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Beauty in the eye of the beholder: the two blue opsins of lycaenid butterflies and the opsin gene-driven evolution of sexually dimorphic eyes

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Summary

Although previous investigations have shown that wing coloration is an important component of social signaling in butterflies, the contribution of opsin evolution to sexual wing color dichromatism and interspecific divergence remains largely unexplored. Here we report that the butterfly *Lycaena rubidus* has evolved sexually dimorphic eyes due to changes in the regulation of opsin expression patterns to match the contrasting life histories of males and females. The *L. rubidus* eye contains four visual pigments with peak sensitivities in the ultraviolet (UV; λ_{max}=360 nm), blue (B; λ_{max}=437 nm and 500 nm, respectively) and long (LW; λ_{max}=568 nm) wavelength range. By combining *in situ* hybridization of cloned opsin-encoding cDNAs with epi-microspectrophotometry, we found that all four opsin mRNAs and visual pigments are expressed in the eyes in a sex-specific manner. The male dorsal eye, which contains only UV and B (λ_{max}=437 nm) visual pigments, indeed expresses two short wavelength opsin mRNAs, *UVRh* and *BRh1*. The female dorsal eye, which also has the UV and B (λ_{max}=437 nm) visual pigments, also contains the LW visual pigment, and likewise expresses *UVRh*, *BRh1* and *LWRh* mRNAs. Unexpectedly, in the female dorsal eye, we also found *BRh1* co-expressed with *LWRh* in the R3-8 photoreceptor cells. The ventral eye of both sexes, on the other hand, contains all four visual pigments and expresses all four opsin mRNAs in a non-overlapping fashion. Surprisingly, we found that the 500 nm visual pigment is encoded by a duplicate blue opsin gene, *BRh2*. Further, using molecular phylogenetic methods we trace this novel blue opsin gene to a duplication event at the base of the Polyommatine+Thecline+Lycaenine radiation. The blue opsin gene duplication may help explain the blueness of blue lycaenid butterflies.

Supplementary material available online at
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Key words: eye evolution, sexual selection, visual pigment, color vision, butterfly, *Lycaena rubidus*.

Introduction

Ever since Darwin's time (Darwin, 1874), there has been intense interest in understanding the origins and evolution of sexually dimorphic traits. Butterflies offer spectacular examples of sexual dimorphism associated with wing color variation between males and females (sexual dichromatism). The use of wing color cues in speciation (Silberglied, 1979) and sexual selection (Obara, 1970) has long been recognized. Sympatric butterfly relatives that look similar from the human perspective are readily distinguished, for instance, by UV-based signals (Silberglied, 1979). Moreover, females have been shown to use species-specific UV reflectance patterns to identify mates (Silberglied and Taylor, 1973; Rutowski, 1977; Robertson and Monteiro, 2005). Nonetheless, discussions of the evolution of butterfly wing colors in relation to visually mediated components of courtship have typically been put forth in the absence of information about sensory receptors.

Previous studies of both butterfly wing color cues and color vision have focused on papilionid, pierid or nymphalid families (Kelber et al., 2003). Little attention, however, has been paid to the youngest of butterfly families, the riodinids or the lycaenids, because they tend to be small and hard to distinguish to the human eye. Lycaenids in particular comprise the second largest of the butterfly families, with more than 4000 species named worldwide, many of which are found in South America (Johnson and Coates, 1999). With a mounting abundance of physiological (Eguchi et al., 1982; Kinoshita et al., 1997), molecular (see below) and behavioral data (Zaccardi et al., 2006) pointing to phenotypically variable butterfly visual systems, it seems increasingly important to consider the

evolution of butterfly wing color in the context of the evolution of their eyes.

Opsins, together with a light-sensitive chromophore, form the visual pigments expressed in the photoreceptor cells (R1-9) of the lepidopteran compound eye. Most lepidopteran eyes contain a minimum of three visual pigments with absorbances that peak at ~350 nm (UV), 440 nm (blue, B) and 530 nm (long wavelength, LW) (reviewed in Briscoe and Chittka, 2001). In the sphingid moth, the painted lady and monarch butterfly, these visual pigments are encoded by paralogous UV, B and LW opsin genes, which were present in the ancestor of all lepidopterans (Briscoe et al., 2003; White et al., 2003; Sauman et al., 2005). Deviations from this basic plan have been described in the most primitive of butterfly families, the papilionids, in which two rounds of duplication of the LW eye opsin gene have occurred (Briscoe, 1998; Kitamoto et al., 1998; Briscoe, 2001); and in the pierids, in which a duplicate blue opsin gene has been reported (Arikawa et al., 2005). In all butterfly species studied, opsin mRNA expression in the eye is similar between the sexes: the R1 and R2 photoreceptor cells express either UV or B opsin mRNAs and the R3-9 photoreceptors express LW opsin mRNAs.

Along with sexually dimorphic wings, sexually dimorphic eyes have likely evolved multiple times in butterflies. To determine whether opsins have in fact played a role in the evolution of sexually dimorphic eyes, we used a combination of physiological, molecular and anatomical approaches to examine the adult eye of the ruddy copper butterfly *Lycaena rubidus* (Lycaenidae), a species with both sexually dimorphic wing coloration and sexually dimorphic eyeshine. The small North American genus *Lycaena* consists of 16 species in which males are often brilliantly colored iridescent copper, blue, red-orange or purple, while females are muted or predominantly gray (Glassberg, 2001; Pratt and Wright, 2002). Females tend to mate only once (Gage et al., 2002) so selection for correct mate choice is intense.

The opsins of *L. rubidus* are also of particular interest, because, rather than the usual three visual pigments, the *L. rubidus* eye contains four with peak absorbances (λ_{max} values) at 360, 437, 500 and 568 nm that are distributed differentially both dorso-ventrally and between the sexes (Bernard and Remington, 1991). As duplicate LW opsin genes have been reported only in the most ancient of butterfly families (Papilionidae), encoding 515, 520 and 575 nm visual pigments (Arikawa, 2003), the 500 and 568 nm visual pigments of the more derived *L. rubidus* represent good candidates for being an independent LW opsin gene duplication. We were therefore interested in investigating whether parallel duplication of LW opsin genes had occurred between papilionid and lycaenid butterflies.

After cloning the *L. rubidus* opsin-encoding cDNAs, we determined by *in situ* hybridization both dorsal-ventral and sex-specific differences in the opsin mRNA expression patterns in the eye. To our surprise, we discovered that the *BRh2* mRNA, which is exclusively expressed in the ventral eye, encodes the 500 nm visual pigment. Rather than being the result of a LW opsin gene duplication, it has actually evolved from a blue opsin gene. We screened eye cDNA libraries from an additional ten butterfly taxa and, using phylogenetic analyses, traced the gene duplication event to the base of the Polyommatine+Theclinine+Lycaenine radiation. Together, the 437 and 500 nm visual pigments may enhance color vision in the blue (400–500 nm) part of the visible light spectrum. We hypothesize that the blue opsin gene duplication and the associated evolution of the 500 nm visual pigment may be causally linked to the radiation of this family that is famous for blue butterflies, including Nabokov's Blues (Tribe Polyommatini) (Nabokov, 1945). While cues from UV, LW and polarized light (Silberglied, 1984; Jiggins et al., 2001; Fordyce et al., 2002; Sweeney et al., 2003) have been recognized as important social signals in butterfly communication, our data suggest that blue cues in blue butterflies may provide an equally important signal for visual communication.

Materials and methods

Reflectance spectra measurements

Procedures for using an epi-microspectrophotometer to make photochemical measurements from butterfly eyeshine were previously described (Bernard, 1983a; Bernard, 1983b; Briscoe et al., 2003; Briscoe and Bernard, 2005). Briefly, an intact *Lycaena rubidus* (Lycaenidae) (either female or male) was mounted in a slotted plastic tube fixed to the goniometric stage, then oriented to set the eye's direction of view to elevation 40° and azimuth 15°. The microscope objective was a Leitz 8X/0.18P. The aperture stop of the illuminator was set to create eyeshine in 20 ommatidia, then 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.

Eyeshine photographs

Photographs of eyeshine were created by exchanging the photometer head for a micro-photographic attachment. The illuminator slide was replaced with a Leitz Mecablitz-III micro-flash. Film was Kodak ASA160 Daylight-Ektachrome. The microscope was focused on the cornea with aperture stop fully open. After 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 flash. 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.

Tissue collection

Tissues used for total RNA extraction and cDNA synthesis were either collected by the authors (*Agriades glandon* de Pruner, *Colias philodice* Godart and *Satyrium behrii* Edwards) or provided as gifts by the following individuals (*Apodemia mormo* Felder & Felder, John Emmel; *Basilarchia arthemis astyanax* Fabricius, Austin Platt; *Bicyclus anynana* Butler,

Antónia Monteiro; *Heliconius melpomene* Linnaeus, Larry Gilbert; *Lycaena rubidus* Behr, Carol Boggs and Ward Watt; *Nymphalis antiopa* Linnaeus, Peter Bryant; *Polyommatus icarus* Rottemburg, Almut Kelber). *Vanessa cardui* Linnaeus were obtained from the Carolina Biological Supply Co (Burlington, NC, USA). Adult *L. rubidus* used for *in situ* hybridization were reared in the laboratory from wild-caught caterpillars collected at Tioga Pass, Mono County, CA, USA or collected from the Rocky Mountain Biological Laboratory, Gothic, CO, USA. Caterpillars were fed *Rumex crispus* leaves until pupation. Adults were fed 20% sugar water and sacrificed 1–2 days after eclosion for tissue fixation and sectioning.

PCR, cloning and sequencing

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA). Adaptor-ligated double-stranded complementary DNA (cDNA) was synthesized from total RNA using the Marathon cDNA Amplification Kit (BD BioSciences Clontech, Mountain View, CA, USA). To obtain the complete cDNA sequences, 3′RACE products were first amplified with a degenerate primer using *ExTaq* DNA polymerase (TaKara Mirus Bio, Madison, WI, USA) under PCR conditions of 2 min at 94°C, then 35 cycles of 30 s at 94°C, 30 s at 50°C and 1 min at 68°C. Bands >500 bp were gel purified (Geneclean I Kit, Q-Biogene, Irvine, CA, USA), then ligated into the pGEM T-easy vector (Promega, Madison, WI, USA). The plasmids were prepared with the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA, USA). Clones were screened by *Eco*RI digest. Clones were cycle sequenced using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the core sequencing facilities at the University of California, Irvine.

Multiplex PCR using opsin-specific primer pairs (supplementary material Table S1) was also performed on 3′RACE clones to identify other potential opsins that had not yet been sequenced as well as to eliminate the ones already sequenced. To obtain the 5′RACE products, gene-specific reverse primers and a touch-down PCR protocol with the BD Advantage Polymerase Kit (BD Biosciences, San Jose, CA, USA) was used as follows: 1 min at 95°C, 5 cycles of [30 s at 95°C, 1.5 min at 68°C], 5 cycles of [30 s at 95°C, 30 s at 65°C and 1.5 min at 68°C], 5 cycles of [30 s at 95°C, 30 s at 60°C and 1.5 min at 68°C], 25 cycles of [30 s at 95°C, 30 s at 55°C and 1.5 min at 68°C] and 10 min at 68°C.

Phylogenetic analysis of opsins

Cloned *L. rubidus* opsin sequences were aligned in MEGA 3.1 (Kumar et al., 2004) together with 44 homologous insect opsin sequences downloaded from GenBank. Only sequences with complete coding regions were used in the alignment. A neighbor-joining tree sampled from 1000 bootstrap replicates was constructed from the amino acid alignment with Poisson correction (MEGA 3.1). A total of 299 amino acid sites were used in the tree reconstruction.

Phylogenetic reconstructions of the lepidopteran blue opsins were performed with PAUP* (Swofford, 2000) using the maximum likelihood method (general-time-reversible, with a gamma substitution correction and a proportion of invariant sites; model parameters estimated from the data). Estimates of the proportion of invariant sites and gamma were then made using this initial tree and used to run 500 ML bootstrap replicates in PhyML (Guindon and Gascuel, 2003; Guindon et al., 2005).

In situ hybridization

Butterfly heads were fixed in 4% phosphate-buffered formaldehyde for 2 h and then immersed in a sucrose/1× PBS (phosphate buffered saline) gradient of 10%–30% for 2 h at each step. The tissues were stored in 30% sucrose at 4°C. Tissues were embedded in OCT freezing compound (Sakura Finetek USA, Torrance, CA, USA) and cryostat-sectioned into 16 μm slices. Riboprobes were generated from cloned opsin templates that were linearized *via* PCR. Sense/anti-sense riboprobes were synthesized from 1 μg template using the DIG RNA Labeling Kit (Roche Applied Science, Indianapolis, IN, USA). The riboprobe yield was quantified by dot blot procedure. Sections were immersed in hybridization buffer for 30 min at 60°C. Approximately 0.05 μg riboprobe per μl hybridization buffer was hybridized to the histologic sections overnight at 60°C. Washing, detection and mounting methods are as described (Briscoe et al., 2003). Slides were viewed under an Axioskop microscope (Zeiss, Thornwood, NY, USA) using bright field illumination. Digital photographs were captured in an Axiocam digital camera (Zeiss) attached to the microscope. Photographs were processed in Adobe Photoshop 7.0 for size, brightness and contrast modification only.

Transmission electron microscopy

The tissue was dissected in 0.08 mol l^{-1} phosphate buffered 2% glutaraldehyde/formaldehyde with 4% sucrose and fixed for 2 h at 4°C. After washing in buffer solution and postfixation with 2% OsO_4 at 4°C for 2 h, the specimens were dehydrated in ethanol and embedded in Epon 812 (Roth, Karlsruhe, Germany). For histological investigations of the filtering pigments, semithin sections were cut and investigated with an Axiophot microscope (Zeiss, Oberkochen, Germany) with differential interference contrast. A F-View II-Camera (Soft Imaging System GmbH, Münster, Germany) was used to collect images. Corresponding ultrathin sections were cut, mounted on grids, and after staining with uranyl acetate and lead citrate for 10 min each, they were examined with an EM 10 electron microscope (Zeiss, Oberkochen, Germany). Images were taken with a ProScan Slow Scan CCD camera (LEO Electron Microscopy, Oberkochen, Germany). All photographs were processed in Adobe Photoshop 7.0 for size, brightness and contrast modification only.

Results

Anatomical overview of L. rubidus ommatidia and non-opsin pigment expression

The basic unit of the butterfly compound eye is the ommatidium, which is comprised of eight elongate

photoreceptor cells (R1-8) and a small basal ninth photoreceptor cell (R9) (Fig. 1A). The visual pigment-containing microvillar membranes produced by each photoreceptor cell are fused into a single optical structure known as a rhabdom (Fig. 1A,B). As some butterflies also have colored filtering pigments that coat the rhabdom and modify the wavelengths of light available to photoisomerize the visual pigments (Ribi, 1978), we used light microscopy to examine the *L. rubidus* adult retina for the presence or absence of non-opsin filtering pigments.

We found two kinds of pigment granules, the first of which was the dark purple pupillary pigment present distally in R1-8 cells everywhere in the eye (data not shown) (Fig. 1A), including the dorsal rim area, that regulates the amount of light entering the eye. The second pigment type was a pink filtering pigment that was absent in dorsal eye ommatidia (Fig. 1B) and in the R5-8 cells of some, but not all, ventral eye ommatidia (Fig. 1C, arrowheads and arrow, respectively). There was no sex difference in the distribution of the two non-opsin filtering pigments.

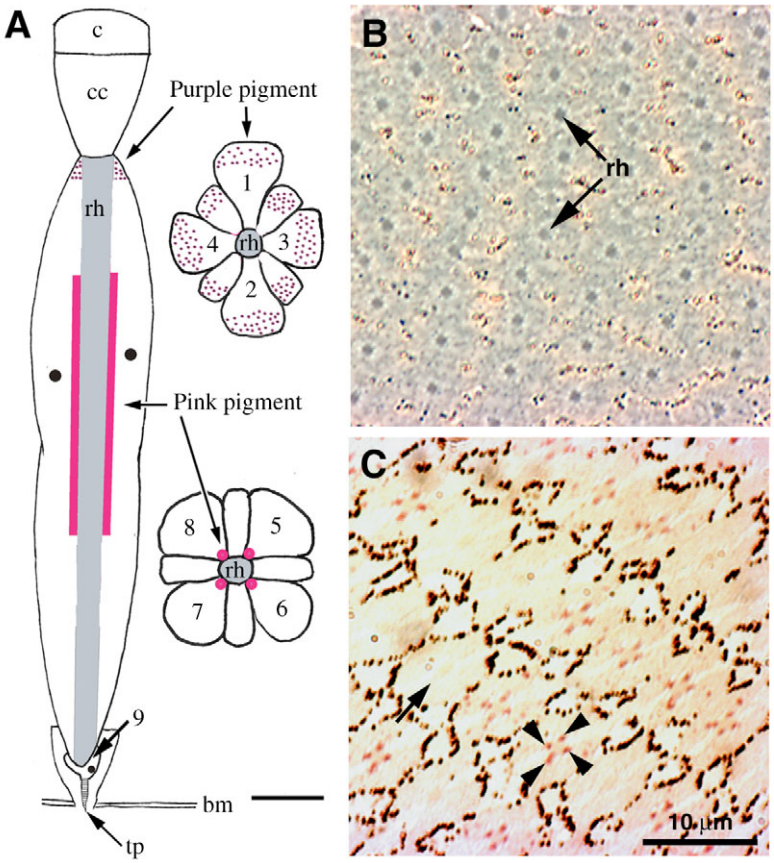


Fig. 1. Anatomical overview of an *L. rubidus* ommatidium and non-opsin pigment expression. (A) Diagram of a typical ommatidium. Longitudinal (left) and transverse (right) view of an ommatidium located in the ventral domain of the eye. Purple pupillary pigments are present in R1-8 photoreceptor cells regulating the amount of light entering the ommatidium. (B) The dorsal eye lacks the pink filtering pigment. Transverse unstained Epon sections were examined by DIC microscopy. Arrows denote rhabdoms (rh). (C) In the ventral eye, most ommatidia contain a pink filtering pigment (arrowheads), which is lacking in other ommatidia (arrow). Basement membrane (bm), cornea (c), rhabdom (rh), tapetum (tp), crystalline cones (cc). Scale bars, 5 μm (A), 10 μm (B).

Eyeshine and visual pigment distribution in dorsal eye

Butterfly eyeshine in *L. rubidus*, a species with sexually dimorphic wing coloration (Fig. 2A,B), is produced by light reflected by multilayered tracheolar mirrors (tapeta) at the base of each ommatidium (Miller, 1979) (Fig. 1A), following its partial absorption by visual pigments in the rhabdom. The coloration of eyeshine is not simply explained, as it depends on the absorbance spectra of all rhodopsins contained with the rhabdom and the reflectance spectrum of the tapetum. It may also be influenced by the presence of non-opsin filtering pigments (Ribi, 1979; Douglas and Marshall, 1999; Stavenga, 2002a; Briscoe and Bernard, 2005). Furthermore, bright illumination can change the coloration of eyeshine by photo-converting rhodopsins to spectrally shifted photoproducts.

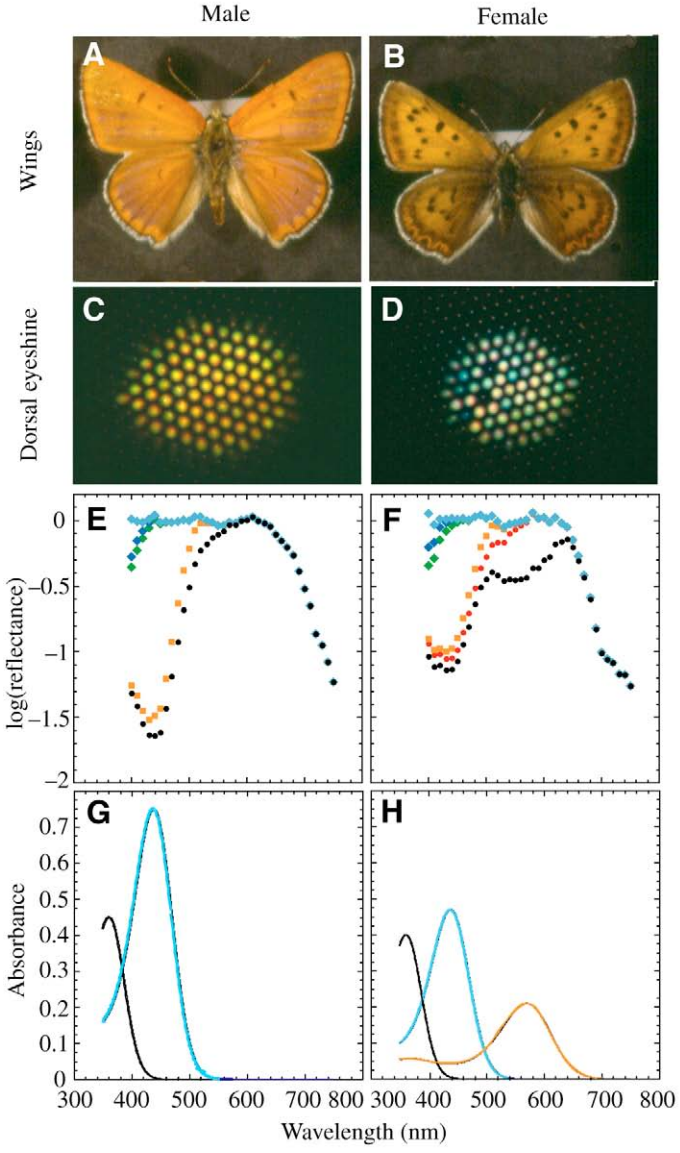
We examined the dorsal eyeshine of adult *L. rubidus* using brightfield epi-illumination and confirmed the substantial differences between the sexes previously reported (Bernard and Remington, 1991). Eyeshine of *L. rubidus* males is predominantly green–orange, while that of females is blue–yellow (Fig. 2C,D). Green–yellow eyeshine has also been reported in dorsal eye of *L. phlaeas* of unspecified sex (Stavenga, 2002b). To determine whether differences in bandwidth of the tapetal reflectance spectra might account for this difference, we used transmission electron microscopy to examine the ultrastructure of the tapeta of male and female dorsal eye. We could not detect, however, any difference in optical design of the tapetum (data not shown). As previously noted, Epon sections of the eye also revealed an absence of the heterogeneously expressed pink filtering pigments in the dorsal eye that was detected in the R5-8 photoreceptor cells of some ommatidia in the ventral eye of both sexes (Fig. 1C). Therefore the dorsal eyeshine pattern is not influenced by differences in tapetal structure or non-opsin filtering pigments.

We next estimated the visual pigment content of the male and female dorsal eyes from experimental reflectance spectra using epi-microspectrophotometric methods, as described (Bernard, 1982; Bernard, 1983a; Bernard, 1983b; Bernard and Remington, 1991; Briscoe et al., 2003). A computational analysis of these spectra revealed two short-wavelength visual pigments in the dorsal eyes of both sexes (λ_{max}=360 and 437 nm) (Fig. 2E–H). In addition, the female dorsal eye has an LW visual pigment (λ_{max}=568) not found in the male dorsal eye (Fig. 2E–H). The ventral eye of *L. rubidus* has been shown previously to contain a fourth visual pigment (λ_{max}=500 nm)

(Bernard and Remington, 1991). Thus, the eyeshine and reflectance spectra data confirm the presence of three visual pigments in *L. rubidus*, which are distributed in sexually dimorphic patterns in dorsal eye.

Opsin sequences and phylogeny

We cloned the full-length cDNAs of four opsin genes, which, based upon phylogenetic analyses (supplementary material Figs S1–S4), represent a UV opsin (*UVRh*, AY587904), two B opsins (*BRh1*, AY587902; BRh2, AY587903) and a LW opsin (*LWRh*, AY587901). The lack of a duplicate LW opsin was unexpected. We note that the short wavelength opsins have a lysine at residue 112 (UVRh) and a glutamate at residue 107 (BRh1 and BRh2), which confer UV-absorbing (lysine) and blue-absorbing (glutamate) spectra (Salcedo et al., 2003). The situation in *L. rubidus* differs markedly from that of the nymphalid butterflies *Vanessa cardui* or the monarch *Danaus plexippus*, in which only three opsin mRNAs are expressed (UV, B, and LW) in the photoreceptor cells of the adult eye (Briscoe et al., 2003; Sauman et al., 2005), or that of the swallowtail butterfly, in which three duplicate LW opsin genes are expressed (Briscoe, 1998; Kitamoto et al., 1998). The *L. rubidus* opsin expression pattern does, however, resemble the situation in *Pieris rapae*, in which duplicate B opsin genes are also expressed in the eye (Arikawa et al., 2005) (but see below).



Sexually dimorphic expression of LWRh

Since our microspectrophotometric results indicate that the dorsal eye of female *L. rubidus* contains a 568 nm visual pigment that is absent from the dorsal eye of males (Fig. 2E–H), we examined the expression of the *LWRh* mRNA to determine if it encodes this LW visual pigment. We found that the expression of *LWRh* mRNA in the retina indeed differed between the sexes. In males, *LWRh* was only expressed in photoreceptor cells R3 through R8 in all ommatidia in the ventral region of the eye, but the transcript was totally absent in the dorsal area (Fig. 3A). In contrast, *LWRh* in females was expressed uniformly in R3-8 of all ommatidia in both the dorsal and ventral regions of the eye (Fig. 3B and inset). We note that the expression of a LW opsin mRNA in the R3-8 photoreceptor cells is the ancestral state for Lepidoptera (Briscoe et al., 2003) (see below), so its absence in dorsal eye of males is unprecedented.

Co-expression of LWRh and BRh1 in female dorsal retina

Next we localized the expression of the gene encoding the blue-sensitive visual pigment (λ_{max}=437 nm), identified through our photochemical experiments in *L. rubidus* (Fig. 2E–H), to the dorsal eye of both males and females. In both sexes, *BRh1* was abundantly expressed in the R3-8 cells of the dorsal eye and, in addition, expressed in the R1 and R2 cells of the ventral eye (Fig. 3C,D). Unexpectedly, in females, we found that the *BRh1* mRNA was co-expressed with the *LWRh* mRNA in R3-8 of all the ommatidia in the dorsal eye (Fig. 3B,D insets). This co-expression pattern was not found in males because of the absence of the *LWRh* mRNA dorsally

Fig. 2. Sex differences (A,C,E,G, males; B,D,F,H, females) in wing color pattern, eyeshine, eye reflectance spectra and visual pigment absorbance spectra. (A,B) (A) UV-reflecting scales (iridescent purple) on the lower forewing and outer hindwing margins of males, (B) Non-UV-reflecting scales on wings of females. Reflectance spectra of both male and female dorsal wings are shown elsewhere (Bernard and Remington, 1991). (C,D) Eyeshine from the dorsal eye of (C) a male and (D) a female showing strongly sexually dimorphic coloration. (E,F) Analysis of experimental reflectance spectra (black dots) from dorsal eye ommatidia of (E) males and (F) females, based on a computational model of pigment content; spectrum was obtained after having stripped the visual pigment with λ_{max}=568 nm (P568) (red circles, female only); metarhodopsin 495 nm (orange squares); 437 nm (green diamonds), retinoid-binding protein 395 nm (dark blue diamonds); and 360 nm (light blue diamonds). (G,H) Absorbance spectra for the visual pigments estimated from epi-microspectrophotometry in the dorsal eyes of males (E, λ_{max}=437 nm and 360 nm) and females (F, λ_{max}=568 nm, 437 nm and 360 nm).

(see Fig. 3B). Of 177 ommatidia counted in the female dorsal eye, in which adjacent tangential sections were stained alternately by *LWRh* and *BRh1* riboprobes, all of the R3-8 photoreceptor cells were labeled by both riboprobes. This co-localization result, along with the results of our microspectrophotometric experiments, indicate that each R3-8 photoreceptor cell in female dorsal eye of *L. rubidus* expresses two different visual pigments. This is a striking finding, because, to our knowledge, there are no other reports of co-expression of a short and long wavelength-sensitive opsin in butterflies.

Dorsal-ventral patterning of UVRh and BRh2 expression

To confirm the identity of the UV visual pigment (λ_{max}=360 nm) detected by microspectrophotometry in *L. rubidus* dorsal eye (see Fig. 2E–H), we examined the distribution of the *UVRh* mRNA. *UVRh* expression also showed variation in its dorsal-ventral distribution across the retina in both males and females, as expression was more abundant in the dorsal area (Fig. 3E,F). The only visual pigment not detected in dorsal eye of either sex was the 500 nm visual pigment that was previously detected by epi-microspectrophotometry to be exclusively in the ventral eye of both sexes (Bernard and Remington, 1991). Consistent with the epi-microspectrophotometric result, we found *BRh2* to be the only opsin transcript exclusively localized to ventral eye (Fig. 3G,H). Therefore, we conclude that *BRh2* encodes the 500 nm visual pigment.

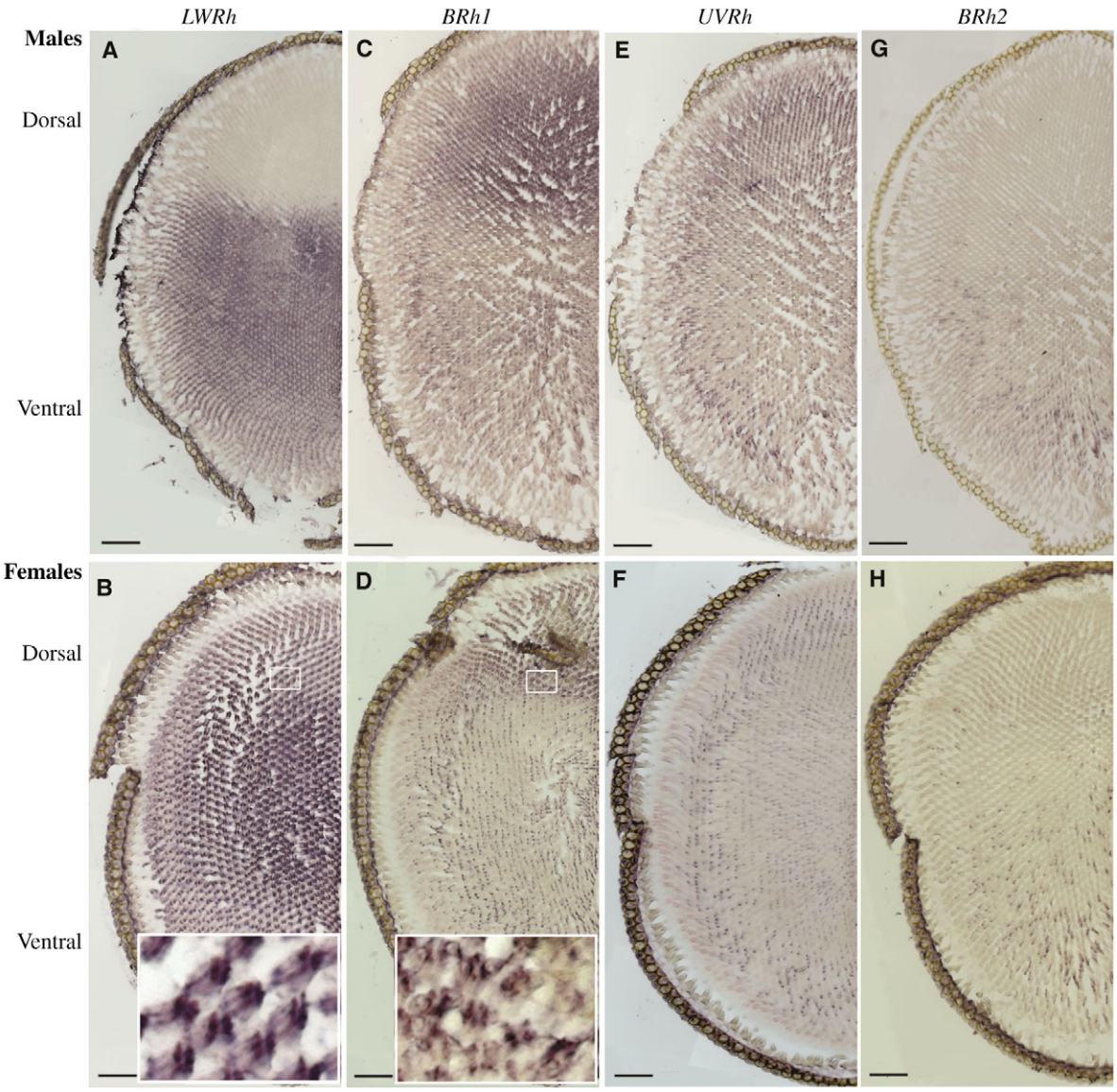


Fig. 3. Sexually dimorphic opsin expression (A,C,E,G, males; B,D,F,H, females). *LWRh* expression in male eye (A) and female eye (B). Inset in B shows expression in R3-8. *BRh1* expression in male (C) and female (D). Inset in D shows expression in R3-8. *UVRh* expression in male (E) and female (F). *BRh2* expression in male (G) and female (H). Tangential sections are shown for each panel. Scale bars, 100 μm.

Non-overlapping expression of UVRh, BRh1 and BRh2

Unlike the unusual *BRh1* mRNA expression in the outer R3-8 photoreceptor cells of the *L. rubidus* dorsal eye, the typical lepidopteran expresses short wavelength opsin mRNAs only in the R1 and R2 cells (Kitamoto et al., 2000; Briscoe et al., 2003; White et al., 2003; Sauman et al., 2005). Closer inspection of *UVRh*, *BRh1* and *BRh2* expression in individual ommatidia indicated that these transcripts are also present in the R1 and R2 photoreceptor cells of both sexes (Fig. 4). In the ventral and dorsal parts of the eye, adjacent sections showed that the three opsin mRNAs have non-overlapping expression in the R1 and R2 photoreceptor cells. In the ventral retina, five different types of ommatidia with respect to non-overlapping *UVRh, BRh1* and *BRh2* expression in R1 and R2 photoreceptor cells can be seen (Fig. 4A–C): ommatidia containing *UVRh-UVRh*, *UVRh-BRh1*, *UVRh-BRh2*, *BRh1-BRh1* and *BRh1-BRh2* (supplementary material Table S2). *BRh2* mRNA expression in both R1 and R2 was also observed in the ventral area, although this ommatidial subtype was rare (data not shown). We note the finding of *BRh1* opsin mRNA in the ventral eye differs from the original report (Bernard and Remington, 1991) in which the 437 nm visual pigment was not found in ventral eye. Our finding is, however, consistent with the report in the same paper of the presence of P437 in the ventral eye of *L. heteronea.* The differences between our finding and that of Bernard and Remington (Bernard and Remington, 1991) may be due to relative differences in visual pigment abundances between species and also to differences in the sensitivity of the detection methods.

Interestingly, the same ommatidia in ventral retina that expressed *BRh2* co-expressed the pink filtering pigment (supplementary material Fig. S5). Since a pink filtering pigment absorbs the short-wavelength part of the light spectrum, the 568 nm spectral sensitivity of the LWRh visual pigment in the R5-8 photoreceptor cells will be modified to have a red-shifted absorbance. It is therefore possible that *L. rubidus* has pentachromatic vision in ventral retina based primarily on four opsins and this pink non-opsin pigment. Filtering pigments of similar effect have been observed for the LW receptors of *Papilio xuthus* and *Pieris rapae* (Arikawa et al., 1999; Wakakuwa et al., 2004), and have been shown to be important for butterfly color vision (Zaccardi et al., 2006).

In the dorsal eye, R1 and R2 photoreceptors expressed predominantly *UVRh* (Fig. 4D), while *BRh2* expression was never observed (Fig. 4F). *BRh1* mRNA was also expressed in R1 or R2 or both, but these ommatidial subtypes were rare (Fig. 4E, insets). Instead, *BRh1* mRNA was predominantly expressed in R3-8 of all ommatidia in the dorsal eye (Fig. 4E, section taken from a different male individual). We

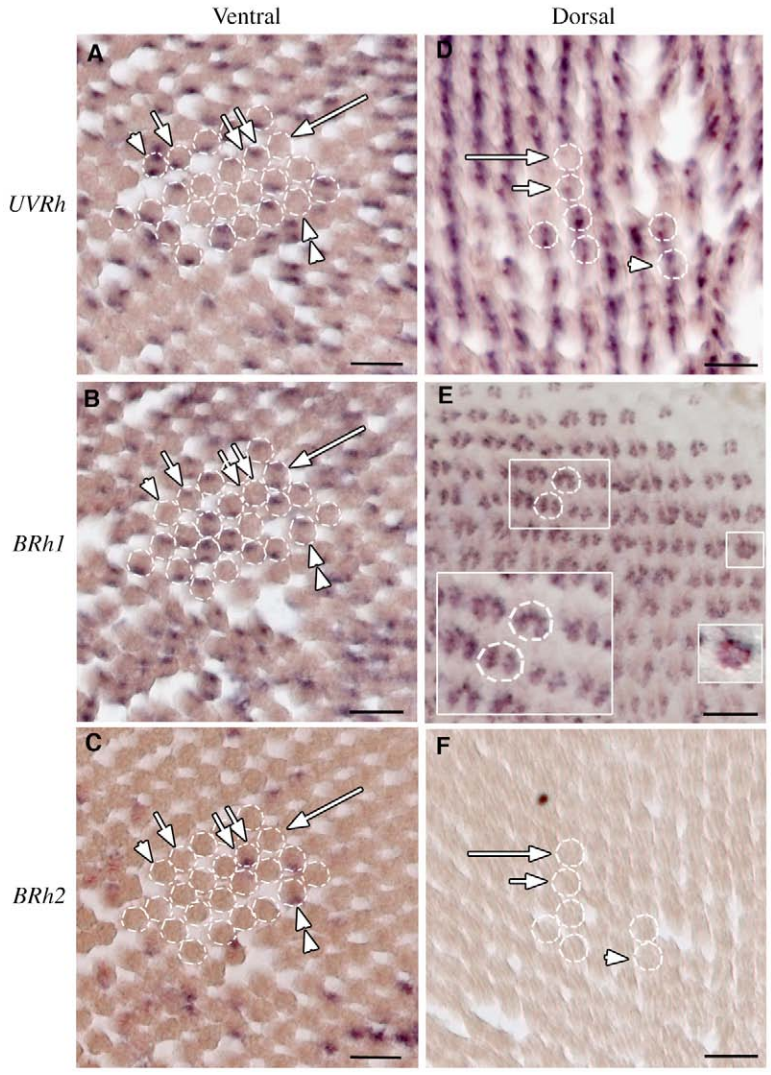


Fig. 4. Non-overlapping *UVRh*, *BRh1* and *BRh2* expression in R1 and R2 photoreceptor cells. (A–C) Ventral views. (A) Tangential section showing *UVRh* expression in R1 and R2 photoreceptor cells in the ventral retina. (B) Section adjacent to A showing *BRh1* mRNA expression in R1 and R2 cells in the ventral retina that was non-overlapping with *UVRh* expression. Broken circles in each panel indicate identical ommatidia in adjacent sections. (C) Section adjacent to B showing *BRh2* expression in R1 and R2 cells in the ventral retina that was non-overlapping with *UVRh* and *BRh1* mRNA expression. Five different types of ommatidia are depicted in each panel: *UVRh-UVRh* (one arrowhead), *UVRh-BRh1* (short arrow); *UVRh-BRh2* (double short arrows); *BRh1-BRh1* (long arrow); *BRh1-BRh2* (double arrowheads). A sixth combination *BRh2-BRh2* was also observed (data not shown). (D–F) Dorsal views. (D) Tangential section showing *UVRh* expression in nearly all R1 and R2 cells in the dorsal retina. (E) Tangential section showing *BRh1* expression in a small number of R1 and R2 (and all R3-R8) cells in the dorsal retina. Inset (big square) shows *BRh1* expressed in R1, as well as R3-8 cells; small square shows *BRh1* expressed in R1-R8 cells. (F) Section adjacent to D showing no *BRh2* mRNA expression in dorsal retina. Broken circles indicate identical ommatidia in adjacent sections. Three different types of ommatidia were found: *UVRh-UVRh* (arrowhead), *UVRh-BRh1* (short arrow), *BRh1-BRh1* (long arrow).

did not examine opsin expression in the dorsal rim area (DRA) ommatidia.

Rhabdoms of the main retina are organized for color vision

In *Drosophila* (Wernet et al., 2003) and monarch butterflies (Sauman et al., 2005), the ommatidia of the DRA have a unique pattern of opsin expression compared to the main retina – this region contains ommatidia with rhabdoms specialized for polarization vision (Labhart and Meyer, 1999; Stalleicken et al., 2006). One possible explanation for the dramatically altered pattern of opsin mRNA expression we identified in the dorsal part of the *L. rubidus* eye is that it represents an expansion of the DRA. To test this hypothesis, we examined the organization of the microvillous membranes that form the rhabdoms in ultrathin sections of ommatidia from the DRA, the dorsal eye, and the ventral eye using transmission electron microscopy (supplementary material Fig. S6). We found that *L. rubidus* do indeed have DRA ommatidia similar to those found in other butterflies (Labhart and Meyer, 1999), characterized by square-shaped rhabdoms that contain perpendicularly oriented microvilli that are specialized for polarized sky light detection (supplementary material Fig. S6A). The rhabdoms from ommatidia of both sexes in the dorsal and ventral parts of the main retina, on the other hand, were circular in shape (supplementary material Fig. S6B,C,D), consistent with the idea that they are organized for detecting the spectral content of light (i.e. colour of an object) (Stalleicken et al., 2006). This architecture was verified by observing the brush-shaped organization of the microvilli in longitudinal sections (supplementary material Fig. S6E).

Origins of a gene and a family of blue butterflies

The unexpected finding of duplicate B opsin genes in *L. rubidus* (Subfamily Lycaeninae) rather than the expected duplicate LW opsin genes led us to investigate their evolutionary origins. Within the true butterflies (Papilionoidea) the current understanding of familial relationships is (Papilionidae+(Pieridae+(Nymphalidae+(Riodinidae+Lycaenidae)))) (Wahlberg et al., 2005), where papilionid and pierid butterflies represent the most basal lineages, and riodinid and lycaenid the most derived. Because the two pairs of B opsin genes identified from pierid (*Pieris rapae*) (Arikawa et al., 2005) and lycaenid butterflies (this study) are from distantly related families, we were interested in testing the hypothesis that the blue opsin genes of pierids and lycaenids evolved independently. We therefore screened eye-specific cDNA libraries from an additional ten butterfly taxa from four families (supplementary material Table S3).

We cloned a total of 14 full-length blue opsin-encoding cDNAs from these ten additional taxa, including homologues of both *BRh1* and *BRh2* in all surveyed lycaenid subfamilies. Only one blue opsin cDNA was detected in each of the seven species of nymphalid surveyed. Neighbor-joining and maximum likelihood analyses using all three nucleotide positions unambiguously indicated that the blue opsin genes of *L. rubidus* evolved independently of the pierid duplicate blue opsin genes (Fig. 5). To our knowledge, no other insects besides pierid and lycaenid butterflies have two blue opsin genes. Our finding of independent duplication events is quite consistent with the very different λ_{max} values (425 nm and 453 nm) of the pierid blue visual pigments (Arikawa et al., 2005) compared to the lycaenid (λ_{max}=437 nm and 500 nm, respectively). Our results also indicate (bootstrap support=100%) that the *L. rubidus* blue opsin gene duplication event occurred before the radiation of the coppers, hairstreaks and blues. (Lycaeninae+Theclinae+Polyommatinae) (Eliot, 1973).

Evolution of a blue receptor and a sexually dimorphic eye

To explain the four major evolutionary innovations we have identified in *L. rubidus* eye (duplicate blue opsin cDNAs, novel R1 and R2 photoreceptor subtypes, co-expression of B and LW opsins and male-only *BRh1* opsin expression in R3-8 cells) we

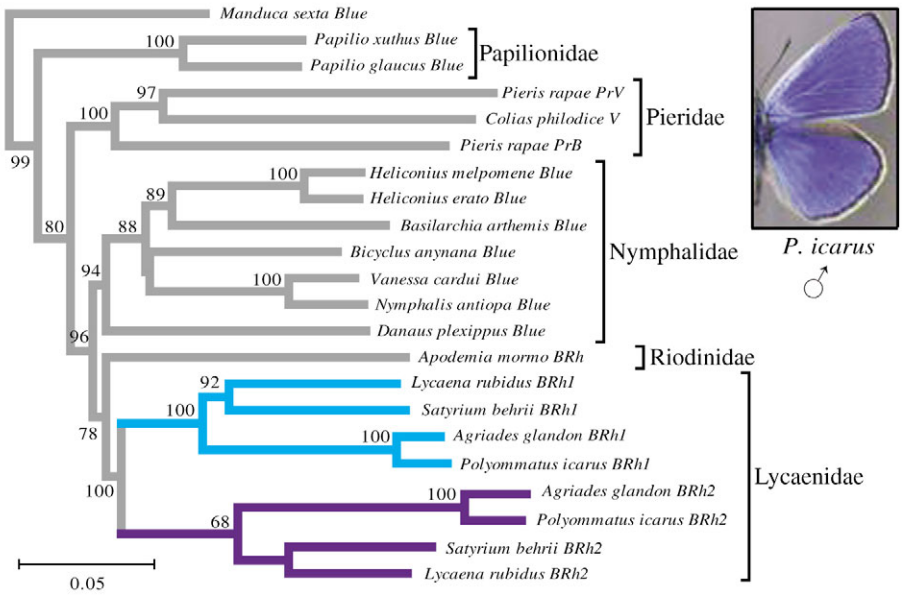


Fig. 5. Phylogeny of lepidopteran blue opsin genes. The tree is based upon a neighbor-joining (NJ) analysis of 1077 nucleotide sites, using Tamura-Nei distance and heterogeneous pattern of nucleotide substitution among lineages. Bootstrap values shown are based upon 500 maximum likelihood (ML) bootstrap replicates determined using the GTR+Γ+I model with estimated gamma shape parameter=0.574 and proportion of invariant sites=0.1474. GenBank accession numbers for sequences are provided in supplementary material Table S3. Inset: Dorsal wing of a male *Polyommatus icarus,* one of the lycaenids surveyed.

propose the following evolutionary scheme (Fig. 6): Nymphalids, the closest outgroup for which gene expression data are available, have three kinds of ommatidia: UV and B opsin mRNAs are expressed in R1 and R2 photoreceptor cells in a non-overlapping fashion, and the LW opsin mRNA is expressed in the R3-8 photoreceptor cells (Briscoe et al., 2003; Sauman et al., 2005; Zaccardi et al., 2006). This pattern is also found in the moth *Manduca sexta* (White et al., 2003), and likely represents the ancestral lepidopteran eye plan. *L. rubidus* differs from nymphalids and moths, in having a duplicate B opsin gene (Fig. 6, Step 1). A duplicated gene can have two fates; either it will have the same function as the ancestral copy, or it will evolve a new function (neofunctionalization) (Force et al., 1999). In *L. rubidus*, the visual pigment encoded

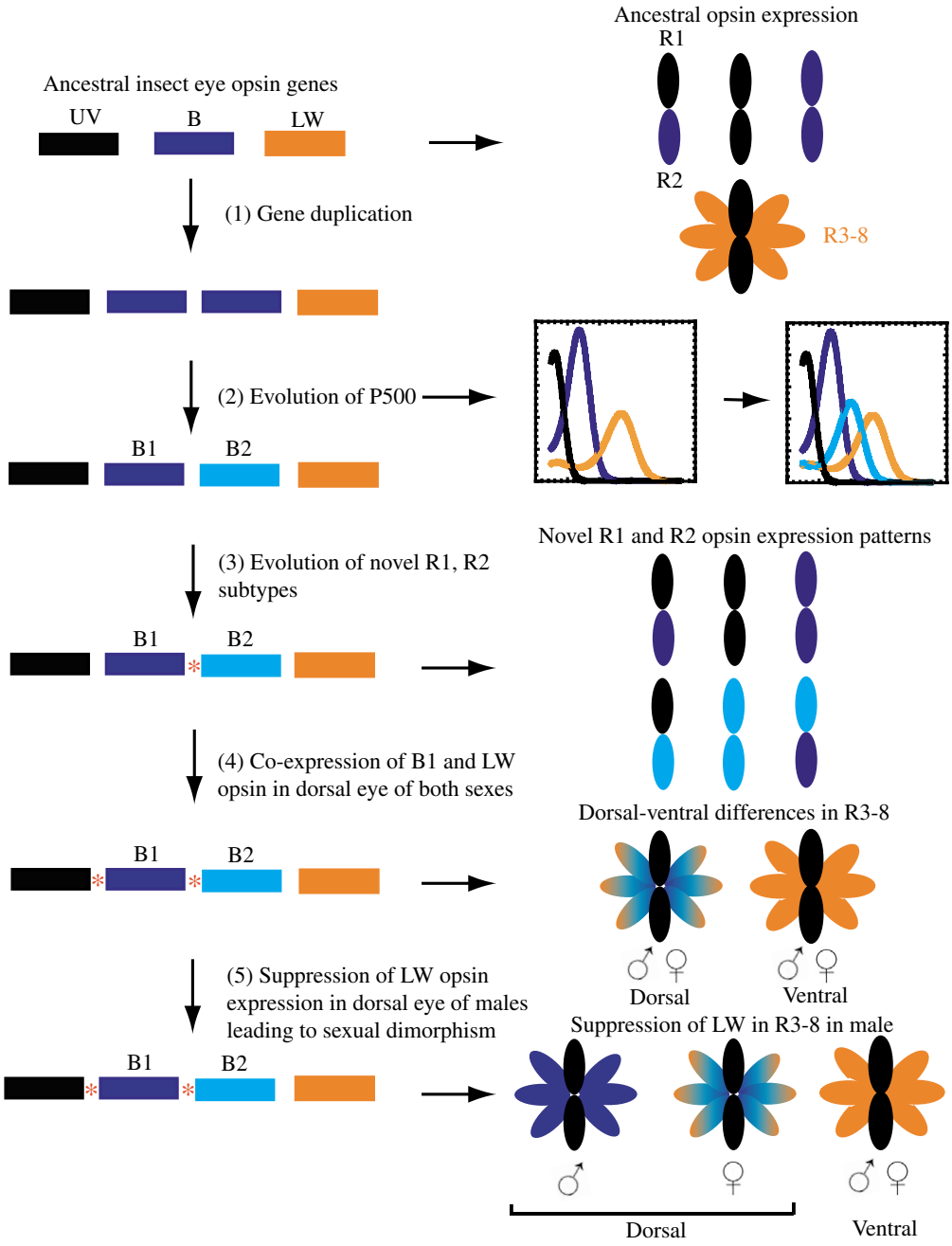


Fig. 6. Evolutionary steps leading to the adaptive evolution of the blue opsin duplication and the evolution of sexually dimorphic eyes. Step (1). Duplication of the blue opsin gene. Step (2) Neofunctionalization of the B2 opsin (BRh2) to an extremely red-shifted (500 nm) peak absorbance. Step (3) Subfunctionalization of the BRh2 expression to a subset of the R1 and R2 photoreceptor cells that are non-overlapping with BRh1 and UVRh. Step (4) Alteration in the regulation of BRh1 (B1) spatial expression to include co-expression with LWRh (LW) the outer R3-8 photoreceptor cells of dorsal eye of both sexes. Step (5) Suppression of LWRh opsin expression in dorsal eye of males only. Color code: black, UV opsin; dark blue, B1 opsin; light blue, B2 opsin; orange, LW opsin.

by the B opsin gene copy (B2) has evolved an extremely red-shifted (500 nm) peak absorbance due to positive selection on the amino acid coding region (Fig. 6, Step 2) (neo-functionalization). The LW opsin also has an unusually red-shifted peak absorbance (568 nm) compared to the typical lepidopteran absorbance (520–530 nm) (White et al., 2003; Vanhoutte and Stavenga, 2004; Briscoe and Bernard, 2005) and probably evolved in a coordinated fashion with the 500 nm visual pigment. The duplicated B2 opsin *BRh2* found only in the ventral retina of both sexes is expressed in a non-overlapping fashion with *UVRh* and *BRh1* in the R1 and R2 photoreceptor cells (Fig. 6, Step 3) (subfunctionalization) (Force et al., 1999). Alteration in the regulation of *BRh1* (B1) spatial expression to include co-expression with *LWRh* (LW) in the outer R3-8 photoreceptor cells of dorsal eye of both sexes probably occurred next (Fig. 6, Step 4). Finally, the expression of *LWRh* opsin was suppressed in the dorsal eye of males only (Fig. 6, Step 5), perhaps due to strong selection for male–male recognition or the recognition of conspecific females (Bernard and Remington, 1991).

Discussion

In this study, we found that *L. rubidus* exhibits a sexually dimorphic distribution of visual pigments and opsin mRNA expression in the dorsal eye, in which males exclusively express UV and B visual pigments in ommatidia specialized for color detection. The highly territorial male *L. rubidus* (Bernard and Remington, 1991) probably use their dorsal eye for dichromatic color vision and detection of flickering moving objects, such as airborne males. Strong male–male interactions seem to be a common theme among lycaenids (e.g. *Polyommatus icarus*) (Lundren, 1977). UV-reflecting 'flickering' wing patterns have also been proposed as a stimulus useful in mating displays (Meyer-Rochow, 1991).

A dorsal eye, which has predominantly UV and B receptors, seems to be a common phenomenon in male insects having sexually dimorphic mates. Such a pattern is found in the male honeybee (Muri and Jones, 1983; Menzel et al., 1991; Velarde et al., 2005), dragonfly *Sympetrum* (Labhart and Nilsson, 1995), mayfly *Atalophlebia* (Horridge and McLean, 1982), *Musca domestica* (Hardie, 1986) and the bibionid fly *Bibio marci* (Burkhart and De LaMotte, 1972; Zeil, 1983). These data suggest that a sexually dimorphic eye may represent the ancestral state in insects. However, other more basal butterflies that have been examined, such as the swallowtail, the painted lady, the heliconian and the monarch butterfly, do not have sexually dimorphic patterns of opsin mRNA expression (Kitamoto et al., 1998; Briscoe et al., 2003; Sauman et al., 2005; Zaccardi et al., 2006). Rather, our data suggest that sexually dimorphic male eyes, expressing predominantly UV and B opsins, may have evolved independently in insects.

The situation in females is different. Our microspectrophotometric data indicate the dorsal eye of females expresses UV, B and LW opsins that, together with the chromophore, produce the 360, 437 and 568 nm visual pigments, respectively. Six outer photoreceptor cells (R3-8) double-labeled by *LWRh* and *BRh1* riboprobes, strongly indicate that these photoreceptors contain two visual pigments. Although the co-expression of two LW visual pigments in one photoreceptor cell has been observed in other arthropods (Sakamoto et al., 1996; Kitamoto et al., 1998), to our knowledge, *L. rubidus* is the first insect species to have two visual pigments of both short and long wavelength spectral types co-expressed in the same photoreceptor cells. Assuming that both visual pigments are involved in phototransduction, the co-expression of *LWRh* and *BRh1* in a single photoreceptor cell would indicate that the receptors have a broad sensitivity from the violet to orange–red spectrum (350–650 nm). Intracellular recordings of the spectral sensitivity of a single photoreceptor cell co-expressing these visual pigments are required to confirm this point. Together with UV receptors, the *L. rubidus* female is outfitted with a receptor type in the dorsal area that would in principle provide trichromatic color vision over a broader part of the spectrum, as compared to the male.

Sexual dimorphic butterfly eyes have likely evolved independently multiple times through a variety of physiological mechanisms. In the case of the small white cabbage butterfly, *Pieris rapae* (Pieridae) (Arikawa et al., 2005), three short wavelength receptors were found, sensitive to ultraviolet (λ_{max}=360 nm), violet (λ_{max}=425 nm) and blue (λ_{max}=453 nm) light, and each expressing a unique opsin. A spectral filtering pigment was found co-expressed with the blue opsin only in males, producing a uniquely narrow blue receptor, highlighting the changes in the spatial expression patterns of non-opsin filtering pigments as a mechanism for producing a sexually dimorphic retina. The situation in pierids, however, differs completely from what we have found in lycaenids with respect to both the physiological basis of the sexual dimorphism and the fate of the duplicate B opsin genes.

The presence of a fourth visual pigment mRNA *BRh2*, together with *UVRh* and *BRh1*, furnishes *Lycaena* with six ommatidial subtypes in the ventral eye area (Fig. 4), twice the number found in any other lepidopteran (Arikawa, 2003; Briscoe et al., 2003; White et al., 2003; Sauman et al., 2005; Zaccardi et al., 2006). Even in *Pieris rapae* (Arikawa et al., 2005), which as noted above has independently evolved a violet receptor from a duplicate blue opsin gene, only three ommatidial types have been reported: those expressing UV-UV, UV-B, and V-V opsin mRNAs. The *Lycaena* ommatidial subtypes are heterogeneously distributed in the ventral region, suggesting that with appropriate neuronal wiring, there may be good spectral discrimination (i.e. blue color vision) in this part of the eye; a hypothesis that can be tested using behavioral experiments.

Conclusions

The eye design of *L. rubidus* is exceptional. We have shown that the novel sex-specific distributions of opsin mRNAs do not resemble that of any other lepidopteran studied. In the female eye, the co-expression of *BRh1*, encoding a blue-sensitive visual pigment (λ_{max}=437 nm), and *LWRh*, encoding

a long wavelength-sensitive (λ_{max}=568 nm) visual pigment, provides an exception to the one-receptor, one-cell rule. We speculate that the expression of the *LWRh* transcript has been suppressed in males due to strong selection for male–male and conspecific mate recognition (Fig. 6). Visual signals appear to play a major role in the interspecific social interactions of lycaenid butterflies, as evidenced by behavioral observations and the unparalleled diversity of wing colors among sympatric species (Lukhtanov et al., 2005). We suggest that the molecular evolution of the 500 nm visual pigment and the novel ommatidial subtypes have likely enhanced color vision in the short wavelength part of the spectrum and have provided a mechanism for the rapid evolution of wing color in the largest of lycaenid subfamilies (Polyommatinae+Theclinae+ Lycaeninae) (Johnson and Coates, 1999).

List of abbreviations

B	blue
BRh1	blue-sensitive rhodopsin (visual pigment)
DIC	differential interference contrast
DRA	dorsal rim area
LW	long wavelength
LWRh	long wavelength-sensitive rhodopsin (visual pigment)
ML	maximum likelihood
NJ	neighbor-joining
R1-8	photoreceptor (retinula) cells
RACE	rapid amplification of cDNA ends
UV	ultraviolet
UVRh	ultraviolet-sensitive rhodopsin (visual pigment)
λ_{max}	wavelength of peak absorbance

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