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Journal

Journal of Experimental Biology, 211(3)

ISSN

0022-0949

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Publication Date

2008-02-01

DOI

10.1242/jeb.012617

The Journal of Experimental Biology 211, 361-369
Published by The Company of Biologists 2008
doi:10.1242/jeb.012617

The lycaenid butterfly *Polyommatus icarus* uses a duplicated blue opsin to see green

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Accepted 14 November 2007

SUMMARY

The functional significance of gene duplication is rarely addressed at the level of animal behavior. Butterflies are excellent models in this regard because they can be trained and the use of their opsin-based visual pigments in color vision can be assessed. In the present study, we demonstrate that the lycaenid *Polyommatus icarus* uses its duplicate blue (B2) opsin, *BRh2*, in conjunction with its long-wavelength (LW) opsin, *LWRh*, to see color in the green part of the light spectrum extending up to 560 nm. This is in contrast to butterflies in the genus *Papilio*, which use duplicate LW opsins to discriminate colors in the long-wavelength range. We also found that *P. icarus* has a heterogeneously expressed red filtering pigment and red-reflecting ommatidia in the ventral eye region. In behavioural tests, the butterflies could not discriminate colors in the red range (570–640 nm). This finding is significant because we have previously found that the nymphalid butterfly *Heliconius erato* has filter-pigment mediated color vision in the long wavelength range. Our results suggest that lateral filtering pigments may not always influence color vision in insects.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/211/3/361/DC1

Key words: lycaenid, color vision, visual pigment, filter pigment, butterfly, opsin.

INTRODUCTION

Photoreceptor cells in the compound eye of the adult butterfly contain the visual pigment rhodopsin, which is composed of an opsin protein and a retinal-based chromophore. Most butterfly eyes only contain 11-*cis*-3-hydroxyretinal as the chromophore, with some also containing a minor amount of 11-*cis*-retinal (Smith and Goldsmith, 1990; Seki and Vogt, 1998). Spectral tuning of the visual pigment's wavelength of peak absorbance, λ_{max}, is achieved through the interaction of the chromophore with critical amino acid residues within the opsin. In many insects such as bees and moths, color vision is based on three classes of photoreceptors with maximal sensitivity in the ultraviolet (UV), blue (B) and long-wavelength (LW) range (for reviews, see Briscoe and Chittka, 2001; Kelber, 2006), that correspond to three classes of opsin protein encoded by distinct UV, B and LW opsin genes.

Unlike bees and hawkmoths, the visual system among butterfly families is highly diverse and their color vision abilities have only begun to be explored (e.g. Kelber and Pfaff, 1999; Kinoshita et al., 1999; Weiss and Papaj, 2003; Ômura and Honda, 2005). This diversity is based upon lineage-specific opsin gene duplications (Briscoe, 1998; Kitamoto et al., 1998; Arikawa et al., 2005; Sison-Mangus et al., 2006; Frentiu et al., 2007) and the presence in the eye of heterogeneously expressed filtering pigments (Stavenga, 2002).

In the eyes of several, but not all, butterfly species, lateral (perirhabdomal) pigment granules are found very close to the rhabdom (Ribi, 1979; Arikawa et al., 1999; Stavenga, 2002; Briscoe et al., 2003; Briscoe and Bernard, 2005). In the described cases, these pigments are red or yellow and thus absorb the short wavelength end of the light spectrum. The rhabdoms of these butterflies are narrow and function as wave-guides, in which a fraction of the wave energy travels outside the rhabdom surface (Nilsson et al., 1988). It is this fraction of light that can be absorbed by the perirhabdomal filter pigment. Since the spatial distribution of light across the rhabdom and surround is not affected, this influences the spectral composition of the light traveling inside the rhabdom that can be absorbed by the opsin pigment. If they absorb light in an appropriate short-wavelength range, perirhabdomal pigments can shift the sensitivity of the photoreceptor into the longer wavelengths (Stavenga 2002; Warrant et al., 2007), which has been shown electrophysiologically in *Papilio xuthus* (Arikawa et al., 1999) and *Pieris rapae crucivora* (Wakakuwa et al., 2004). In *Heliconius erato*, electrophysiological data revealed the existence of a red-sensitive receptor over 30 years ago (Struwe, 1970; Swihart and Gordon, 1971; Swihart, 1972). These butterflies are now known to express only one LW opsin pigment in the eye. They possess perirhabdomal filter pigments that most likely shift the sensitivity peak of receptors in a sub-set of ommatidia (Zaccardi et al., 2006).

If signals from two receptors with different spectral sensitivity are compared neurally, color vision is possible. For *H. erato* only, behavioral evidence has been obtained demonstrating that photoreceptors expressing the same opsin pigment but differing in sensitivity as a result of perirhabdomal filter pigment are used for color vision (Zaccardi et al., 2006). More behavioral evidence is needed to better understand the evolution of such systems, particularly since it has been suggested that variation of ommochrome pigments in butterfly wings may be genetically linked to variation in lateral filtering pigments in the eyes, thus co-

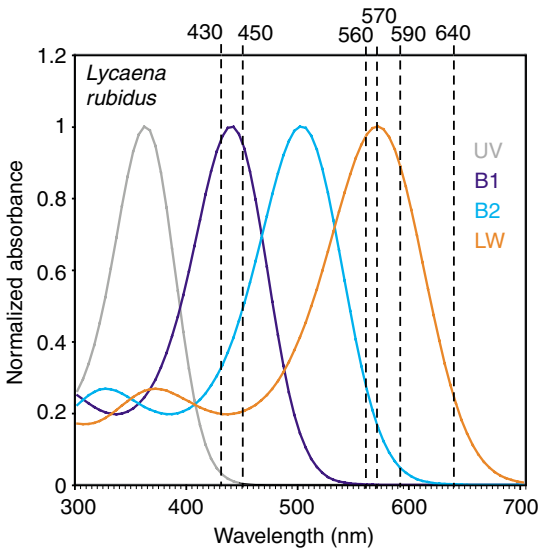

Fig. 1. Normalized absorbance spectra for the visual pigments of *L. rubidus* based on the Bernard template for idealized spectra (see Palacios et al., 1996) and λ_{max} values measured by epi-microspectrophotometry (Bernard and Remington, 1991). The identities of the pigments are indicated by color (UV, gray; B1, dark blue; B2, light blue; LW, orange). The vertical broken lines correspond to the wavelengths of the color stimuli used in the behavioral tests.

evolution of mating signals and photoreceptors may have occurred (Kronfrost et al., 2006).

In the present study, we have focused on the visual system of the lycaenid butterfly, *Polyommatus icarus*. Current understanding of vision in lycaenid butterflies is mostly based on studies of a related butterfly, *Lycaena rubidus* (Bernard and Remington, 1991; Sison-Mangus et al., 2006). In the compound eye of this animal, four opsin genes are expressed – one UV (*UVRh*), duplicate B, B1 (*BRh1*) and B2 (*BRh2*), and one LW (*LWRh*) opsin, producing visual pigments with λ_{max} corresponding to 360 nm, 437 nm, 500 nm and 568 nm, respectively (Fig. 1). The ommatidia of the lycaenid compound eye contain nine photoreceptor cells, R1–9 (Fig. 2). The ventral eye of *Lycaena rubidus* contains six classes of ommatidia that differ according to the opsins expressed in the R1 and R2 cells: UV-UV, UV-B1, UV-B2, B1-B2, B1-B1 or B2-B2 (Fig. 2C). The R3–8 cells of the ventral retina express the LW opsin. In addition, *L. rubidus* eyes contain a red filtering pigment that is found exclusively in the ventral area and is located in the R5–8 cells, but only in ommatidia in which B2 is also present (Sison-Mangus et al., 2006). Thus, it is possible that in addition to the four purely opsin-based photoreceptor classes just described, there is a fifth class in the ventral eye that is based upon the LW opsin together with the red filtering pigment. This additional spectral class could, in principle, extend color vision abilities of the animal in the long wavelength range.

The dorsal eye of *L. rubidus,* by contrast, is sexually dimorphic. In males, the R3–8 cells exclusively express the B1 opsin while in females, the R3–8 cells co-express the B1 and LW opsins. The R1 and R2 cells of the dorsal eye of both males and females express almost entirely UV-UV, with a minor number of ommatidia expressing the UV-B1 or B1-B1 combination (Sison-Mangus et al., 2006). The highly territorial male *L. rubidus* probably use their dorsal eye for dichromatic color vision and the detection of sympatric males (Bernard and Remington, 1991).

Like *L. rubidus*, *P. icarus* males are highly territorial and engage in intense male–male interactions (Lundgren, 1977). Their eyes also express the duplicate blue opsins, B1 and B2, found in *L. rubidus* (Sison-Mangus et al., 2006). In addition, there is sufficient evidence that *P. icarus* use ultraviolet signals to select their mates (Burghardt et al., 2000; Knüttel and Fiedler, 2001) but proof of color vision is lacking.

In the present study, we examined color vision in *Polyommatus icarus* in the long-wavelength range. First, we asked whether *P. icarus* extends its color vision in the red range, possibly *via* the effects of a perirhabdomal filtering pigment. Secondly, we asked whether the putatively blue-green-absorbing visual pigment encoded by the duplicate blue opsin, B2, is used for color vision in the context of feeding.

MATERIALS AND METHODS

Light microscopy

To see the overall distribution of filtering pigments in the eyes, heads of wild-caught *Polyommatus icarus* Rottemburg 1775 were severed in half under daylight illumination and were fixed and embedded in Epon resin according to methods described earlier (Zaccardi et al., 2006). The eyes were oriented sideways such that the first few cuts showed tangential sections of the ommatidia in the lateral part of the eye and the later cuts were longitudinal sections of the frontal part of the eye. Some eyes were also sectioned more frontally to obtain tangential sections of ommatidia in this part of the eye.

Eye-shine photographs

Two dark-adapted female *P. icarus* eyes were photographed using the method described earlier (Zaccardi et al., 2006) to look at the eye-shine of different ommatidial types. Eye-shine is light that initially passes through the rhabdom and is not absorbed by the visual pigments. Once the light reaches the tapetum basal to the rhabdom, it is reflected back to be absorbed by the visual pigments. Any unabsorbed light leaving the eye is seen as eye-shine. All butterfly eyes examined so far reflect eye-shine (Miller and Bernard, 1968; Bernard and Miller, 1970; Stavenga, 2002), except for those in the most basal family, the Papilionidae (Miller, 1979) and in the pierid genus, *Anthocharis* (Takemura et al., 2007). Animals were immobilized with wax and placed with the centre of curvature of one eye in the centre of a goniometer. This way, eye-shine of ommatidia looking dorsally (D), laterally (L) or anteriorly (A) could be taken as well as pictures of ommatidia looking into intermediate directions. Light from a 45 W xenon lamp was focused on the centre of curvature of the eye for illumination of a large patch of ommatidia. A microscope cover glass was used as a semi-transparent mirror to allow observation from the direction of the incident light (orthodromic illumination). Photographs were taken with the shutter open for 0.1 s with intervals of 10 s to avoid pupil closure. Pictures were taken using a digital camera and a Zeiss Luminar 25 mm objective.

PCR, cloning and sequencing of opsin genes

A cDNA library was synthesized from the total RNA of three female *P. icarus* heads and screened for opsin transcripts following the procedures described (Sison-Mangus et al., 2006). Briefly, 3′RACE (rapid amplification of cDNA ends) products were amplified using the degenerate primer (5′ GAA CAR GCW AAR AAR ATG A 3′) and cloned. Then, 95 plasmids were screened by *Eco*RI digestion. 24 plasmids with inserts were sequenced. Gene-specific reverse primers (UV, 5′ TTT GCA AGT CAC GGC TGG

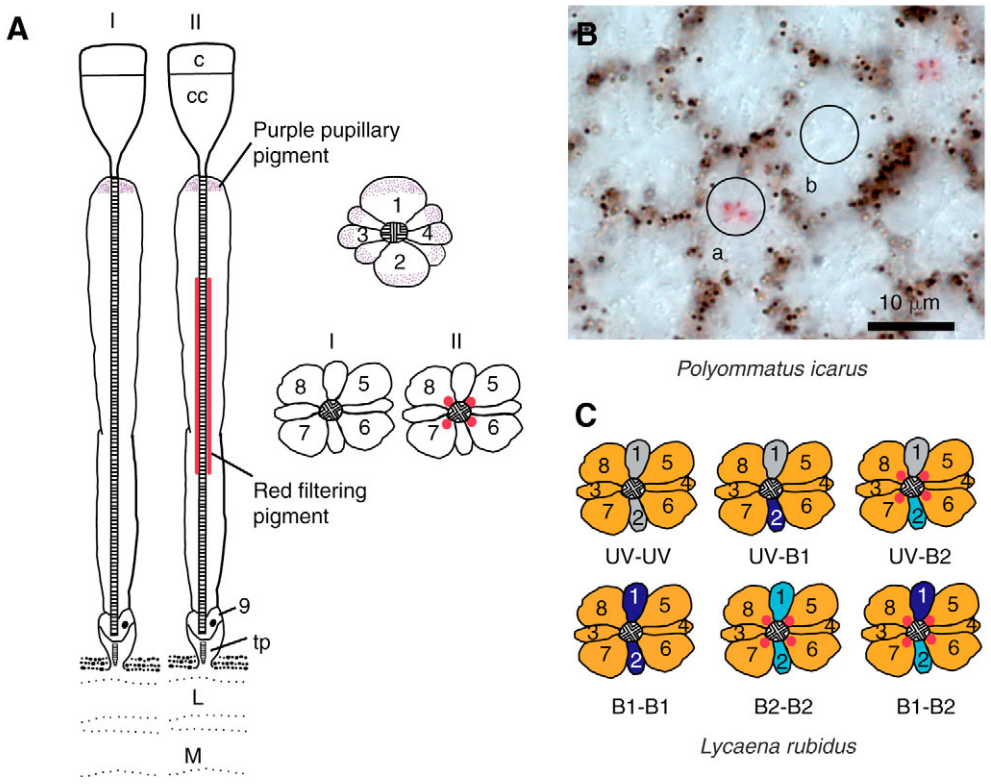

Fig. 2. Ommatidia, filtering pigment distribution and pattern of opsin gene expression in lycaenid butterflies. (A) Longitudinal (left) and tangential views (right) of the two types of ommatidia in the ventral eye of *P. icarus*, non-pigmented (I) and red-pigmented (II). Purple pupillary pigments are also present distally all in R1–8 photoreceptor cells regulating the amount of light entering each ommatidium. c, cornea; cc, crystalline cone; 9, the ninth photoreceptor; tp, tapetum; L, lamina; M, medulla. (B) Red-filtering pigment in the lateral eye of *P. icarus* is present in some ommatidia (a) and absent in others (b). (C) Cartoon of the six ommatidial subtypes found in the ventral retina of *L. rubidus* with respect to the non-overlapping expression of *UVRh* (UV, gray), *BRh1* (B1, dark blue) and *BRh2* (B2, light blue) mRNAs in R1 and R2 photoreceptor cells. The *LWRh* mRNA (orange) is expressed in the R3–8 cells. Note: the red-filtering pigment (red dots) of *L rubidus* is coordinately expressed in the same ommatidia as those expressing *BRh2*. Experimental data upon which the cartoon is based are from Sison-Mangus et al. (Sison-Mangus et al., 2006).

TAT C 3′; LW, 5′ GCT CGG TAC TTA GGA TGG CTT ATG 3′ and 5′ AAT GTG CAA CTT CTA ACC CGA TAC 3′) were then designed and used to amplify 5′RACE products. The remaining 3′RACE plasmid clones were further screened for opsin-specific gene inserts *via* multiplex PCR. Three opsin-specific primer pairs were mixed in one PCR cocktail and plasmids that did not amplify any product were sequenced.

Phylogeny reconstruction

P. icarus opsin genes were aligned with other homologous lepidopteran opsins downloaded from GenBank in Mega 3.0 (Kumar et al., 2004). The gene trees were reconstructed by neighbor-joining analysis of nucleotides using Tamura–Nei distance, heterogeneous pattern of nucleotide substitution among lineages and complete deletions of gaps. The reliability of the tree was sampled with 1000 bootstrap replicates.

Animals, training and test conditions

Two behavioural experiments were performed for this study. The first experiment (red color vision) was performed during September 2001, the second experiment (green color vision) during July and August 2006. For the red color vision experiment, 122 pupae of *P. icarus* were obtained from the offspring of four females from the same wild population in Bavaria, Germany, using breeding methods described by Burghardt and Fiedler (Burghardt and Fiedler, 1996). For the green color discrimination experiment, two groups of *P. icarus* animals obtained from pupae were used, the F_1 generation of butterflies caught in Lund, Sweden and those of *P. icarus* butterflies from Bavaria, Germany. Butterflies caught in Lund were placed in a butterfly cage and allowed to lay eggs on bird's foot trefoil *Lotus corniculatus*. Caterpillars were collected and fed *ad libitum* with young *L. corniculatus* leaves. They were grown in a high humidity chamber above 25°C, and food was changed daily until pupation. Pupae from both groups were kept in an open plastic box maintained at high humidity with 16 h:8 h light:dark photoperiod inside the butterfly cage.

The experimental cage for behavioral testing was 70 cm wide, 60 cm deep and 50 cm high. For the red color vision test, the cage was illuminated from above by two 18 W Osram Biolux tubes (Osram, Hamburg, Germany) and one 40 W Philips 09N tube (Philips Lighting, Hamburg, Germany), in a 14 h:10 h light:dark regime. For the green color tests, three 18 W Osram Biolux tubes were used. The light intensity during training and tests corresponded to 100 cd m^{-2}. After lights on in the morning, the temperature in the cage increased from 22°C to 30°C within 1 h.

Red color discrimination test

To establish color vision, the animal must be able to detect differences in the spectral composition of two stimuli irrespective of the relative intensities (Kelber et al., 2003). To test if the

butterflies had color vision abilities in general, and whether they could discriminate colors in the red range, naïve (newly eclosed) animals were trained on an array of 18 feeders that were positioned on a 10 cm×10 cm horizontal black board. A feeder consisted of a light emitting diode (LED) surrounded by a short piece of transparent tubing that served as reservoir for 10% (w:w) sucrose solution. For an exact description and picture of the feeder board, see Kelber and Pfaff (Kelber and Pfaff, 1999). Only the six yellow (590 nm) feeders contained sucrose solution, and the six red (640 nm) and six blue (430 nm) feeders were empty. The butterflies easily learned to land on the feeder tubes and extend their proboscis into the feeders. Although all animals were trained and tested together, for individual identification each animal was marked by a number on the wing. During tests, only two colors (yellow and either red or blue) were used; LEDs of the third color were switched off. Each landing of a butterfly on a feeder was counted as a choice. Landings on feeders where another butterfly was sitting were avoided by gently removing butterflies from feeders after landing. The intensities of the rewarded color (yellow) and the unrewarded color (red or blue, in respective tests) were adjusted such that the ratio of the intensities of rewarded (+) and unrewarded color ranged from 0.1 to 4. Animals were tested for ca. 1 h per day, and tests with different colors and intensity ratios were performed in a random order. Not all animals survived long enough to be tested. Data from the entire population were pooled, and G-tests (Sokal and Rohlf, 1995) performed to test for statistical significance of results.

Green color discrimination test

We wished to investigate whether *P. icarus* uses one of its duplicated blue opsins (*BRh2*, encoding a visual pigment with possibly a λ_{max} ~500 nm as in *Lycaena rubidus*) in discriminating colors in the green part of the light spectrum (see Fig. 1). Since *P. icarus* only possesses one LW opsin (that may produce a visual pigment with a λ_{max} ~568 nm as in *L. rubidus*), we hypothesized that the animal utilizes its *BRh2* visual pigment to extend its color discrimination in the green range.

In these experiments, animals were also trained to discriminate a rewarded (+) color from an unrewarded color. Each stimulus was produced by light from a lamp with adjustable intensity, passed through a narrow-band (10 nm) interference filter and presented on a feeder of 20 mm diameter. Both feeders were presented on the same feeder plate. The behavioral apparatus and experimental method we used are described in more detail elsewhere (Zaccardi et al., 2006), but there were some differences. In the present study, the feeder plate was oriented horizontally instead of vertically, which allowed the small butterflies to land more easily on the feeders. Additionally, the floor of the cage was covered with black paper, which dissuaded the animals from perching on the floor. Naïve butterflies were trained to find 10% sugar solution on the positive feeder 6–8 h after eclosion. The naïve butterflies were first offered cotton soaked in sugar solution. Once their proboscis was extended, the animals were brought to the positive feeder and allowed to feed *ad libitum*. Training continued for 3–7 days until the animals flew freely toward the positive feeder (supplementary material Movie 1). The light intensity ratio of 0.1 was used in training the butterflies.

Four colors were used in the experiments. These were produced using filters transmitting light at 450 nm, 560 nm, 570 nm and 590 nm, respectively. In each experiment, two color stimuli with varying light intensity combinations were presented simultaneously to the animals. We used three different ratios of 0.1, 1 and 10 between the light intensity of the rewarded and unrewarded stimulus for the two color stimuli, ranging between 9×10^{15} and 1.2×10^{17} photons sr^{-1} cm^{-2} s^{-1}.

We first trained the animals to discriminate between 450 nm (blue) and 590 nm (yellow), with the latter designated as the rewarded (+) stimulus. Only one individual at a time was allowed to approach the feeder. A choice was registered if the animal extended its proboscis. The animal was allowed to feed for 3 s and then gently removed from the feeder. Tarsus extension was not counted as a choice and, if displayed, the animal was gently removed from the feeder. Between choices, the feeders were covered with black cardboard and the side and light intensity ratio between rewarded (+) and unrewarded (–) stimuli changed in a pseudorandom manner. The feeders were cleaned with water after each individual session to remove sugar or odor traces. An individual must have made a minimum of ten choices for each of three intensity combinations before being trained for the next experiment.

In the subsequent tests, we wanted to determine the color vision limit of the animal between the green and yellow colors. The same individuals that were able to discriminate colors in the first experiment were continuously trained to 590 nm as the rewarded (+) stimulus. They were then tested for their ability to discriminate this wavelength from either 560 nm (–) or 570 nm (–). The number of choices made by an individual for each intensity combination was tested for statistical significance using the test of binomial proportions of P=0.5, compared in the table of critical values for tests of proportions [table Q (Rohlf and Sokal, 1995) p. 107] using the two-tailed significance level (α) of 0.05.

RESULTS

Filtering pigment expression in the ventral eye

In addition to the visual pigments, many butterfly eyes contain lateral filtering pigments (Stavenga, 2002) and we were interested in whether or not *P. icarus* eyes contain a filtering pigment like *Lycaena rubidus* that could possibly extend its color vision capability into the red range. Indeed, we found a red filtering pigment in the *P. icarus* eye which was also present proximally in the R5–8 photoreceptor cells and only expressed exclusively in a sub-set of ommatidia in the ventral eye (Fig. 2B). In tangential sections, the distance between pigment spots was 0.9±0.15 μm (Fig. 2B). No pigment was seen over the most distal 30 μm of the rhabdom, pigmentation extended over a length of 55 μm, and the basal 45 μm were pigment free again (see supplementary material Fig. S3). We also noticed a difference in the distribution of these pigment granules between the lateral and anterior sides of the eye. More ommatidia with red pigmentation were observed in the latter than in the former (data not shown). Another pigment, the purple pupillary pigment, which regulates the amount of light entering the ommatidium, was seen in all ommatidia and was present more distally (data not shown) in the R1–8 photoreceptor cells.

Red-reflecting ommatidia in the ventral retina

There was a notable heterogeneity of eye-shine color in the retina of *P. icarus* (Fig. 3). Most remarkable was the difference between dorsal and ventral eye-shine. The dorsal retina was dominated by yellow-reflecting ommatidia while the ventral retina exhibited yellow- and red-reflecting ommatidia. Interestingly, the red-reflecting ommatidia were much more abundant in the anterior (frontal) part of the eye than in the lateral side of the eye. These eye-shine data correspond rather well with the location and frequency of filtering pigment granules observed from the Epon eye

section in Fig. 2. Undoubtedly, red-reflecting ommatidia contain the filtering pigment whereas yellow ones do not. The presence of red ommatidial eye-shine suggested that the animal may have red-sensitive photoreceptors in the ventral retina.

P. icarus has only one LW opsin

We previously reported that, like *L. rubidus*, *P. icarus* has duplicated B opsin genes, *BRh1* and *BRh2* (see Sison-Mangus et al., 2006). To identify other candidate photoreceptors in the *P. icarus* retina, we screened head-specific cDNA. We cloned two more full-length opsin encoding cDNAs which, based upon phylogenetic analysis, represent UV- and LW-absorbing pigments (see supplementary material Figs S1, S2; Fig. 4). The four opsins in *P. icarus* were found to be orthologous to those of *L. rubidus,* whose gene sequence similarity ranges from 86–89%. Since *P. icarus* contains the same four opsin genes as found in *L. rubidus,* we assume that the pattern of opsin expression in the adult compound eye of both lycaenids is similar. Namely, in the ventral retina, the R3–8 cells likely express the LW opsin while the R1 and R2 cells likely express either UV-UV, UV-B1, UV-B2, B2-B1, B1-B1 or B2-B2 (Fig. 2C). Together with the red filtering pigment, it is therefore possible that the *P. icarus* ventral eye contains five spectral classes of receptor suitable for color vision.

Red color discrimination is absent in *P. icarus*

In an experiment where the butterflies were allowed to feed *ad libitum* from yellow (590 nm) feeders but did not find any food in red or blue feeders, they learned to reliably discriminate yellow from blue, independent of intensities. The animals did not learn to visit the yellow feeders very well. In tests with the training intensities, they chose the yellow color in about 70% of the landings (70 animals, G-test, $P<0.05$ for all intensity ratios). This choice frequency did not change with changing intensity ratios (Fig. 5A) and was similar for both sexes (not shown). In tests with yellow (590 nm) versus red (640 nm) (Fig. 5B), the choice frequency changed dramatically, if the intensity ratio was changed. From this result, we conclude that the animals use color vision to discriminate yellow of 590 nm from blue of 430 nm but not to discriminate yellow from red of 640 nm. For this latter task, the animals obviously use a brightness cue for discrimination, a cue that does not allow them to choose the correct color when intensities are changed.

Green color discrimination with *LWRh* and *BRh2* opsins

We confirmed the result of the previous experiment that *P. icarus* have color vision because they can discriminate the rewarded color of 590 nm (+) from the unrewarded color, 450 nm (Fig. 6A). All individuals tested chose 590 nm nearly all the time regardless of

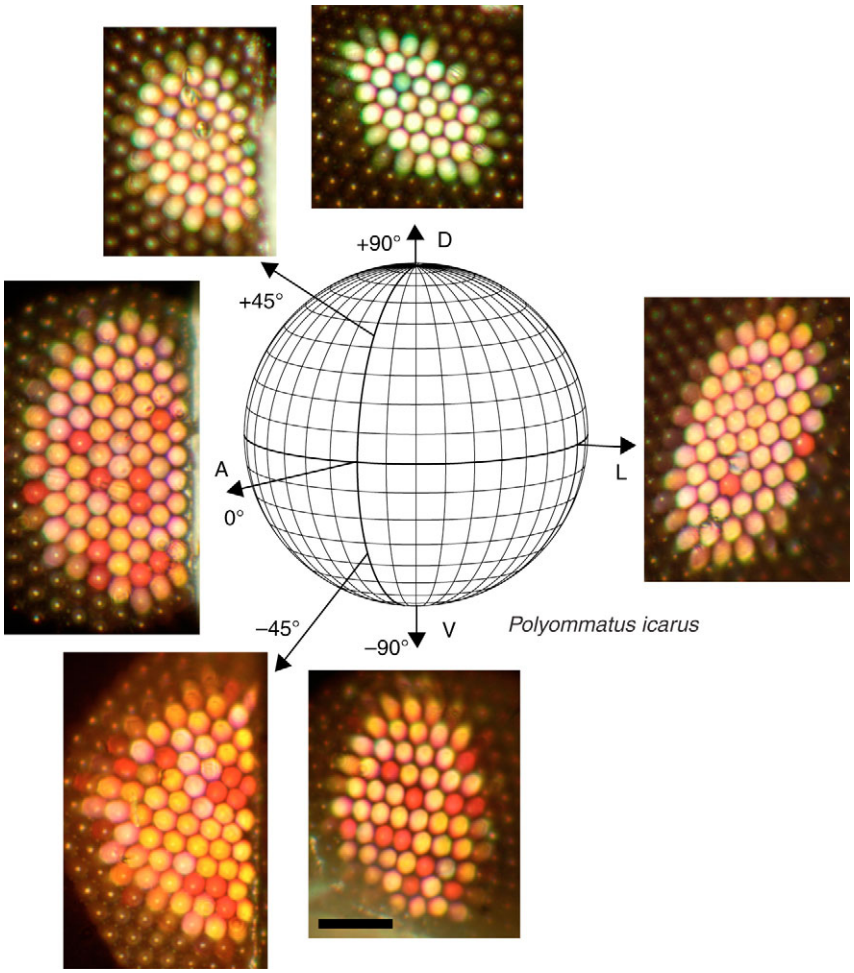

Fig. 3. Eye-shine of a female *P. icarus*. Ommatidia looking into the dorsal direction (D) reflect yellow while yellow- and red- reflecting ommatidia are concentrated in the area looking ventrally (V). Red-reflecting ommatidia are present in greater number in the eye region looking anteriorly (A) than in the eye region looking laterally (L). Scale bar, 50 μm.

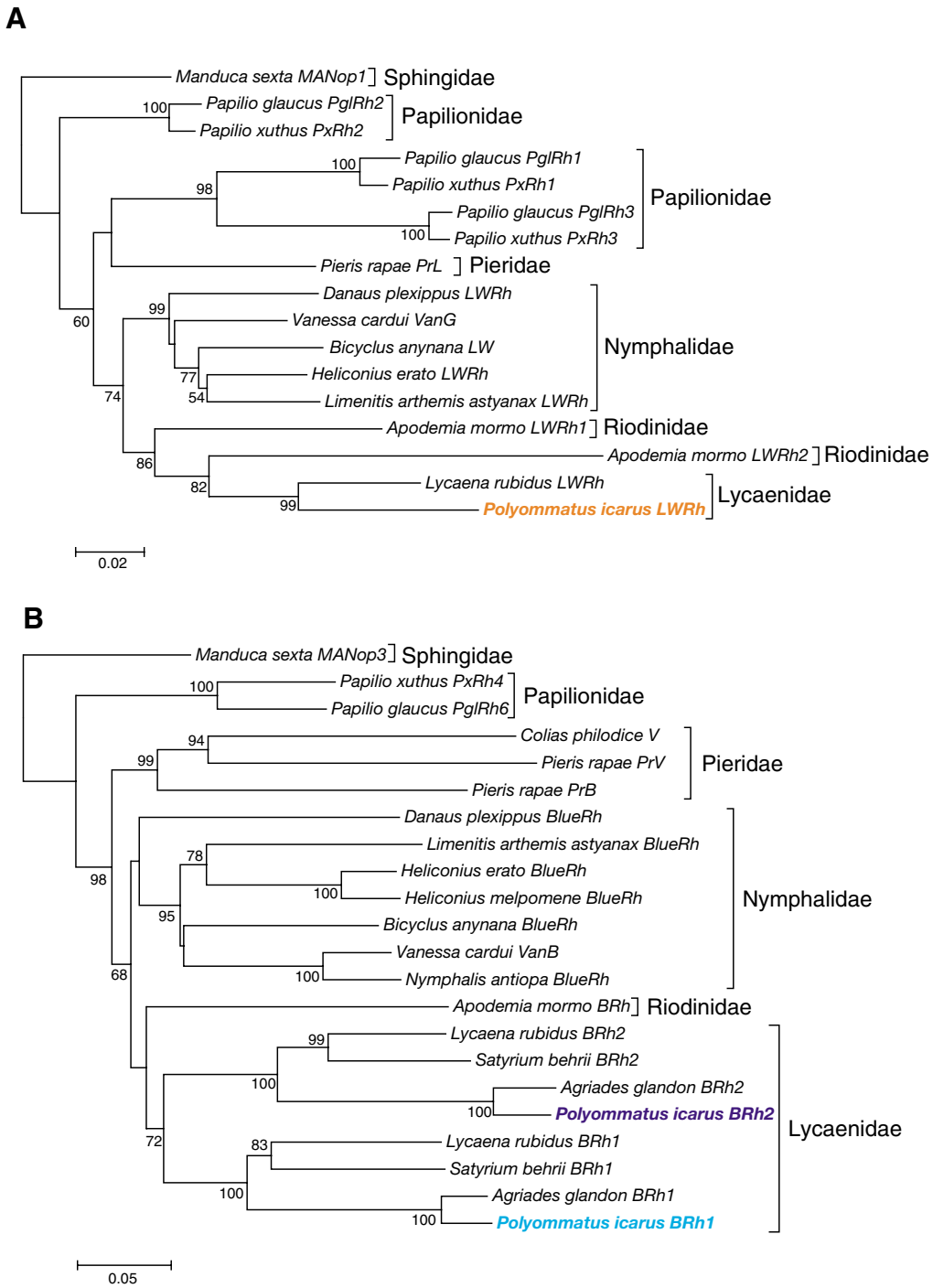

Fig. 4. Phylogenies of lepidopteran LW and B opsin genes. The tree is based upon a neighbor-joining analysis of nucleotide sites using Tamura–Nei model of evolution with a correction for heterogenous patterns of nucleotide substitution among lineages. The reliability of the tree was tested using 1000 bootstrap replicates. Only bootstrap support values >50% are shown. (A) The LW opsin gene tree was reconstructed using the first and second nucleotide positions (742 sites). (B) The B opsin gene tree was reconstructed using all nucleotide positions (1055 sites). GenBank accession numbers are as follows: *Agriades glandon* (*BRh1*, DQ402502; *BRh2,* DQ402503); *Apodemia mormo* (*BRh*, AY587906; *LWRh1*, AY587907; *LWRh2*, AY587908); *Bicyclus anynana* (Blue, AY918894; *LW*, AY918895); *Colias philodice* (*V*, AY918899); *Danaus plexippus* (*Blue*, AY605544; *LW*, AY605545); *Heliconius erato* (*Blue,* AY918906; *LW*, AY918907); *Heliconius melpomene* (*Blue*, AY918897); *Limenitis arthemis astyanax* (*Blue*, AY918902; *LWRh*, DQ212962); *Lycaena rubidus* (*LWRh*, AY587901; *BRh1*, AY587902; *BRh2*, AY587903); *Manduca sexta* (*Manop1*, L78080; *Manop3*, AD001674); *Nymphalis antiopa* (*Blue*, AY918893); *Papilio glaucus* (*PglRh1*, AF077189; *PglRh2*, AF077190; *PglRh3*, AF067080; *Blue*, AF077192); *Papilio xuthus* (*PxRh1*, AB007423; *PxRh2*, AB007425; *PxRh3*, AB007425; *PxRh4*, AB028217); *Pieris rapae* (*PrB*, AB208675; *PrV*, AB208674; *PrL*, AB188567); *Polyommatus icarus* (*BRh1*, DQ402500; *BRh2*, DQ402501); *Satyrium behrii* (*BRh1*, DQ402498; *BRh2*, DQ402499), and *Vanessa cardui* (*VanG*, AY613986; *VanB*, AY613987). GenBank accession number of the newly cloned *Polyommatus icarus LWRh* is EU088114 (orange).

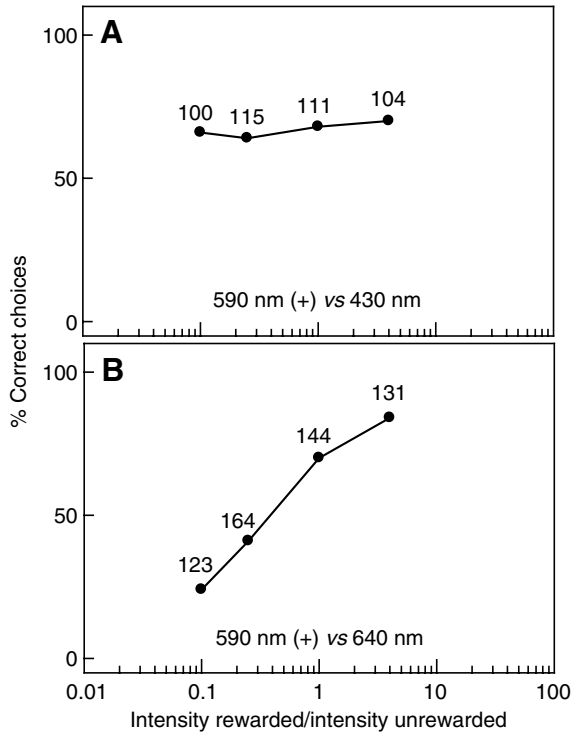

Fig. 5. Red color discrimination. Percent choices of *P. icarus* for the rewarded yellow, as a function of the ratio between the intensities of the rewarded and the unrewarded color. The number of choices made for each test and intensity ratio is indicated. (A) Choices made by 70 animals in tests with yellow and blue. G-test, $P<0.05$ for all intensity ratios. (B) Choices made by 86 animals in tests with yellow and red. G-test, $P<0.05$ for all ratios of intensity except 0.25. Note that the choice frequency for the rewarded color is significantly lower than chance in the test with 0.1 intensity ratio.

whether the light intensity was dimmer, equal or brighter than 450 nm (6 animals, at least 10 choices for each light intensity combination, $P<0.05$ for each animal in each intensity combination). However, in the second experiment, the same animals failed to discriminate between 590 nm (+) and 570 nm (Fig. 6B) by choosing the brighter color most of the time. Only at a brighter intensity did they choose 590 nm (+) correctly ($P<0.05$). This indicates that the animals are not capable of using color vision in discriminating between the two colors, using brightness as a cue instead, just as they did with the yellow and red colors in the previous experiment. Because the animals failed in this experiment, the animals were next tested for discrimination of 590 nm (+) from 560 nm (Fig. 6C). Only three animals survived long enough to reach the minimum number of choices. Nonetheless, these animals demonstrated that they could discriminate 590 nm (+) from 560 nm more than 75% of the time at the dimmer intensity and, significantly, at equal and brighter intensities (two of three animals, $P<0.05$ for 1 and 10 intensity ratio combinations; Fig. 6C). Since only three individuals survived the test, a new batch of individuals was trained to do the same experiment, using 560 nm (+) instead as the rewarded color. These animals were trained to discriminate between 560 nm (+) *versus* 590 nm directly after eclosion. As expected, the animals were able to discriminate 560 nm (+) from 590 nm regardless of light intensity (Fig. 6D) (eight animals, at least ten choices for each intensity combination; $P<0.05$ for each animal in each intensity combination). This experiment therefore demonstrates that the color vision limit of *P. icarus* in the green range is between 560 and 570 nm. Since *P. icarus* has only one LW opsin, and does not extend its color vision to the red range (Fig. 5B, Fig. 6B) using the red filtering pigment, we conclude that *P. icarus* is using its B2 opsin (*BRh2*, probably with an absorbance peak around 500 nm) in discriminating colors extending up to the green wavelength range.

The results of the behavioural experiments show clearly that *P. icarus* can use color vision to discriminate rewarding from unrewarding stimuli. The differences in choice frequencies between both color discrimination experiments (70% correct choices in the first set of experiments and 90% correct choices in the second) are most certainly due to the differences in training and testing methods, in which a large number of animals were tested in the first experiment and the motivated specimens were selected during individual training in the second experiment. Together, both experiments show that *P. icarus* can discriminate colors and that the long-wavelength limit of color vision lies between 560 and 570 nm.

DISCUSSION

The behavioral results in this paper reveal two insights into the evolution of color vision in butterflies. First, the presence of a red filtering pigment in the retina of butterflies does not always extend color vision into the red range. This is different from the situation in *Heliconius erato*, where behavioral color vision extends into a wavelength range (620 nm), in which the blue receptor has no sensitivity (Zaccardi et al., 2006), and from *Pieris rapae*, where electrophysiology proved two long-wavelength receptor types with different spectral sensitivities (620 nm and 640 nm) (Wakakuwa et al., 2004). Second, gene duplication of opsins, followed by the gain of new function, serves as a powerful evolutionary mechanism in fine-tuning the color vision capability of the animal.

Our eye-shine and anatomical data suggested that *P. icarus* may have color vision in the red range. The eye possesses several classes of ommatidia, one of which is widely distributed in the ventral retina and is red-reflecting. The red reflection from this class of ommatidia is most likely produced by the red filtering pigment, which is also found in the ventral retina. The red reflection could not result from another LW opsin because we found only one LW opsin transcript. Our hypothesis that the red-reflecting ommatidia might have contained a red-sensitive photoreceptor is based upon the interpretation of Stavenga (Stavenga, 2002). If their signals were compared neurally, the receptor with red filtering pigment, and the receptor without it, could allow the animal to extend its color vision into the red range.

Our behavioral tests, however, reveal that *P. icarus* has no color vision in the red range because it could not distinguish yellow from red. There are several possible explanations for this finding. First, the lateral filtering pigment may not in fact shift the sensitivity of the LW receptors as much as it does in other butterfly eyes. The pigment is only expressed along 55 μm of the rhabdom length, beginning 30 μm proximal from the rhabdom tip, and it is possible that most light is absorbed by the visual pigment in the distal-most part of the rhabdom. Second, the lateral filtering pigment could change the sensitivity of the photoreceptors, but the signals for different ommatidia are not compared neurally. Neural lateral connections that are specific for different ommatidial types have so far only been studied in the lamina of *Papilio* (Takemura et al., 2005). Signals from R3–8 photoreceptors of neighboring ommatidia may be pooled such that the pigment-induced spectral information is equalized and thereby rendered useless. Another

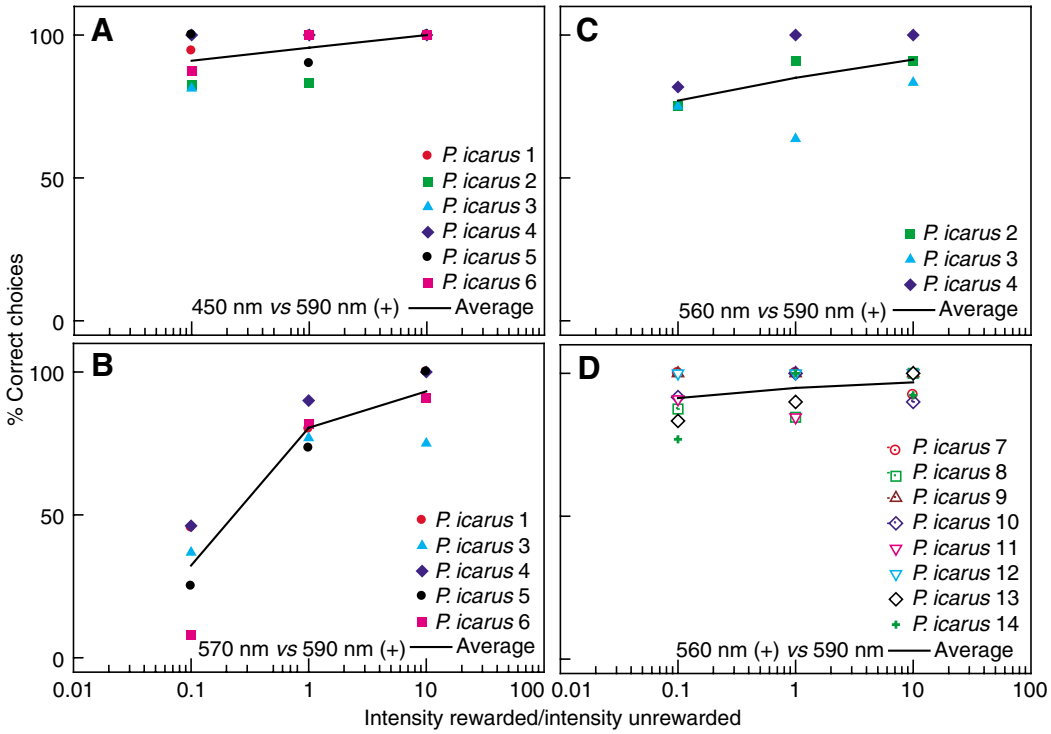

Fig. 6. Green color discrimination. Percent choices of *P. icarus* for the rewarded four training colors, in relation to the ratio between the intensities of the rewarded (+) color and unrewarded color. Each symbol represents one individual animal's performance and the line, the average. (A) Six *P. icarus* trained to 590 nm as the rewarded color and 450 nm as the unrewarded color. All choices differ significantly from chance (P<0.05). (B) Five of the same individuals as in A trained to 590 nm as the rewarded color and 570 nm as the unrewarded color. Only choices at intensity ratio of 10 differ significantly from chance (P<0.05) and not the choices at intensity ratios of 0.1 and 1 (P>0.05). (C) Three of the same individuals as in A trained to 590 nm as the rewarded (+) color and 560 nm as the unrewarded color. Correct choices were made more than 75% of the time at intensity ratio of 0.1 and the choices of individuals 2 and 4 differ significantly from chance at intensity ratios of 1 and 10 (P<0.05). (D) Eight new individuals trained at 560 nm as the rewarded color and 590 nm as the unrewarded color. All choices made by every single individual differ significantly from chance (P<0.05).

possibility is that pigment-induced spectral information is preserved in another behavioral context, such as in the discrimination of oviposition substrates or mate detection. A color vision channel using this information would have to compare signals from both types of ommatidia. While there is no direct proof of such a neural process at present, the possibility is viable.

Lateral filtering pigments have long been described as spectral filters (Ribi, 1978). In *Papilio* and *Pieris*, filtering pigments come in different colors and spatial distributions (Arikawa, 2003; Wakakuwa et al., 2004), and in *Papilio*, are coordinately expressed with particular opsins. The effect of these pigments in *Pieris* as spectral filters has been demonstrated by electrophysiology (Wakakuwa et al., 2004). In *Papilio*, red receptors are used for color vision in both the context of oviposition and food choice (Kelber, 1999; Kelber and Pfaff, 1999). It is difficult to test, however, the behavioral effects of the filtering pigments in *Papilio* independently from that of the co-ordinately expressed opsin. Behavioral and electrophysiological studies on other butterfly species known to have lateral filtering pigments in the retina (e.g. Wakakuwa et al., 2004; Sauman et al., 2005) are needed to confirm the difference between our result in this study and our previous findings in *Heliconius erato* (Zaccardi et al., 2006).

Our results also show that the visual pigments encoded by the blue opsin duplicate gene, *BRh2*, and the LW opsin gene, *LWRh*, function together for green color discrimination in *P. icarus*. Elucidating the function of the B2 pigment is of interest because this is a unique case of a visual pigment in insects that evolved from a blue opsin gene and is red-shifted by 63 nm compared to its 437 nm paralogue (Fig. 1). Because lycaenids only have one LW opsin transcript that may also be somewhat red-shifted in peak absorbance (568 nm as in *L. rubidus*) compared to their estimated ~540±10 nm (mean ± s.e.m.) ancestral pigment (Frentiu et al., 2007), we hypothesize that the B2 receptor, after gene duplication, co-evolved with the LW-absorbing visual pigment to shift the limit of color discrimination capability of the animal towards longer wavelengths. Color discrimination in this range of the spectrum may not only facilitate flower discrimination, but it may also help detect suitable oviposition substrates (e.g. Kelber, 1999; Kelber, 2001).

It is of interest to note that the only other butterfly we are aware of whose eye contains a visual pigment with a peak absorbance similar to B2 is the riodinid *Apodemia mormo* (Bernard et al., 1988). Unlike the 500 nm (B2) pigment of *Lycaena*, however, the 505 nm pigment of *A. mormo* is produced by a duplicate LW opsin, *LWRh1* (Frentiu et al., 2007). Intriguingly, the LW opsins of *L. rubidus* and *P. icarus* are homologous to the *LWRh2* opsin of *A. mormo,* which encodes a 600 nm pigment (Bernard 1979; Frentiu et al., 2007), but a homolog of the *A. mormo* 505 nm (*LWRh1*) pigment in lycaenids is missing. The convergent evolution of two visual pigments with λ_{max} in the 500 nm range implies strong selection for this trait.

It is intriguing that butterflies from different lineages, such as the papilionids and lycaenids, follow a different manner of modifying their color vision system to achieve good color

discrimination in the green range. Papilionids use duplicated LW opsins, one of which is a red receptor, to have a better green color discrimination for the purpose of oviposition (Kelber, 1999) while the lycaenids have a duplicated blue opsin, that is maximally sensitive in the blue-green (at least in *L. rubidus*), to be able to discriminate green colors. Hence, we conclude that natural selection has hit upon alternative strategies for producing color vision in the green part of the visible spectrum in butterflies.

We thank Emily Yee, Kristina Jeppsson, Rita Wallen, Shimako Kawauchi, Jim Strother, Will Van Trump, Marianne Porter and Matthew McHenry for their technical assistance, Alexander Beiter for help with breeding butterflies and Dan-Eric Nilsson for generously allowing us to use his ophthalmoscope. We also thank Steven Reppert, Gary Bernard and Robert Reed for helpful discussions. This work was supported by a Faculty Mentor Program award to M.S.M., NSF IBN-0346765 and IOS-0646060 to A.D.B. and a research grant from the Swedish Research Council to A.K.

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