band of the LW rhodopsin’s absorbance spectrum. An estimate of $\lambda_{\text{max}}$ is obtained by least-squares fitting to template absorbance spectra (Palacios et al. 1996). Free parameters determined by regression are density and $\lambda_{\text{max}}$ of rhodopsin and a free constant to fit the level of the long-wavelength tail. Error estimates for these data are complex because of wavelength-dependent error mechanisms, such as stray light and the presence of photoproducts. Therefore normal statistical error analysis that assumes Gaussian distributions is not
valid. On the basis of many experiments, not just the ones that produced Figure 1, $\lambda_{\text{max}}$ estimates are accurate to approximately $\pm 1.5$ nm. This method was used for Hermeuptychia hermes R530, Inachis io R530, Limenitis archippus floridensis R514, L. arthemis astyanax R545, Junonia coenia R510, and Nymphalis antiopa R534.

If dark-recovery is allowed to continue, later difference spectra are also partial bleaches, but of smaller amplitude. Comparisons among those spectra may also be used to estimate the L absorbance spectrum. This method was used for Agraulis vanillae R555 and Limenitis weidemeyerii R530.

Rhodopsins of butterfly species that have large eyes can be present in two-way optical densities of more than 2. Thus, reflectance of eyeshine at wavelengths in the neighborhood of $\lambda_{\text{max}}$ may be so small that the measured reflectance spectrum is influenced substantially by stray light, overestimating reflectance of the dark-adapted eye in that spectral range. Substantial photo-conversion to high densities of metarhodopsin can create a similar problem at shorter wavelengths. An effective method for dealing with this stray-light problem is to partially bleach the eye of L rhodopsin, waiting until certain that all metarhodopsin has gone, measure a new reference spectrum that is not polluted by stray light, perform substantial photo-conversion of L rhodopsin, wait, then measure a difference spectrum for the new partial bleach. We used this method for Anartia jatrophae R530, Heliconius hecale R560, Siproeta steneles R522, and Limenitis archippus archippus R514.

When laboratory temperatures are low, the rate of metarhodopsin decay from the rhabdom is so slow that it is not practical to wait for complete decay. It is possible, however, to use non-linear least squares regression to analyze difference spectra from
those experiments. Free parameters determined by regression are densities and lambda-max for both rhodopsin and metarhodopsin, plus a free constant to fit the level of the long-wavelength tail. If the lambda-max of metarhodopsin is well known, the fitted contribution of metarhodopsin to an experimental difference spectrum can be stripped from it to estimate the absorbance spectrum of LW rhodopsin. In some cases there is also a small accumulation of photoproduct RBP395 which is also stripped to yield the putative rhodopsin absorbance spectrum [For examples, see Fig. 4A of Briscoe et al. (2003), and Fig. 2E of Sison-Magnus et al. (2006)]. We used this method for *Anartia jatrophe* R565, *Apodemia mormo* R600, *Archaeoprepona demophon* R565, *Asterocampa leilia* R530, *Danaus plexippus* R545, *Euphydryas chalcedona* R565, *Heliconius charithonia* R550, *Limenitis lorquini* R530, *Neominois ridingsii* R515, and *Oeneis chryxus* R530.

**OPTOPHYSIOLOGY**

Butterfly photoreceptor cells contain intracellular granules that move centripetally in response to bright illumination and bleed light from the rhabdom by scattering and absorption, creating an effective pupillary response (Stavenga et al., 1977). This intracellular pigment migration is mediated exclusively by photo-isomerization of the rhodopsin contained within that same cell’s rhabdomere. Thus, the pigment granules can be used as an intracellular probe of the physiological responses to light from that cell. Each butterfly rhabdom contains several spectral types of receptor, so the most general pupillary response contains contributions from all spectral types. Pupillary sensitization, however, makes it possible to isolate responses from only one spectral type and measure its spectral sensitivity function over most of its visible spectrum. The trick is to measure
pupillary responses with a steady beam of spectral content and intensity adjusted so that it partially light-adapts the pupil of only one spectral type but leaves the pupils of other spectral types completely dark-adapted. The sensitivity of light-adapted pupils to brief monochromatic stimuli is much greater than sensitivity of dark-adapted pupils. In butterflies, where this effect of pupillary sensitization was first recognized, sensitivity differences can be as large as 1.4 log-units (Bernard, 1978). A description of optophysiology as an experimental method is found in Bernard, 1982.

This is a particularly good method for estimating $\lambda_{\text{max}}$ of L rhodopsin because the long-wavelength tail of the log-sensitivity function decreases linearly. In the case of Heliconius erato we used a steady measuring beam filtered by a 633 nm interference filter and a Hoya R64 cutoff filter. Stimuli were 10 second monochromatic flashes separated by 120 second dark periods. Using randomized sequence of wavelengths every 10 nm between 470 nm and 670 nm, the quantum flux was adjusted to achieve a 10% criterion response at each wavelength. Non-linear least squares regression to a template log-sensitivity function over that wavelength range produced an estimate for $\lambda_{\text{max}}$ of 555 nm $\pm$ 1 nm with 0.07 log-unit standard deviation of regression.

Another experimental situation ideal for optophysiology is the characterization of rhodopsins whose $\lambda_{\text{max}}$ is in the neighborhood of 500 nm. Their metarhodopsin photoprodut has essentially the same $\lambda_{\text{max}}$ as that of the native rhodopsin, so photochemical difference spectra are very weak and their presence is very difficult to determine using epi-MSP. Fortunately, the female and male Apodemia mormo have different regional distribution of four rhodopsins over their eyes. Both sexes possess R600, but the female has none just above the eye’s equator. That region contains a lot of
R505 in photoreceptor cells that exhibit pupillary responses, which was discovered during optophysiological experiments and confirmed using ERG (Bernard et al., 1988). The measuring beam was yellow, using a 580 nm interference filter and a Schott KV550 cutoff filter. Criterion responses of 5% to 20 second flashes were achieved over the wavelength range 400 nm to 605 nm. Least squares regression to log-sensitivity produced an estimate for $\lambda_{\text{max}}$ of 504 nm $\pm$ 1 nm with 0.08 log-unit standard deviation of regression. This is consistent with the 1988 estimate of R505.

**Supplementary References:**


Palacios AG, Goldsmith TH, Bernard GD. 1996. Sensitivity of cones from a cyprinid fish 

beholder: the two blue opsins of lycaenid butterflies and the opsin gene-driven 

Stavenga DG, Numan JAJ, Tinbergen J, Kuiper JW. 1977. Insect Pupil Mechanisms - II: 