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Eyeshine and spectral tuning of long wavelength-sensitive rhodopsins: no evidence for red-sensitive photoreceptors among five Nymphalini butterfly species

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Summary

Spectral tuning of rhodopsins commonly refers to the effects of opsin amino acid substitutions on the wavelength for peak sensitivity of the rhodopsin absorption spectrum. Nymphalini butterflies provide an opportunity for identifying some of the amino acid substitutions responsible for insect rhodopsin spectral tuning because the majority of photoreceptor cells (R3-9) in the adult retina express only a single long wavelength-sensitive (LWS) opsin mRNA transcript. Therefore, the opsin genotype can be directly correlated with its phenotype. We determined the LWS opsin gene sequence from cDNA of the mourning cloak *Nymphalis antiopa,* and from genomic DNA of the malachite *Siproeta stelenes* and the peacock *Inachis io*. Using an epi-microspectrophotometer we examined each butterfly's eyeshine for photochemical evidence of multiple LWS rhodopsins and found only one. We then performed partial-bleaching experiments to obtain absorbance spectra for the LWS rhodopsins of all three species as well as from another nymphalid, the buckeye *Junonia coenia.* The isolated LWS opsin gene sequences varied in length from 1437–1612 bp and encode rhodopsins R522 (*S. stelenes*), R530 (*I. io*), R534 (*N. antiopa*) and, together with a previously published sequence, R510 (*J. coenia*). Comparative sequence analysis indicates that the *S. stelenes* rhodopsin is slightly blue-shifted compared to the typical 530 nm lepidopteran rhodopsin because of the presence of a S138A substitution at a homologous site that in mammalian MWS/LWS rhodopsins causes a 5 nm blue-shift. The difference in peak absorption between R522 of *S. stelenes* and R530 of *Inachis io* is therefore largely accounted for by this substitution. This suggests that spectral tuning mechanisms employing the S138A may have evolved in parallel in mammalian and butterfly MWS/LWS rhodopsins across 500 million years of evolution.

Key words: color vision, rhodopsin, photoreceptor, spectral tuning, Lepidoptera, *Inachis io, Junonia coenia, Nymphalis antiopa, Siproeta stelenes, Vanessa cardui.*

Introduction

Adult butterflies use their compound eyes to extract visual information from the illuminated environment. Along with olfaction, they use visual cues to select mates, detect food for themselves and for their offspring, and in some instances, to initiate long-distance migratory flight (Silberglied, 1984). Much work in recent years has advanced our understanding of the physiological, anatomical, and molecular basis of butterflies' visually mediated behavior. It is becoming clear that butterfly visual systems are very diverse, and that behaviors which are shared by all butterflies are probably mediated by anatomically and physiologically heterogeneous mechanisms. Behavioral studies show that female *Papilio* butterflies use color vision, the ability to discriminate wavelengths of light independent of brightness, when making decisions about where to oviposit (Kelber, 1999), and in *Pieris* butterflies as well, multiple receptors are involved in mediating oviposition (Kolb and Scherer, 1982; Scherer and Kolb, 1987). Both color and polarized light cues are used by male *Heliconius* butterflies in choosing mates (Jiggins et al., 2001; Sweeney et al., 2003). Both sexes of *Papilio* butterflies use color vision when foraging for flower nectar (Kelber and Pfaff, 1999; Kinoshita et al., 1999), and female *Battus philenor* butterflies can be trained to associate one color with a nectar source and another color with an oviposition site (Weiss and Papaj, 2003). Some butterflies also use a time-compensated sun compass and polarized light to orient during long distance migratory flights (Mouritsen and Frost, 2002; Reppert et al., 2004). Less well known is the molecular basis of spectral tuning differences in the color receptors used by butterflies to mediate some of these complex behaviors.

There is great diversity among butterfly species, in the wavelength for peak sensitivity (λ_{max}) of rhodopsins found in photoreceptors of the compound eyes. Typically, rhodopsins are categorized according to the range of wavelengths within which their λ_{max} values fall: short wavelength (SW, 300–400 nm), middle wavelength (MW, 400–500 nm) and long wavelength (LW, 500–600 nm). At the molecular level, it is clear that these arbitrary physiological classifications roughly correspond to three major clades of rhodopsin apoproteins (opsins): UV, blue and long wavelength. Different butterfly species express different numbers of opsins, depending upon the family. For instance, papilionid butterflies have six opsins, one UV, one blue and four LW rhodopsins (Briscoe, 1998; Briscoe, 2000; Kitamoto et al., 1998), five of which are expressed in the retina (Kitamoto et al., 2000). The nymphalid butterfly, *Vanessa cardui,* by contrast, has only three opsins expressed in the retina, one UV, one blue and one LW (Briscoe et al., 2003), two of which (UV and LW) are also expressed in the remnants of the larval stemmata in the adult optic lobe (Briscoe and White, 2005). The butterfly *Pieris rapae* has apparently only one major LW opsin transcript expressed in the R3-8 photoreceptor cells (Wakakuwa et al., 2004). Phylogenetic analysis of the opsin gene family indicates that two duplications of an ancestral LW opsin gene occurred along the papilionid lineage since papilionids and nymphalids shared a common ancestor (Briscoe, 2001; Wakakuwa et al., 2004). This is evident in the large differences in opsin expression pattern between papilionid and nymphalid adult eyes. In *V. cardui* (and apparently the pattern is the same in the moth *Manduca sexta*; White et al., 2003), the UV and blue opsins are expressed in a non-overlapping fashion in the R1 and R2 cells, and the LW opsin is expressed in the R3-9 cells in all ommatidia of the main retina.

The fact that the R3-9 cells of *Vanessa cardui* (Nymphalini) retina express a single LW opsin and that nymphalid butterfly eyes have a mirrored tapetum makes quantitative study of the relationship between Nymphalini LW opsin genotype and phenotype especially attractive. The tapetum is a specialized manifold of the tracheal system that subtends the photoreceptor cells of each ommatidium. It is composed of alternating layers of air and cytoplasm, spaced in a regular way, that function optically as an interference filter, reflecting broad-band light (300–700 nm) as in *V. cardui* (Briscoe et al., 2003), or reflecting relatively narrow-band light (320–590 nm) as in *Junonia coenia* (Bernard, unpublished). The ommatidial tapetum is the reason why most butterfly eyes (excluding papilionids) exhibit colorful eyeshine (Bernard and Miller, 1970; Stavenga, 2002a,b, 2001), and the reason why epi-microspectrophotometric measurements of rhodopsin absorption spectra can be made (see below).

In addition to the membrane-bound rhodopsins, photoreceptor cells of butterflies may contain intracellular red, orange or yellow photostable pigment granules that function optically as lateral filters, bleeding short-wavelength light from the rhabdom and thereby red-shifting both the spectral sensitivity of photoreceptors and the reflectance spectrum of eyeshine. Red intracellular pigment granules, which are responsible for saturated red eyeshine, were first described in photoreceptor cells of Pieridae (Ribi, 1979), as intracellular granules packed densely around rhabdomeres of proximal retinular cells. The substantial red-shifts caused by similar red and yellow photostable filtering pigments on spectral sensitivity of *Papilio xuthus* photoreceptor cells were measured electrophysiologically (Arikawa et al., 1999). Effects of deep-red and pale-red filtering pigments on *Pieris* photoreceptor cells (Qiu et al., 2002) were described more recently.

In the present study, we examined the LW opsin genes from a group of closely related butterflies in the Nymphalini subfamily in order to further our understanding of the molecular basis of spectral tuning. The long wavelength class of rhodopsin is particularly notable in butterflies because it is so very diverse (compared to the heavily studied hymenopterans; reviewed by Briscoe and Chittka, 2001). Because of the recent work on the spatial expression patterns of opsins described above, the LW opsin genotype can be correlated unambiguously to its λ_{max} phenotype. Quantitative epi-microspectrophotometry of butterfly eyeshine can determine the number of LW rhodopsins and their absorbance spectra, and can detect the presence or document the absence of colored photostable filtering pigments.

Materials and methods

Epi-microspectrophotometry

Rhodopsins of a nymphalid ommatidium are contained within a long, thin rhabdom waveguide that is illuminated efficiently by the cornea and cone (Nilsson et al., 1988) at one end and terminated optically by an interference reflector at the other end. Light propagates down the rhabdom waveguide, being partially absorbed by rhodopsins as it goes. The light that reaches the proximal end of the rhabdom is reflected at the tapetum and propagates back up the rhabdom, again being partially absorbed. Light that survives this double pass through the rhabdom is collected by the cone and cornea, then passes out of the eye where it is observable as a narrow beam of eyeshine. Viewed in a bright-field, incident-light microscope with repeated bright-white flashes, two striking optical effects are observable: (1) the intensity of eyeshine decreases rapidly with time during each flash, and (2) the coloration of eyeshine changes during each flash. The first effect is the pupillary response, mediated by intracellular migration of pigment granules within photoreceptor cells (Stavenga, 1979). The second effect is photochemical, caused by changes in absorption spectra accompanying the photo-isomerization of rhodopsins to metastable photoproducts (Bernard, 1983a). The epi-microspectrophotometric apparatus shown in Fig. 1 was developed by exploiting these optical effects to measure the spectral properties of both photoreceptors and rhodopsins of completely intact insects.

Procedures for using eyeshine to make photochemical measurements from butterfly eyes have been described

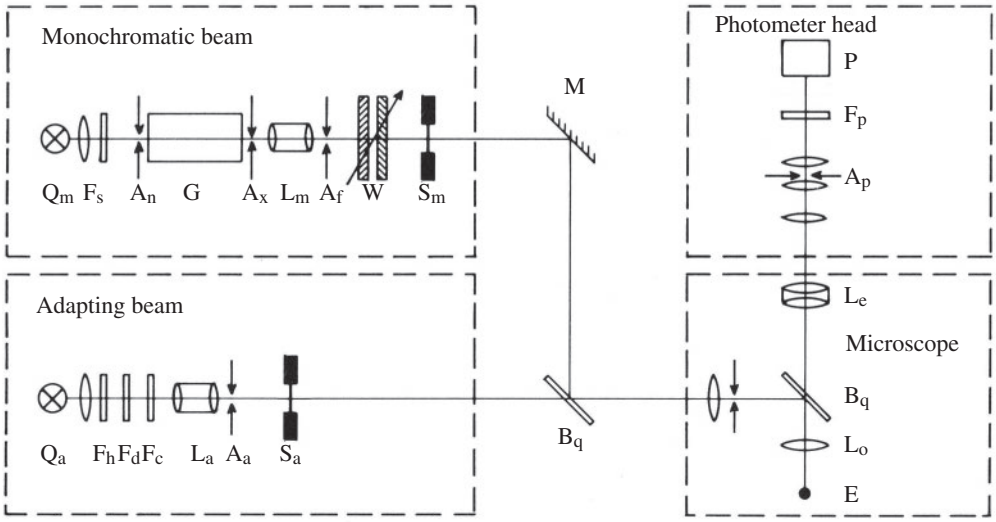

Fig. 1. Diagram of the epi-microspectrophotometric apparatus. The monochromatic beam contains: Q_m, Ealing 150 W optically stabilized Xenon-arc illuminator; F_s, heat filter and stray light filter; G, computer-controlled monochromator with entrance slit A_n and exit slit A_x; L_m, quartz condenser; A_f, field aperture; W, computer-controlled counter-rotating neutral density wheels; S_m, computer-controlled Uniblitz shutter; M, mirror. The adapting beam contains: Q_a, 45 W quartz-iodine illuminator; F_h, heat filter; F_d, neutral filter; F_c, cut-off filter; L_a, telephoto lens; A_a, field aperture; S_a, Uniblitz shutter. The two collimated beams are combined in quartz beam-splitter B_q and inserted into the Leitz Pol-Opak epi-illuminator equipped with 620-257 illumination slide designed for low stray-light. A, aperture diaphragm; L_o, microscope objective, either Leitz 8X/0.18P acromat/215 or Zeiss Luminar-25 mm/0.15; L_e, GF10X eye piece; E, eye of completely intact butterfly mounted on Leitz UT-4 goniometric stage. The Leitz UV-MPV photometer head contains A_p, adjustable/centrable photometer diaphragm; F_p, stimulus-blocking filter; P, cooled Hammamatsu R928 photomultiplier.

previously (Bernard, 1983a,b; Briscoe et al., 2003). Briefly, a completely intact butterfly was mounted in a slotted plastic tube fixed to the goniometric stage, oriented to select an eye region of approximately 20 ommatidia for study, then the microscope objective was focused on the deep pseudopupil for optimal collection of eyeshine and reduction of stray light. After at least 1 h in the dark, the reflectance spectrum of eyeshine was measured with a series of dim monochromatic flashes (see Fig. 3). This was used as a reference spectrum against which difference spectra were computed following treatment with photo-isomerizing flashes.

Our experimental goal was to measure the difference spectrum for a partial bleach of the rhodopsin that is most sensitive at long wavelengths, then characterize it by λ_{max}, the wavelength of maximal absorbance. This was achieved using the epi-microspectrophotometric apparatus (Fig. 1) with the adapting beam equipped with one of a series of 3 mm Schott (Duryea, PA, USA) cutoff filters in position F_c, and illuminating at full intensity for 5 s, and measuring the reflectance spectrum several minutes after the flash. This was started with filter RG665 but if the flash caused no measurable photochemical change, the filter was changed to RG645, then RG630, RG610, RG590, RG570 successively, until the procedure of flashing and spectral measuring created a difference spectrum that revealed modest photo-conversion of rhodopsin to its blue-shifted photoproduct (λ_{max} approximately 490 nm). Then that filter was used to deliver multiple flashes separated by dark periods as described by Bernard (1983b), to partially bleach the eye of its LW-sensitive rhodopsin. Bleaching of the rhabdom is possible because metarhodopsin photoproducts decay from the rhabdom more rapidly than rhodopsin content regenerates. The photoproduct-free difference spectrum for this partial bleach yields directly a two-way absorbance spectrum. Least-squares fitting to template absorbance spectra (Stavenga et al., 1993) yields an estimate of λ_{max}.

Photographs of butterfly eyeshine

The apparatus depicted in Fig. 1 was modified for photomicrography by exchanging the MPV photometer with a Leitz 543-040 microphotographic attachment, Leica MDa camera body, and GF16X eyepiece. The film used was Kodak ASA160 Daylight-Ektachrome. The illuminator slide was replaced with a Leitz Mecablitz-III microflash. The microscope objective was 8×/0.18P, the back focal plane of which was filled by the Leitz Opak epi-illuminator. An intact butterfly was mounted in a slotted plastic tube fixed to the goniometric stage, then oriented to set the eye's direction of view. The microscope was adjusted to center the eyeshine spot in the field of view, focused on the cornea. After at least several minutes of dark-adaptation, the shutter of the camera was opened long enough for the eye to be flashed at full intensity by the Mecablitz strobe light. Repeated photos from the same spot required several minutes of dark-adaptation between flashes to ensure full recovery from pupillary responses prior to each photo.

PCR, cloning and sequencing

Genomic DNA templates of *Inachis io* and *Siproeta stelenes* were gifts from Dr Andrew Brower. The locality where the *S. stelenes* specimen was collected is given in Brower and DeSalle (1998). Wild-collected eggs of *Nymphalis antiopa* were kindly provided by Dr Peter Bryant and the hatched larvae were fed on willow leaves (*Salix exigua*) collected in the San Joaquin Freshwater Marsh Reserve maintained by the University of California Natural Reserve System. Two sets of primers were used in PCRs to obtain fragments of the long wavelength opsin, 80 (5′-GAA CAR GCW AAR AAR ATG

A-3′) and OPSRD (5′-CCR TAN ACR ATN GGR TTR TA-3′), which amplifies a short fragment of the gene. Once the gene-specific fragment had been cloned, species-specific reverse primers were designed to amplify a longer fragment (*Inachis* RD 5′-CAG ATA GTG GCA AGA GGA GTG AT-3; *Siproeta* RD 5′-TCG AAG ATA CCG GAA TAG TTG AT-3′), in combination with the forward primer LWFD (5′-CAY YTN ATH GAY CCN CAY TGG-3′). For *Nymphalis antiopa,* total RNA was extracted from adult head tissue using TRIZOL and a cDNA library was synthesized using the Marathon cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA, USA). 3′RACE products were obtained by pairing primer 80 with an adaptor primer and 5′RACE products were obtained by use of a gene-specific reverse primer (*Nymphalis* RD 5′-GCA GTT TCG AAG ATA CCA GCA TAG-3′). In all cases, the following PCR conditions were used: 94°C for 1 min then 35 cycles of 94°C for 30 s, 50°C for 1 min, 68°C for 1 min. In all PCR reactions, overlapping pieces of the gene were amplified and cloned independently. PCR products were purified with the GeneClean Kit, and then cloned using the pGEM T-easy Vector System II (Promega, Madison, WI, USA). For each cloned PCR product, two to six clones were purified (Eppendorf) and sequenced using the ABI Big-Dye Terminator Reaction Kit v. 3.1 in forward and reverse directions and run on an ABI Automated Sequencer (Foster City, CA, USA).

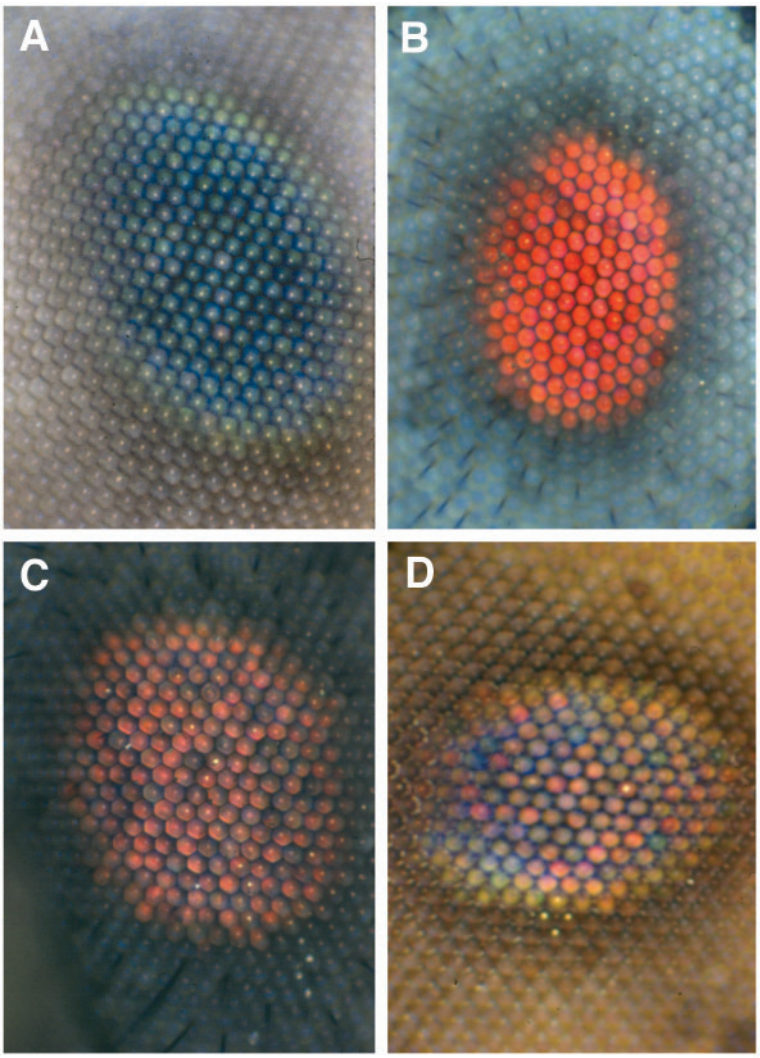

Fig. 2. Eyeshine from Nymphalini butterfly compound eyes. Epi-illumination of a dark-adapted eye showing light reflected from ommatidia viewing the incident illumination. (A) *Junonia coenia* The whitish appearance of the eye is likely due to light reflecting screening pigments located distally within the primary pigment cells (see Yagi and Koyama, 1963; Stavenga, 2002a) (B) *Vanessa cardui*. (C) *Nymphalis antiopa.* (D) *Siproeta stelenes*.

Phylogenetic analysis

Translated amino acid sequences were aligned by eye in MacClade 4.0 (Maddison and Maddison, 2000). We tested first plus second nucleotide positions, third nucleotide positions, and the translated protein sequences for composition homogeneity among lineages using the disparity index test (Kumar and Gadagkar, 2001). We found that third nucleotide positions appear to have evolved in a significantly non-homogeneous fashion in all nymphalid opsins and all other papilionid and moth sequences while in nymphalids, third nucleotide positions have evolved homogeneously (data not shown). Therefore, because the protein sequences and first plus second positions were homogeneous, first plus second nucleotide positions or amino acids were used in the phylogenetic analyses. Both maximum parsimony (MP) and neighbor-joining (NJ) analyses were conducted (PAUP*, MEGA 3.0) (Kumar et al., 2004). A total of 145 parsimony-informative amino acid sites were included, and a stepmatrix of amino acid changes derived from a larger data set of G protein-coupled receptors (Rice, 1994; see supplementary material in Spaethe and Briscoe, 2004) was employed as a weighting scheme to account for unequal probabilities of amino acid change in the MP analysis. The reliability of the MP trees was tested by bootstrap analysis in PAUP* (Swofford, 1998). For the NJ analysis, a total of 532 aligned nucleotide sites (first plus second positions) were used with Tamura-Nei correction (which takes into account both transition/transversion and GC content biases; Nei and Kumar, 2000) and complete deletion of gaps.

Results

Eyeshine coloration is diverse within Nymphalini

Eyeshine coloration was found to vary among the species, with the most dramatic difference observed between *Junonia coenia* and the other examined members of the Nymphalini tribe, *Vanessa cardui*, *Nymphalis antiopa* and *Siproeta stelenes*. The buckeye *J. coenia* has homogeneous blue eyeshine (Fig. 2A) due primarily to the presence of a tapetum that has unusually low reflectivity for wavelengths greater than 590 nm. By contrast, the *V. cardui* retina is

characterized by homogeneous orange eyeshine (Fig. 2B), while both *N. antiopa* and *S. stelenes* share eyeshine (Fig. 2C,D) that is more heterogeneous in color than either *V. cardui* or *J. coenia*. *Inachis io* has been previously characterized as also having homogeneous orange eyeshine (Stavenga, 2002a). No ommatidia having saturated red eyeshine were observed, indicating the absence of any red-sensitive photoreceptor cells in the surveyed species. As well, there were no major dorsal-ventral differences in the appearance of eyeshine in these species.

Log-reflectance spectra

Eyeshine log-reflectance spectra were measured from a medio-ventral region of the dark-adapted eyes of nymphalid butterflies *Inachis io*, *Junonia coenia*, *Nymphalis antiopa*, and *Siproeta stelenes* as well as the pierid butterfly *Pieris rapae*. These data are presented in Fig. 3 in addition to previously published (Bernard, 1983b; Briscoe et al., 2003) spectra for *Vanessa cardui*.

Absorbance spectra

The λ_{max} value of the LW rhodopsin expressed in the retina was estimated to be 522 nm in *Siproeta stelenes*, 530 nm in *Inachis io*, 534 nm in *Nymphalis antiopa*, and 510 nm in *Junonia coenia*. One-way absorbance spectra of these rhodopsins, determined from partial bleaches, are shown in Fig. 4.

All five species were subjected to the photochemical series described in Materials and methods. In all cases the first measurable photochemical difference spectra were well fitted by photo-conversion of the visual pigment reported in the preceding paragraph. There is no evidence at all for visual pigments of greater λ_{max} in eyes of these five species.

Long wavelength opsin sequences

The primer combination 80-OPSRD yielded one band for both *Inachis io* and *Siproeta stelenes* that was 316 and 320 bp in length, respectively. The gene-specific reverse primers designed from these sequences in combination with LWFD primer also yielded a single PCR band for each template. In total, we obtained a 1512 bp genomic fragment of the long wavelength-sensitive opsin gene from *Siproeta stelenes* (GenBank accession no. AY740908) and a 1437 bp genomic fragment from *Inachis io* (AY740906). Each of the genomic sequences contained six exons and introns (ranging from 69–158 bp in length), and encompassed seven transmembrane domains. From *Nymphalis antiopa* cDNA we also sequenced overlapping 3′ and 5′RACE products, which when combined formed a 1612 bp full-length opsin cDNA (AY740907). This sequence included both a start and a stop codon and encodes a protein that contains 378 amino acids. The translated protein sequences are shown in Fig. 5 aligned with those of other nymphalid species.

Opsin phylogeny and evolution of eyeshine in nymphalids

Phylogenetic analysis of the opsin-coding region using the Neighbor-Joining (NJ) algorithm yielded a tree with good (50-100%) bootstrap support in all but one node (Fig. 6). The branching pattern of the NJ tree was identical in all respects to the 50% majority-rule consensus MP tree except for the placement of the two *Pieris* opsins; in the MP tree they form a sister clade to the *Papilio Rh1-Rh3* clade. For instance, all included nymphalid opsin sequences were recovered with 92% bootstrap support, providing further evidence for the previous

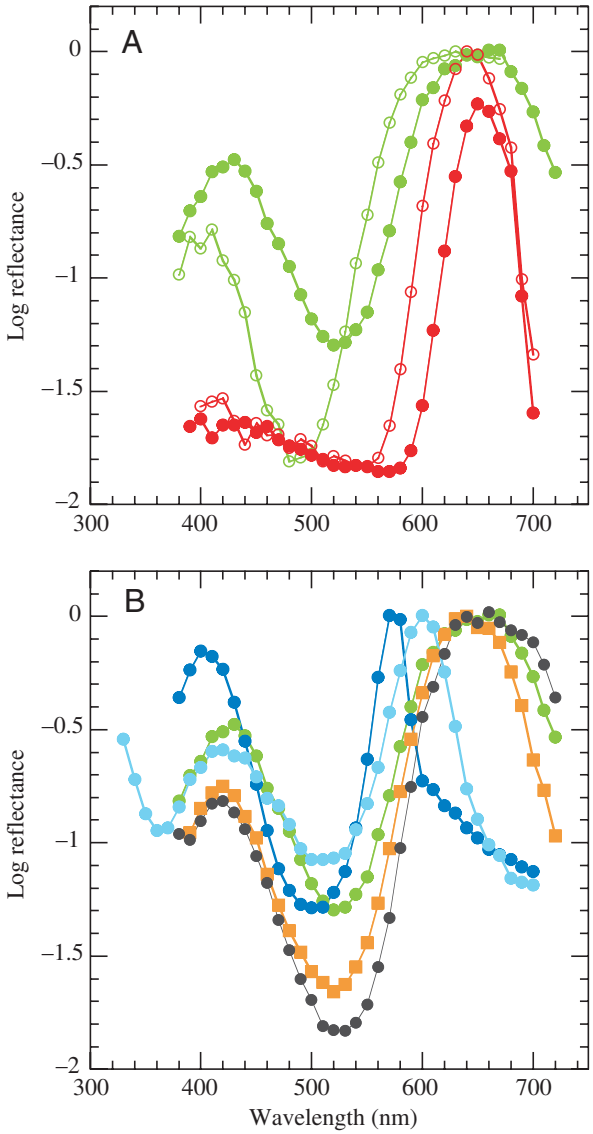

Fig. 3. (A) Log-reflectance spectra of eyeshine measured from a medio-ventral region of the dark-adapted eyes of *Vanessa cardui* (green filled circles) and *Pieris rapae* (red filled circles). The open circles are spectra following substantial photo-conversion of LW rhodopsin to blue-shifted metarhodopsin, for *Vanessa* (green open circles) and *Pieris* (red open circles). (B) Log-reflectance spectra of eyeshine measured from a medio-ventral region of the dark-adapted eyes of nymphalid butterflies *Vanessa cardui* (green filled circles), *Nymphalis antiopa* (black filled circles), *Inachis io* (orange filled squares), *Siproeta stelenes* (cyan filled circles), and *Junonia coenia* (blue filled circles).

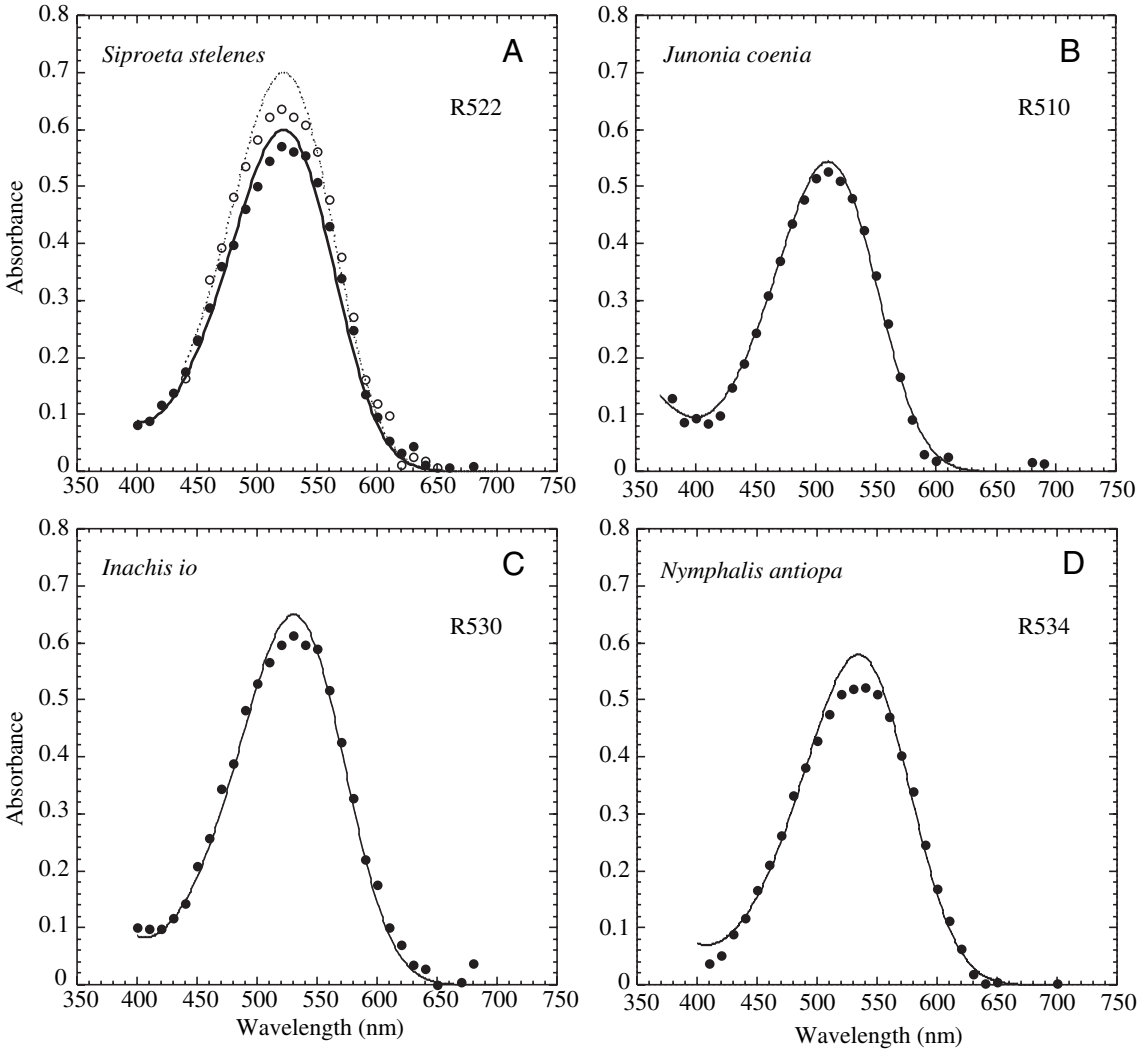

Fig. 4. Absorbance spectra derived from partial bleaches of long wavelength rhodopsins in eyes of completely intact butterflies. The solid curves are idealized spectra based upon the A_1–SSH template (Stavenga et al., 1993). (A) R522 of *Siproeta stelenes*; dorsal retina, filled dots and unfilled dots ventral retina of the same animal. (B) R510 of *Junonia coenia*, from the medio-ventral retina. (C) R530 of *Inachis io*, from the ventral retina just below the acute zone. (D) R534 of *Nymphalis antiopa*, from the medio-equatorial retina. Absorbance units are one-way, base-10 densities of rhabdom content, averaged over all illuminated ommatidia.

observation of Briscoe (2001), that two novel gene duplication events have occurred within papilionids since papilionid and nymphalid butterflies shared a common ancestor. The opsin sequences obtained from *Inachis io* and *Siproeta stelenes* genomic DNA cluster with the opsin sequences obtained from retina-derived cDNAs of other Nymphalini tribe members, i.e. *Vanessa* and *Junonia* (bootstrap support 98%), indicating the homology of the genomic sequences with the majority LW opsin expressed in the retina of related butterflies. We also repeated the phylogenetic analysis including the *Bicyclus anynana* LW opsin fragment (Vanhoutte et al., 2002) that had been cloned from RNA extracted from eye tissue and found similar results with this shorter alignment.

Mapping of eyeshine characteristics onto the opsin phylogeny indicates that the orange eyeshine observed in both *Vanessa cardui* and *Inachis io* (Stavenga, 2002a) and 'blue-orange' eyeshine observed in *Siproeta stelenes* and *Nymphalis antiopa*, is probably a result of their common ancestry (Fig. 6), while the blue eyeshine observed in *Junonia coenia* is a derived trait among the sampled Nymphalini.

Butterfly spectral tuning sites are shared with vertebrate cone opsins

We were interested in identifying candidate spectral tuning sites responsible for the observed variation in λ_{max} values among the Nymphalini rhodopsins. These species have been chosen in part because their short evolutionary history would narrow the number of variable sites. We also limited our comparisons to those rhodopsins for which λ_{max} values had been measured on our experimental apparatus, in order to reduce one source of experimental uncertainty – a difference in experimental paradigm.

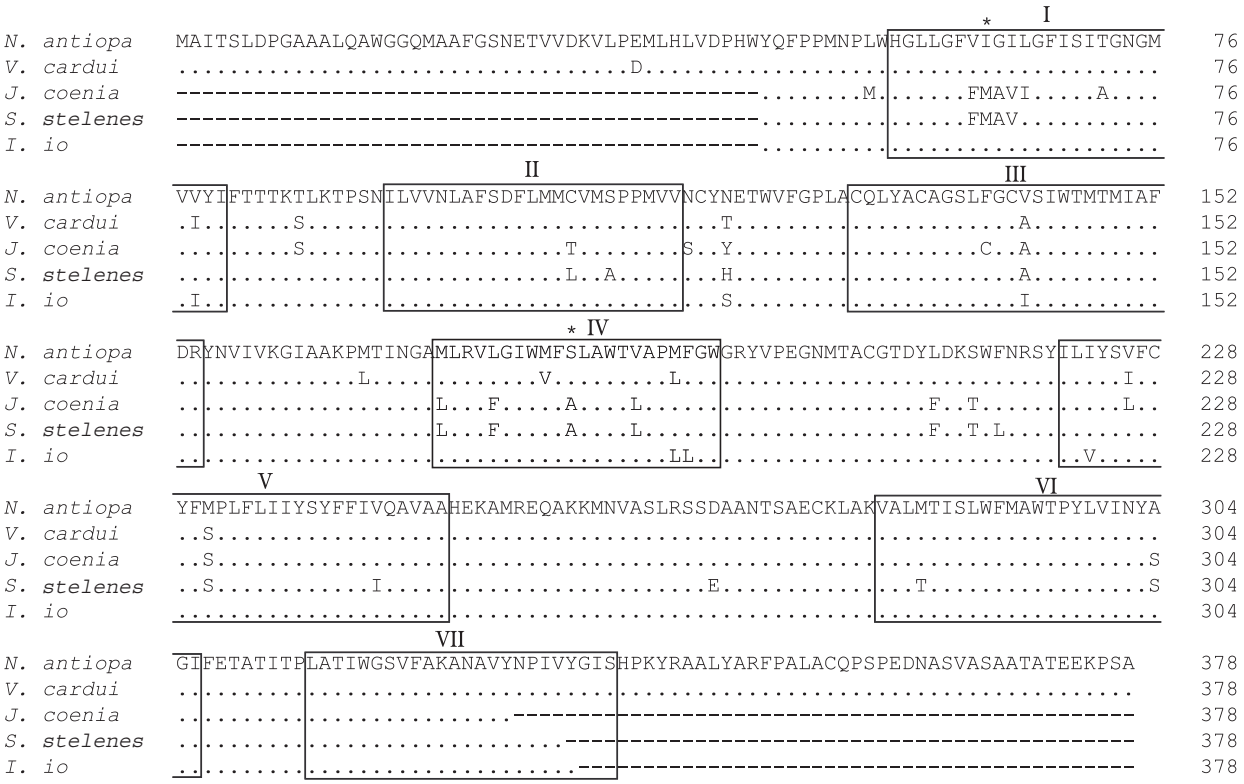

Fig. 5. Alignment of the deduced amino acid sequences of the nymphalid LW opsin. Blocks indicate the location of the transmembrane domains (I–VII) within the opsin protein sequence. Asterisks indicate candidate spectral tuning sites of known spectral shifts among vertebrate rhodopsins.

The maximum parsimony-reconstructed amino acid substitutions that occurred along the blue-shifted *Junonia-Siproeta* branch are shown in Table 1. Amino acid residues that on the basis of homology modeling (Briscoe, 2002) are found within the binding pocket facing the chromophore are also indicated. Notably, we found two amino acid sites that have known spectral tuning effects in the vertebrate rhodopsins. The first occurs in transmembrane domain I, is part of the region immediately surrounding the Schiff base, and corresponds to amino acid 44 in bovine rhodopsin (Palczewski et al., 2000). Mutagenesis of this site causes a 3 nm blue-shift in rhodopsin absorption spectrum (Andres et al., 2003). The second site is found in transmembrane domain IV and corresponds to amino acid 180 in human red cone opsin (or 164 in bovine rhodopsin). Mutagenesis of the serine residue at this site to an alanine causes a 5 nm blue-shift (Asenjo et al., 1994). Both blue-shifted rhodopsins in our data set, those of *Junonia coenia* (R510) and *Siproeta stelenes* (R522) have alanine at this site. Therefore, we suggest that the 8 nm difference between R522 of *Siproeta stelenes* and the ancestral R530 rhodopsin is largely accounted for by the substitution at this site and that of the former site. Six other amino acid sites have also changed along the blue-shifted opsin branch (Table 1). While most of these are likely to be neutral mutations with respect to spectral tuning, it is interesting to note that specific mutations at two of these sites, (amino acids 45 and 46 in bovine rhodopsin) along with the two sites mentioned above (amino acids 44 and 164), are responsible for the human eye disease, autosomal dominant retinitis pigmentosa (Andres et al., 2003; Briscoe et al., 2004; Rodriguez et al., 1993; Sung et al., 1991).

Discussion

Absence of saturated red eyeshine in Nymphalini

Coloration of ommatidial eyeshine is a complex phenomenon involving absorption by rhodopsins and their photoproducts contained in the rhabdom, filtering by photostable pigment granules contained in the photoreceptor cells, and reflections by the tracheolar tapetum. Eyeshine can be used to assess the possibility that the butterfly retina contains colored, photostable filtering pigments that are capable of increasing the number of spectral receptor types to include red-shifted photoreceptors (Stavenga, 2002b). Both visual inspection of eyeshine patterns and epi-microspectrophotometry are necessary, however, to determine quantitatively the number of long wavelength-sensitive rhodopsins present and to confirm the presence or absence of photostable filtering pigments.

The eyeshine in *Vanessa cardui* arises from a tapetum that has a broad, flat reflectance across the visible spectrum, decreasing above 680 nm, and ommatidia that express ultraviolet (UV), blue (B) and green (G) absorbing rhodopsins. This combination of tapetal reflection and rhodopsin absorption creates orange eyeshine (Bernard, 1983b; Briscoe et al., 2003), the dark-adapted log-reflectance spectrum of

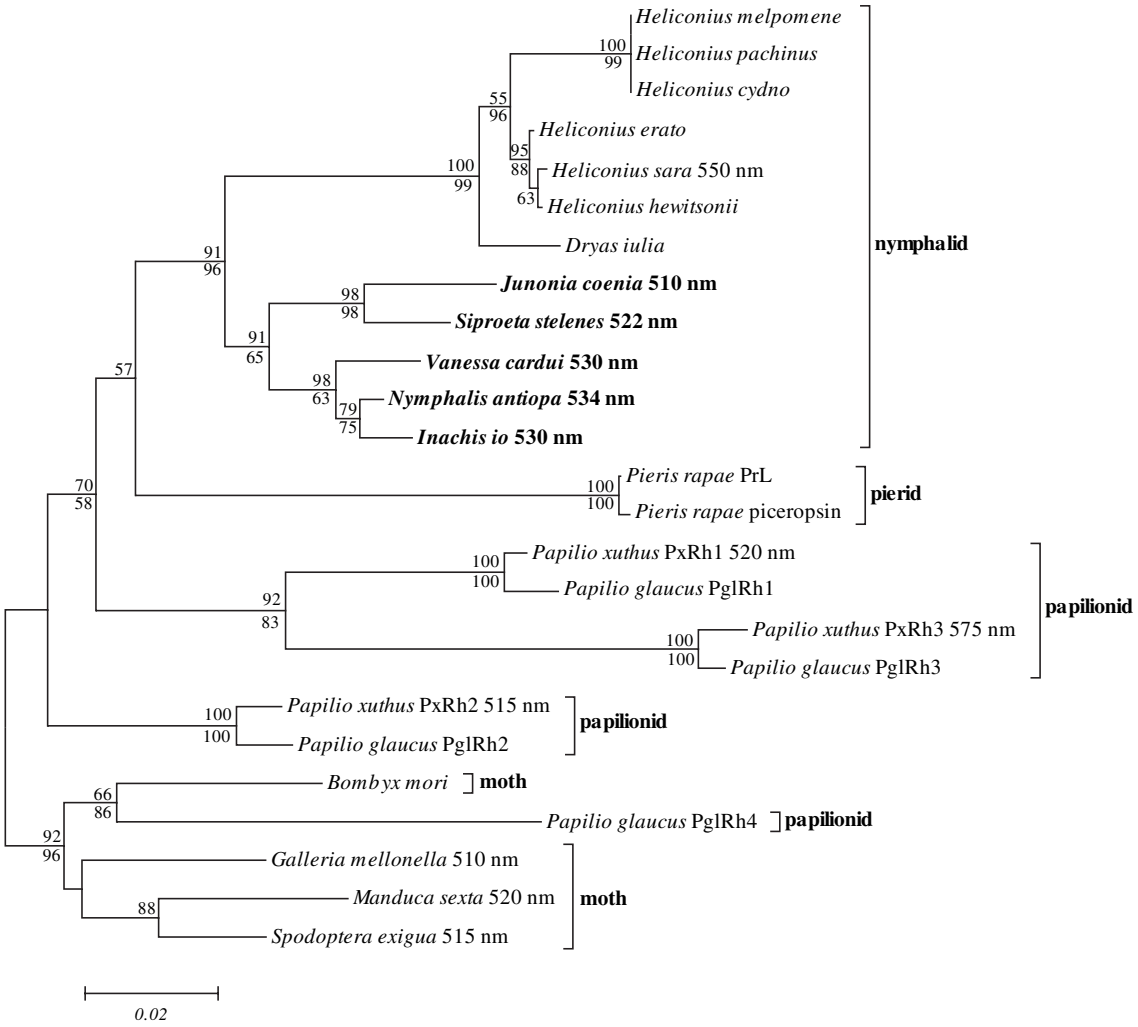

Fig. 6. Phylogeny of butterfly opsins. The neighbor-joining tree (shown) was constructed using a first plus second nucleotide sites and Tamura-Nei correction. Bootstrap support values (out of 500 replicates) for the neighbor-joining tree are shown above the nodes and bootstrap support (out of 100 replicates) for the stepmatrix-weighted maximum parsimony tree are below. References for additional λ_{max} values are given in Arikawa et al. (1999) and Briscoe (2001). GenBank accession numbers are: *Heliconius sara* (OPS1, AF126753; Hsu et al., 2001), *Manduca sexta* (1, L78080; 2, L780801; 3, AD001674; Chase et al., 1997), *Papilio glaucus* (PglRh1, AF077189; PglRh2, AF077190; PglRh3, AF067080; PglRh4, AF077193; PglRh5, AF077191; PglRh6, AF077192; Briscoe, 2000), *Papilio xuthus* (PxRh1, AB007423; PxRh2, AB007424; PxRh3, AB007425; Kitamoto et al., 1998), *Galleria mellonella* (AF385330), *Precis coenia* (AF385332), *Spodoptera exigua* (AF385331), *Vanessa cardui* (AF385333; Briscoe, 2001), *Bombyx mori* (AB064496; Shimizu et al., 2001).

which is shown in Fig. 3A (filled green circles). The filled red circles in the same figure show a log-reflectance spectrum of *Pieris rapae* (ventral) cherry-red eyeshine. Note that the reflectance of *Pieris* is very low for wavelengths less than 600 nm. Its spectrum does not exhibit a large secondary maximum in the blue, as does *Vanessa*. The optical basis for these striking spectral differences is that *Pieris* retinular cells contain short-wavelength absorbing, lateral-filtering granules and *Vanessa* retinular cells do not.

The spectrum shown by the open green circles in Fig. 3A is an example of a photochemical change in the *Vanessa* reflectance spectrum caused by photo-conversion of R530 to its photoproduct M490 (data from fig. 1c/A of Bernard, 1983b). A similar photo-conversion of the LW rhodopsin R563 of *Pieris* (Wakakuwa et al., 2004) exhibits quite a different reflectance spectrum, shown by the open red circles in Fig. 3A. Here, the expected decrease in reflectance in the neighborhood of 500 nm, associated with accumulation of metarhodopsin, is not observable because the lateral-filtering granules of *Pieris* are so effective in absorbing short-wavelength light that enters the rhabdom. None is reflected.

Log-reflectance spectra for all five Nymphalini species we examined showed prominent secondary maxima in the blue. Although not shown, substantial measurable decreases in reflectance, associated with accumulation of metarhodopsin following photo-conversion of LW rhodopsin, was measured in all five species. We conclude that these five species have no lateral-filtering granules in their photoreceptor cells.

Table 1. *Amino acid replacements associated with wavelength shifts*

Amino acid site		Wavelength	
Butterfly	Bovine	510–522 nm	530–534 nm
62[a]	43	F	V
63[b]	44	M	I
64[c]	45	A	G
65[d]	46	V	I
173	154	L	M
183[e]	164	A	S
214	N/A	T	S
304	274	S	A

[a]Amino acid 43 is part of the region that surrounds the Schiff base (Palczewski et al., 2000).

[b]M44T substitution in bovine rhodopsin causes a 3 nm blue-shift (Andres et al., 2003).

[c]F45L substitution in human rhodopsin causes autosomal dominant retinitis pigmentosa (ADRP) (Sung et al., 1991).

[d]L46R substitution in human rhodopsin causes ADRP (Rodriguez et al., 1993).

[e]Corresponds to human red cone pigment amino acid 180; S180A substitution causes a 5 nm blue-shift (Asenjo et al., 1994).

Most of the Nymphalini species we examined visually had eyeshine that was similar to that of *Vanessa* (Fig. 2). Tapetal reflectance for *Nymphalis*, *Inachis* and *Vanessa* is high in the red, out to at least 680 nm (Fig. 3B). An exception is *Junonia,* which has blue eyeshine owing to a modification of the tapetal structure in which wavelengths of light above 600 nm are poorly reflected. *Siproeta* is an intermediate case in which tapetal reflectance is low for wavelengths greater than 620 nm.

Orange eyeshine has been reported in *Inachis io* and *Polygonia c-album* (Stavenga, 2002a), which are both members of the Nymphalini subfamily. Our phylogenetic analysis of the LW opsin gene as well as phylogenetic studies of the *EF1alpha* and *wingless* genes (Wahlberg et al., 2003) indicates that *Nymphalis antiopa* is most closely related to *Vanessa cardui*. We also find that *V. cardui* shares orange eyeshine with *Nymphalis antiopa* and *Inachis io.* This observation, in combination with previous studies (Stavenga, 2002b) that found that most Nymphalini butterflies have yellow-orange eyeshine, tentatively suggests that the orange eyeshine may be the ancestral Nymphalini eyeshine state, whereas the distinctive blue eyeshine we find in *Junonia coenia* is a trait of recent origin that likely represents a change in tapetal development. We also note that we did not observe any dramatic dorsal-ventral differences in the eyeshine of any of the included species, as has been reported for other nymphalids, e.g. *Bicyclus anynana*, *Pararge aegeria*, *Hypolimnas anthedon* (Stavenga, 2002b; Stavenga, 2002a). With an expanded collection of butterfly eyeshine data mapped onto a robust phylogeny of the nymphalids (e.g. Wahlberg et al., 2003), it would be interesting to estimate how frequently dorsal-ventral eyeshine patterning differences have evolved within butterflies in order to begin to identify ecological factors correlated with their evolution.

From the notable lack of saturated red ommatidial eyeshine, and measured reflectance spectra that have high reflectivity in the blue, we conclude that there is no evidence for the presence of photostable filtering pigments within the surveyed Nymphalini. From our photochemical experiments with multiple cutoff filters we conclude that there is only a single long wavelength-sensitive rhodopsin present. Indeed an extensive PCR-based screen of the eye-specific cDNA of *Nymphalis antiopa* indicates the presence of only three opsins, UV, blue and LW (M. P. Sison-Mangus and A.D.B., unpublished data). This result suggests that the color vision system of *Junonia, Nymphalis*, *Siproeta*, *Polygonia*, *Inachis* and *Vanessa* (the Nymphalini 'eye ground plan') is probably trichromatic in its most fundamental aspects, like that of the hawkmoth, *Manduca sexta* (White et al., 2003), a prediction that can be tested behaviorally using the paradigms of Kelber et al. (2003) and Kinoshita et al. (1999). [The dorsal retina, which in *V. cardui*, expresses almost entirely UV and green-sensitive opsins, may be employed for brightness or contrast discrimination, while the ventral retina, which expresses all three opsins (Briscoe et al., 2003), is probably functionally trichromatic.]

Spectral tuning of the long wavelength sensitive rhodopsins of Nymphalini

Since we found no evidence of either a second LW rhodopsin in any of the investigated species, or of red lateral-filtering pigments that would produce two or more receptor types from one rhodopsin, our observations have permitted the exploration of the relationship between the peak absorbance of the rhodopsin and its genotype.

Intriguingly, we found evidence that the amino acid substitution homologous to S180A in visual pigments of human cones causes a similar blue-shift of absorption spectrum in the visual pigments of butterflies. This suggests that some of the same polymorphic sites that are involved in generating red-green color vision in New World primates may be segregating in butterflies.

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