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Color discrimination in the red range with only one long-wavelength sensitive opsin

Guillermo Zaccardi[1,*], Almut Kelber[1], Marilou P. Sison-Mangus[2] and Adriana D. Briscoe[2]

[1]*Vision Group, Department of Cell and Organism Biology, Lund University, Helgonavägen 3, S-22362 Lund, Sweden and* [2]*Comparative and Evolutionary Physiology Group, Department of Ecology and Evolutionary Biology, University of California, Irvine, CA 92697, USA*

*Author for correspondence (e-mail: guillermo.zaccardi@cob.lu.se)

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Summary

The basic precondition for color vision is the presence of at least two receptor types with different spectral sensitivities. The sensitivity of a receptor is mostly defined by the opsin-based visual pigment expressed in it. We show here, through behavioral experiments, that the nymphalid butterfly *Heliconius erato*, although it expresses short and medium wavelength opsins and only one long wavelength opsin, discriminates colors in the long-wavelength range (590 nm, 620 nm and 640 nm), whereas another nymphalid, *Vanessa atalanta*, despite having color vision, is unable to do so. In the eyes of *H. erato* we identified filtering pigments very close to the rhabdom which differ between ommatidia and produce the yellow and red ommatidial reflection seen under orthodromic illumination. The eyes of *V. atalanta* lack the filtering pigments, and reflect a homogeneous orange. We hypothesize that the filtering pigments found in the eyes of *H. erato* may shift the spectral sensitivity peak of the long wavelength receptors in some ommatidia towards longer wavelengths. The comparison of the signals between the two new receptor types makes color discrimination in the red range possible. To our knowledge, this is the first behavioral proof of color vision based on receptors expressing the same opsin.

Key words: color vision, opsin, filter pigment, insect, butterfly, *Heliconius erato.*

Introduction

Color vision enables animals to reliably detect and recognize important objects such as food sources, hosts or mating partners (for reviews, see Menzel, 1979; Kelber et al., 2003). It arises from the comparison of signals from at least two types of photoreceptor with different spectral sensitivities. Depending on the number and sensitivities of photoreceptor types, the spectral range of color vision differs between species and is usually narrower than the total range of vision. Most insects have a short wavelength (S) receptor sensitive in the ultraviolet (UV) range, a medium wavelength (M) receptor sensitive in the blue range and only one long wavelength (L) receptor sensitive in the green/yellow range. In the case of the honeybee, the best studied example, these receptors have peak sensitivities that correspond to 344 nm (S), 436 nm (M) and 544 nm (L) (Peitsch et al., 1992). Moths with a receptor set similar to that of the honeybee are incapable of discriminating between lights of 590 and 630 nm by means of colors but instead they used the intensity of the stimuli (Kelber and Hénique, 1999).

The spectral sensitivity primarily depends on the visual pigment expressed in the receptor. The three insect receptor types correspond to three major clades of the insect opsin phylogenetic tree (Fig. 1). In bees, moths and butterflies, each ommatidium has six (or seven) receptors expressing L opsin, and two receptors expressing either both M opsin, both S opsin or one M opsin and one S opsin: *Vanessa cardui* (Briscoe et al., 2003); *Danaus plexippus* (Sauman et al., 2005); *Pieris rapae* (Arikawa et al., 2005; Wakakuwa et al., 2004); *Manduca sexta* (White et al., 2003); *Bombus terrestris* (Spaethe and Briscoe, 2005); *Apis mellifera* (Wakakuwa et al., 2005). Papilionid butterflies have evolved a fourth, red-sensitive opsin (Fig. 1) expressed in four receptors in a subset of ommatidia (Arikawa, 2003), and behavioral experiments have proved that this enables them to discriminate colors in the L range, e.g. spectral lights of 590 and 640 nm wavelength (Kelber and Pfaff, 1999).

The presence of molecules other than opsins in the receptor cells can filter the light traveling inside the rhabdom, modifying the spectral sensitivity of the receptor (Goldsmith and Bernard, 1974; Kong et al., 1980). In butterflies (with the exception of papilionids) a tapetum basal to the rhabdom reflects either broadband light (300–700 nm) as in *Vanessa cardui* (Briscoe et al., 2003), or relatively narrow-band light (320–590 nm)

(Arikawa et al., 2005; Douglas and Marshall, 1999; Stavenga, 2002a) (G. Bernard, personal communication). In the latter case, it can thus modify the spectral sensitivity of the photoreceptors. However, the effect of a tapetum is limited since it only affects the spectral composition of light during the second pass through the retina, and most light absorption happens on the first pass, which is before light reaches the tapetum.

Different filtering pigments associated with different photoreceptors that express the same opsin can result in photoreceptors with different spectral sensitivities that can be used for color vision. Additional photoreceptors resulting from this kind of filtering would not extend the total spectral sensitivity of the animal – that is defined by the sensitivities of the opsin pigments – but it may extend the range of color vision

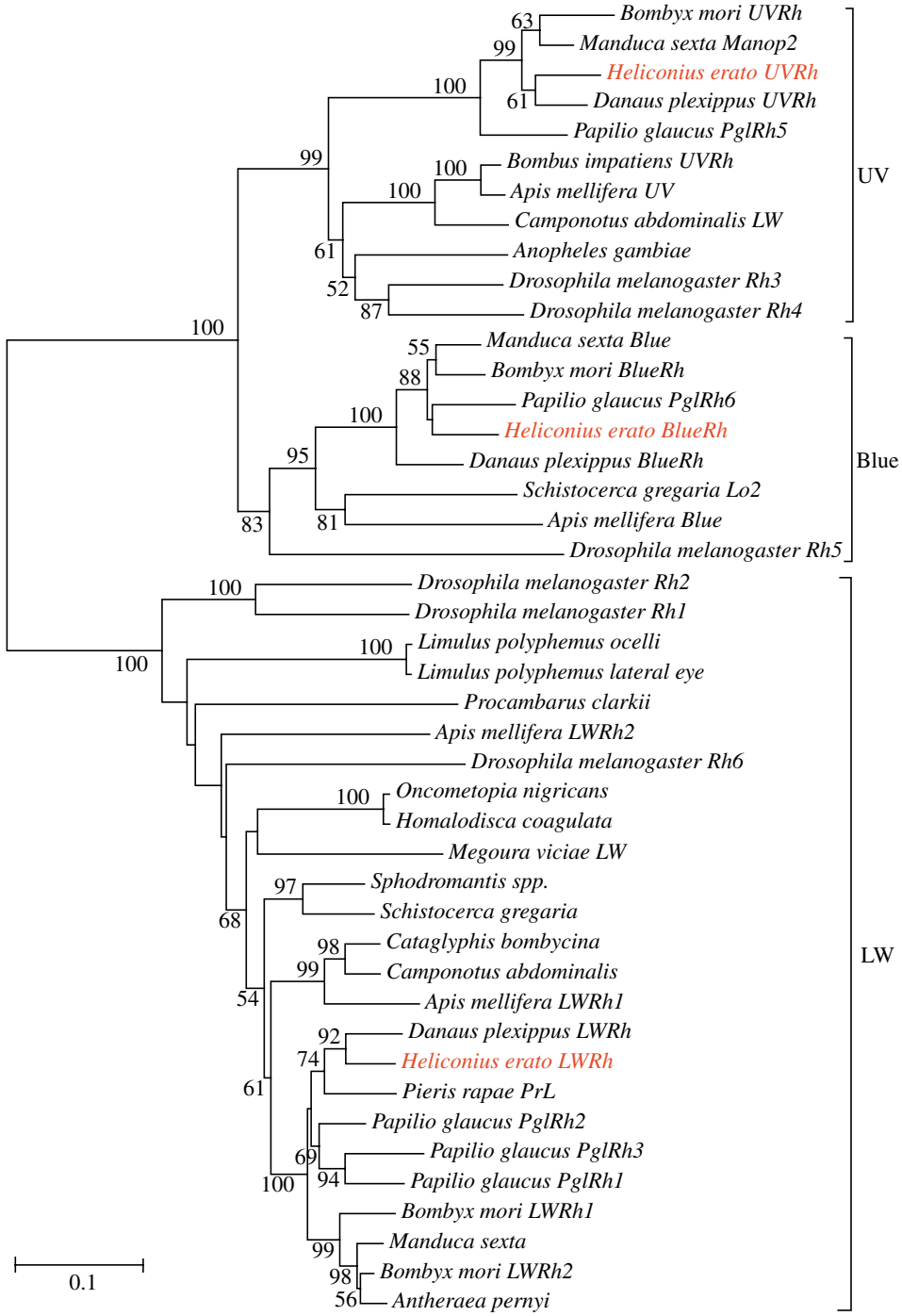

Fig. 1. Arthropod opsin gene phylogeny. The phylogeny is based upon a neighbor-joining analysis of first and second nucleotide positions using the Tamura–Nei model of evolution with a correction for heterogeneous patterns of evolution among lineages. A total of 631 nucleotide sites were used. Numbers at the junctions indicate bootstrap replicates out of 1000 (given as a percentage) in which a particular node is supported. Red indicates cloned *Heliconius erato* opsin cDNAs. GenBank accession numbers for the sequences used in the reconstruction are as follows. Chelicerates: *Limulus polyphemus* (lateral eye, L03781; ocelli, L03782). Crustaceans: *Procambarus clarkii* (S53494). Insects: *Anopheles gambiae* [accession no. is given as supplementary information in Hill et al. (Hill et al., 2002)]; *Antheraea pernyi* (AB073299); *Apis mellifera* (*UV*, AF004169; *Blue*, AF004168; *LW*, U26026); *Bombyx mori* (*LWRh1*, AB064496), *Camponotus abdominalis* (*LW*, U32502; *SW*, AF042788); *Cataglyphis bombycinus* (*LW*, U32501; *SW*, AF042787); *Danaus plexippus* (*UVRh*, AY605546; *BlueRh*, AY605545; *LWRh,* AY605544); *Drosophila melanogaster* (*Rh1*, K02315; *Rh2*, M12896; *Rh3*, M17718; *Rh4*, M17730; *Rh5*, U67905; *Rh6*, Z86118); *Homalodisca coagulata* (AY588065); *Manduca sexta* (*Manop1*, L78080; *Manop2*, L78081; *Manop3*, AD001674); *Megoura viciae* (*UV*, AF189715; *LW* AF189714); *Papilio glaucus* (*PglRh1*, AF077189; *PglRh2*, AF077190; *PglRh3*, AF067080; *PglRh5*, AF077191; *PglRh6*, AF077192); *Oncometopia nigricans* (AY725781); *Pieris rapae* (*PrL*, AB177984); *Schistocerca gregaria* (*Lo1*, X80071; *Lo2*, X80072); *Sphodromantis* spp. (X71665). Full-length nucleotide sequences for the *Bombyx mori* UV, blue and L opsin coding regions were obtained using a tBlastx search of GenBank whole genome sequences (wgs), manually removing the introns in MacClade, and then comparing the coding sequences with partial *B. mori* opsin cDNAs reported in (Shimizu et al., 1998). The bar indicates the number of substitutions/site.

(i.e. the range of wavelengths that can be discriminated). The colored oil droplets found in some birds had earlier been thought to play this role in color vision (Walls, 1942; King-Smith, 1969) but it is now known that each colored oil droplet is associated with a specific opsin in the receptor. They act as a cut-off filter, narrowing the spectral sensitivity of the cones, rather than increasing spectral types (Vorobyev, 2003). The same principle applies to the lateral filtering pigments in the receptors of *Papilio xuthus* (Arikawa et al., 1999). However, in the eyes of the male small white butterfly, *Pieris rapae*, the pale-red or deep-red pigment clusters that surround the rhabdoms of different ommatidia act as long-pass filters, creating receptors with peak sensitivity at 620 or 640 nm, but both contain the same opsin (Qiu et al., 2002; Qiu and Arikawa, 2003; Wakakuwa et al., 2004). In these cases, color discrimination could theoretically be extended by means of filtering pigments but direct behavioral evidence confirming this is missing. Different corneal filters in different ommatidia found in tabanid flies and grasshoppers have the same potential to create new receptor types (Kong et al., 1980; Lunau and Knüttel, 1995).

The ability to discriminate colors in the red range seems to be very useful. It can increase the number of flower species that can be distinguished and facilitate the finding of better hosts for larvae. This seems to be the case in *Papilio* butterflies. These animals use color vision not only when foraging for flowers (Kelber and Pfaff, 1999; Kinoshita et al., 1999) but also when making decisions about where to oviposit (Kelber, 1999). Color discrimination in the L range enables them to choose the optimal host for their offspring. The same probably applies to *Pieris* butterflies (Scherer and Kolb, 1987; Kelber, 2001; Weiss and Papaj, 2003).

All species investigated so far within the third butterfly family, Nymphalidae, have only three opsin genes (Sauman et al., 2005; Briscoe and Bernard, 2005) but more than three receptor types have been reported from several species (Bernard, 1979; Kinoshita et al., 1997; Stavenga et al., 2001) (for a review, see Briscoe and Chittka, 2001). The sensitivity curves recorded from the nymphalid butterflies *Polygonia c-aureum* and *Sasakia charonda* show clearly that the long-wavelength cut-off of all L receptors coincide, whereas peak sensitivities differ by as much as 50 nm. This can only result from filtering, not from multiple opsins (Kinoshita et al., 1997).

We have chosen to investigate whether color vision extends into the L range, in two species of the nymphalid family, *Vanessa atalanta* (Linnaeus 1758; subfamily Nymphalinae) and *Heliconius erato* (Linnaeus 1758; subfamily Heliconiinae). Both species have red areas on their wings, thus color discrimination in the red range could be useful for mate detection as well. In *H. erato* only three receptor types have been described so far, with λ_{max} at 370, 470 and 570 nm (Fig. 2) (Struwe, 1972). However, electroretinograms (Bernhard et al., 1970; Swihart and Gordon, 1971) and electrophysiological recordings in the brain (Swihart, 1972) give indications that a second L receptor (λ_{max} at 620 nm) may exist. It has long been known that *Heliconius* uses color to find food (Swihart, 1971), and both color and polarized light cues are used by males in choosing mates (Jiggins et al., 2001; Sweeney et al., 2003). *V. atalanta* has three receptors with λ_{max} at 360, 470 and 530 nm similar to the congener *V. cardui* (Fig. 2) (Briscoe et al., 2003) (G. Bernard, personal communication). We have performed behavioral experiments, characterized the opsin genes and their expression in the retina, and studied the eye glow and lateral filtering pigments. In short, we prove, for the first time using behavioral studies, that an insect with only S, M and L opsin pigments can discriminate colors in the red range. This is not explained by the sensitivities of the opsins alone and it is probably due to the shift in the receptors' sensitivity caused by the presence of lateral filtering pigments.

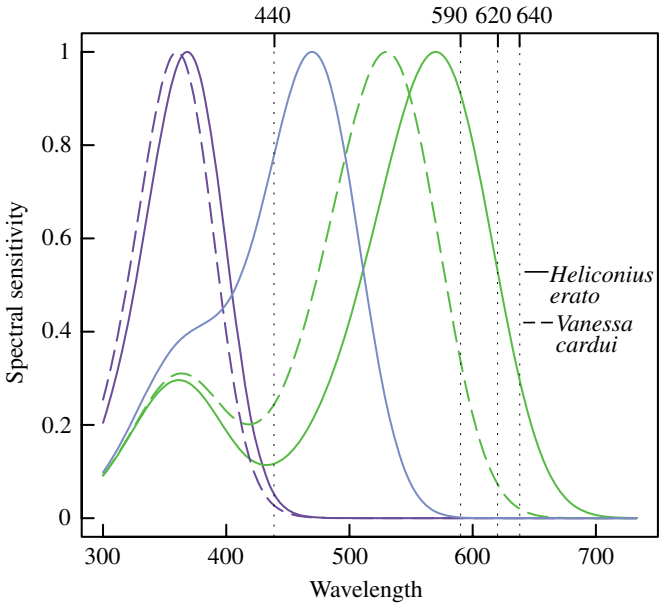

Fig. 2. Spectral sensitivity of the photoreceptors calculated from the sensitivity maxima given in Struwe (Struwe, 1972) for *H. erato* (solid lines) and Briscoe et al. (Briscoe et al., 2003) for *V. cardui* (broken lines) using the template from Stavenga et al. (Stavenga et al., 1993). Peak sensitivity values for the short, medium and long wavelength visual pigments are identical between *V. cardui* and *V. atalanta* (not shown; G. Bernard, personal communication). Observe that both species have the medium wavelength photoreceptor peaking at 470 nm therefore the curves for both species coincide. The vertical dotted lines correspond to the wavelengths of the stimuli used.

Materials and methods

Animals

Heliconius erato butterflies were bought as pupae from a professional breeder (Stratford Butterfly Farm, England). The pupae were hung on pieces of cardboard in plastic boxes and kept at high humidity at 28–30°C under a 12 h:12 h light:dark regime. *Vanessa atalanta* butterflies were reared in the lab from animals captured locally around Lund (Sweden), and

allowed to oviposit on nettle leaves (*Urtica dioica*), its natural host, which was also collected around Lund. The caterpillars were kept in plastic boxes and the nettle leaves were changed every day. When *V. atalanta* butterflies pupated they were hung on pieces of cardboard in the same manner as *H. erato*. The pupae were kept under a 20 h:4 h light:dark regime at 25–28°C and high humidity. Once the adults of both species emerged, they were individually marked on the wings and released in a flight cage. The butterflies were fed with 20% (w/w) sucrose solution on the positive stimulus during the training and test. On average, only about half the animals would eclose from the pupa. Of these animals, not all survived long enough to finish the experiment. We started with 15 *H. erato* in one experiment, but we could only collect sufficient data with nine of them. In a second experiment, where we started training seven animals, four died before the end of the experiment. With *V. atalanta*, only three out of nine animals survived until the experiment was finished.

Behavioral experiments

The experiments with *H. erato* were performed in an indoor cage (2 m×1.60 m×2.8 m) constructed from metal pipes and covered with gauze except for the ceiling that was made of a thin plastic sheet. The cage was illuminated by 26 fluorescent tubes (Osram Biolux 965, 65 W; München, Germany) distributed around and above the cage. The intensity of the illumination was approximately 100 cd m^{-2} in the center of the cage. The temperature was 30°C, and the light regime was set at 12 h:12 h light:dark. The experiments with *V. atalanta* were performed in a smaller cage (75 cm×50 cm×60 cm) illuminated by four fluorescent tubes (Philips TLD 965 18 W; Eindhoven, The Netherlands). The light intensity in the center of the cage was roughly the same as in the big cage. The light regime was 20 h:4 h light:dark, and the temperature between 20 and 25°C.

The stimuli were presented vertically in an apparatus (Fig. 3A,B) consisting of a black metal plate measuring 20 cm×10 cm. Two light guides were attached to the plate connected to two independent cold light sources (Schott KL 2500, Mainx, Germany). Each light beam passed through an interference filter and a transparent Plexiglass feeder disk (Fig. 3B,C). These feeders were built such that the sucrose solution could be placed away from the center and the animals were not able to see the drop of sugar solution (Fig. 3C). Frontally two colored disks of 3 cm in diameter separated by 6 cm were visible. We used four different colors as stimuli. The colors were obtained by means of four narrow band (10 nm bandwidth) interference filters, transmitting light of 440 nm, 590 nm, 620 nm and 640 nm. Fig. 2 shows these wavelengths together with the sensitivity curves of each receptor in both species. The light intensities could be adjusted electrically, by changing the energy delivered to the light bulb, and mechanically, by means of a diaphragm. Using these two possibilities together, a large range of intensities (between 3.19×10^{15} and 2.75×10^{17}; quanta s^{-1} steradian^{-1} cm^{-2}) could be attained.

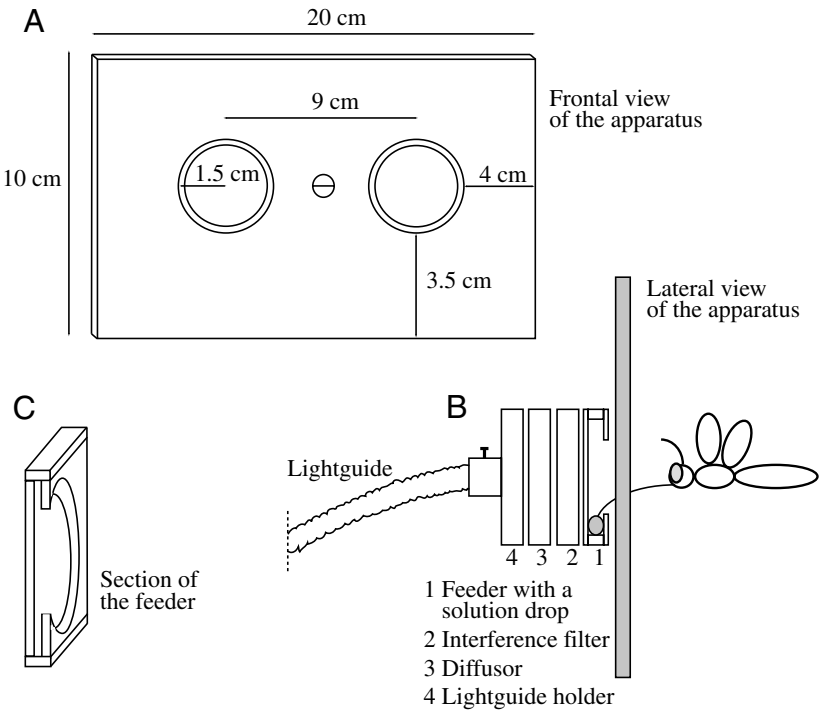

Fig. 3. Schematic views of the apparatus used to train and test the butterflies to the different colors. (A) Frontal view of the apparatus. (B) Lateral view of the apparatus. (C) The feeder disk.

Training and test

The butterflies were fed for the first time between 6 h and 8 h after they emerged. A drop of sugar water was placed on the feeder illuminated with the positive color (+); the other feeder (–) was always kept empty. Each butterfly was grasped by the wings and the proboscis was unrolled with a thin needle until it touched the sugar solution. Immediately the butterfly started to drink. This procedure was repeated twice a day. After 3–4 days, the animals flew towards the apparatus by themselves and were able to find the rewarded color (+). From this moment, the choices were registered. Each time an animal approached and touched a feeder, either with its proboscis or with a tarsus, a choice was registered. If an animal touched the feeder more than once during an approach, only the first touch was counted. In further data analysis, we only included animals that made a minimum number of 15 choices with each intensity combination, thus in total at least 60 choices.

We first trained both species to discriminate a yellow or red (590 nm or 620 nm) and a blue (440 nm) color, to determine whether the

animals used color vision at all. The animals that were able to discriminate these colors were then trained to a second pair of wavelengths. Animals did not survive long enough to be trained to three wavelength combinations. Therefore, *H. erato* that were trained to two combinations of long wavelengths were not initially trained with blue and yellow. These *H. erato* were first trained to 620 nm (+) *vs* 590 nm. When they reached the minimum number of choices, the same individuals were trained to 620 nm (+) *vs* 640 nm. *V. atalanta* were first trained to 620 nm (+) *vs* 440 nm, and once the animals reached the minimum number of choices, the same individuals were trained to 620 nm (+) *vs* 590 nm. Only one animal at a time was allowed to visit the apparatus. Each butterfly was allowed to drink for 1–2 s and then gently forced to leave for a new choice. Between choices the intensities and/or the position of the stimuli were changed in a pseudorandom way. Five different ratios of the physical intensities of +/– were used: 0.01, 0.1, 1, 10 and 100 (i.e. +100 times less intense than –, +10 times less intense than –, equal intensities and +10 times more intense than the –, +100 times more intense than –, respectively), but only three or four of them were used with one wavelength combination. This was achieved by changing the intensities of both the rewarded and the unrewarded stimulus. This schema resulted in an average of 10 choices per animal daily.

The performance of each animal with each intensity combination was evaluated separately by comparing the numbers of choices this animal made with the critical value corresponding to two-tailed significance levels (α) of 0.05 for a binomial proportion of P=0.5 [equal number of choices for each color; Rohlf and Sokal (Rohlf and Sokal, 1995), p. 107, Table Q].

Light microscopy

Under daylight illumination the heads of 10-day old *H. erato* were severed in two halves and the pieces were put in 4% PFA (paraformaldehyde) in 0.1 mol l^{-1} phosphate-buffered saline (pH 7.2) for 1 h. The eyes were dehydrated in an alcohol series (50%, 75%, 96%, 100%), finally immersed in 100% acetone and embedded in Epon resin (Agar Scientific, Agar Scientific Ltd, Essex, UK) After hardening the resin at 60°C for 48 h the eyes were cut laterally in 10 μm thick sections using a microtome and mounted on a slide. In this way the ommatidia that in the living animal pointed sideways, pointed directly at the observer in the microscope. Some *H. erato* were dark adapted by keeping them in total darkness for 30 min at room temperature and then for 20 min in total darkness at 4°C. After that, the head of the animal was cut off under red light (produced by attaching a 660 nm cut-off filter to the tip of a light guide connected to a Schott KL 2500 lamp) in order to avoid light adapting the eyes. *V. atalanta* butterflies were captured locally around Lund (Sweden) and were treated in the same way as the light adapted *H. erato*.

Eye glow

At least four dark-adapted *H. erato* and *V. atalanta* eyes were photographed with a Nikon camera connected to a modified epi-illumination microscope. In this instrument, the incident light applied to the eye is channeled by the facet lens and crystalline cone into the light-guiding rhabdoms (see Land, 1984; Miller and Bernard, 1968; Stavenga, 2002b). When the dark-adapted eye is illuminated with strong light and observed from the same direction (orthodromic illumination), a bright eye-glow is seen for a few seconds. Light reaching the ommatidial tapetum is reflected and guided back through the rhabdom. When not absorbed there, it leaves the eye again and is then observable as the eye glow. The butterflies were restrained by waxing the thorax and head to a support but were otherwise alive and intact. The animals were oriented such that pictures could be taken in the frontal-lateral part of the eye. The eye glow was photographed by leaving the shutter of the camera open and delivering flashes of between 0.1 s and 0.5 s with intervals of 10 s. In this way, the eye glow could be photographed without the pupil closing. As a light source we used a xenon arc lamp that supplied 1.4×10^{-4} W cm^{-2}. The objective lens was a Zeiss Luminar 25 mm (3.5/A 0.15). To study the spectral sensitivity of the pupil response, we illuminated the eye with lights of different wavelengths obtained by means of interference filters (680 nm, 650 nm, 620 nm, 590 nm; 10 nm bandwidth) and observed pupil closure.

PCR, cloning and sequencing

The *H. erato* opsins were isolated by the polymerase chain reaction (PCR) using a combination of degenerate and gene-specific primers. cDNA template was prepared from RNA extracted from whole head tissue (Trizol; Gibco-BRL, Gaithersburg, MD, USA) and synthesized using the Marathon cDNA Amplification Kit (BD Biosciences Clontech, Mountain View, CA, USA). PCR products were ligated into the pGEM-T Easy cloning vector (Promega, Madison, WI, USA) and plasmids were screened by *Eco*RI digestion for inserts of the correct size. Plasmid DNA was cycle sequenced using the Big Dye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the core sequencing facilities at the University of California, Irvine. In this initial screen, we sequenced more than 100 clones, and then designed opsin gene-specific primers to use in a multiplex PCR. To ensure that all opsins had indeed been recovered, approximately 120 additional plasmids were screened using multiplex PCR in which three pairs of opsin gene-specific primers were combined in a single PCR reaction. Only those plasmids that did not amplify with those primers were then sequenced.

Phylogenetic analysis

To identify the homology of the cloned *H. erato* opsins, we conducted a BLAST search and downloaded a total of 41 full-length arthropod opsin genes from GenBank including representatives of all available insect orders (six total), as well as chelicerates and crustaceans. Only first and second nucleotide positions were used as third positions were saturated (A.D.B.,, unpublished observation). A gene tree was

reconstructed using the neighbor-joining algorithm with Tamura-Nei distance, heterogeneous rates among lineages and complete deletion of gaps as implemented in MEGA 3.0 (Kumar et al., 2004). Robustness of the tree was assessed by bootstrap analysis (1000 replicates).

Cryosectioning

The adult butterflies were placed at 4°C for 1 h before being killed by a swift severing of the head with a scalpel. Subsequently, the head was cut in half. The eyes were fixed in 4% PFA in 1× phosphate-buffered saline (pH 7.2) for 2 h to 4 h at room temperature and stepped through a sucrose gradient (10%, 20%, 30%). Then the tissue was cryostat sectioned into 8–12 μm slices at –18°C and placed on a slide. The eyes were oriented such that the ommatidia that in the living animal pointed directly to the front, pointed directly at the observer.

Riboprobe synthesis and in situ hybridization

Starting with 1 μg of purified PCR product (amplified from plasmid DNA), digoxigenin-UTP-labeled RNA probes (riboprobes) complementary to the mRNAs of the visual pigments were synthesized by using a DIG RNA labeling kit (Roche Diagnostics, Mannheim, Germany). After the synthesis, the riboprobes were precipitated with 4 mol l^{-1} LiCl and 100% ethanol. A dot blot procedure was used to quantify the amount of riboprobe. Typically, 10 ng/μl of riboprobe was obtained after this procedure.

The slides with the sections were then incubated in hybridization buffer (0.3 mmol l^{-1} NaCl, 2.5 mmol l^{-1} EDTA, 20 mmol l^{-1} Tris-HCl, pH 8.0, 50% formamide, 10% dextran sulfate, 100 g ml^{-1} yeast tRNA and 1× Denhart's medium) (Sakamoto et al., 1996) in a humid chamber for 30 min at 60°C. The labeled probe was diluted in the hybridization buffer (1:75), corresponding to approximately 0.013 ng of probe per ml of hybridization buffer. The sections were incubated in the diluted probe overnight at 55–60°C in a humid chamber and then washed with 2×, 1× and 0.1× standard saline citrate and 0.1% Tween 20, for 10 min each. The probes were identified in the histologic sections by incubation with an anti-digoxigenin alkaline phosphatase-conjugated antibody (Boehringer Mannheim), diluted in 1× phosphate buffer plus Tween 20 (1:1000) for 2 h. The probes were detected by a colorimetric reaction produced by Nitro Blue tetrazolium (5-bromo-4-chloro-3-indolylphosphate) and 10% Tween 20 in alkaline phosphatase developing solution. An Axioskop microscope (Zeiss, Thornwood, NY, USA) equipped with an AxioCam Hrc digital camera (Zeiss) was used to collect images. Image data were recorded in ZeissVision 3.1 software on a personal computer at 2,060×2,600 pixel resolution.

Results

Behavior

In order to investigate whether *H. erato* or *V. atalanta* have color vision in the L range, we first determined that the animals were capable of discriminating a rewarded color from an unrewarded color. Fig. 4A shows that *V. atalanta* (N=3) was able to discriminate a rewarded light stimulus of 620 nm (+) from 440 nm. The animals chose it significantly more often than the unrewarded one independently of the intensity (three animals, at least 15 choices in each intensity combination, P<0.05 for each animal in each intensity combination). When the same animals were trained to discriminate 620 nm (+) from 590 nm (Fig. 4B), the situation was completely different. The animals always approached the brighter stimulus, indicating that the wavelength was not the relevant feature in this test. Even with an intensity ratio of one, the animals chose the unrewarded color (590 nm). As the peak sensitivity of the green receptors of *V. atalanta* is similar to that of its close relative *Vanessa cardui* [530 nm; G. Bernard, personal communication (Briscoe et al., 2003)], it is more sensitive to light of 590 nm than to light of 620 nm. If both lights have the same physical intensity, 590 nm is thus supposed to look brighter for *V. atalanta* than 620 nm. The fact that the choice frequencies massively change with the relative intensities of the stimuli clearly disproves the use of color vision.

Similar to *V. atalanta, H. erato* was also able to discriminate a rewarded stimulus of 590 nm (+) from 440 nm (figure not shown). The same animals were later trained to discriminate 590 nm (+) from 620 nm. However, the performance of *H. erato* in discriminating the 620 nm (+) light from 590 nm was strikingly different from that of *V. atalanta*. The three animals trained to discriminate 590 nm (+) from 440 nm first and from 620 nm later, chose 590 nm significantly more often than 620 nm, independent of the relative intensities (three animals, minimum of 15 choices per animal and intensity combination, P<0.05 for each intensity combination and animal). The same was true for *H. erato* (N=9) that were trained to discriminate 620 nm from 590 nm without prior training with other wavelengths (Fig. 4C). The choices of the animals were based only on the wavelength and were independent of the intensity (P<0.05, for each animal and intensity combination). These animals were then trained to discriminate lights of 620 nm (+) from 640 nm (Fig. 4D). To the L receptor of *H. erato* (peak sensitivity at 570 nm), a light of 620 nm must look brighter than an equally intense light of 640 nm (Fig. 2). Therefore, we presented the 620 nm (+) light 100× darker than the 640 nm light (an intensity ratio of 0.01). Even in this critical test four out of nine animals chose the correct color significantly more often than 50% [Fig. 4D; butterflies nos. 3 (20/26), 4 (20/26), 5 (23/34), 6 (22/31), correct choices/total choices]. P<0.05 for each of these four animals). Our results indicate that *H. erato* has color vision in the L range, whereas *V. atalanta* does not.

A similar behavioral result was reported in *Papilio aegeus* (Kelber and Pfaff, 1999). In this study it was shown that *Papilio* butterflies could discriminate colors in the red range (590 nm *vs* 620 nm). In *Papilio*, this ability is based on duplicated L opsins that these animals possess (Arikawa, 2003). In contrast, only one L opsin has so far been found in the compound eyes of *Heliconius* (Hsu et al., 2001) and the M opsin is not sensitive to light of 620 nm (Fig. 2).

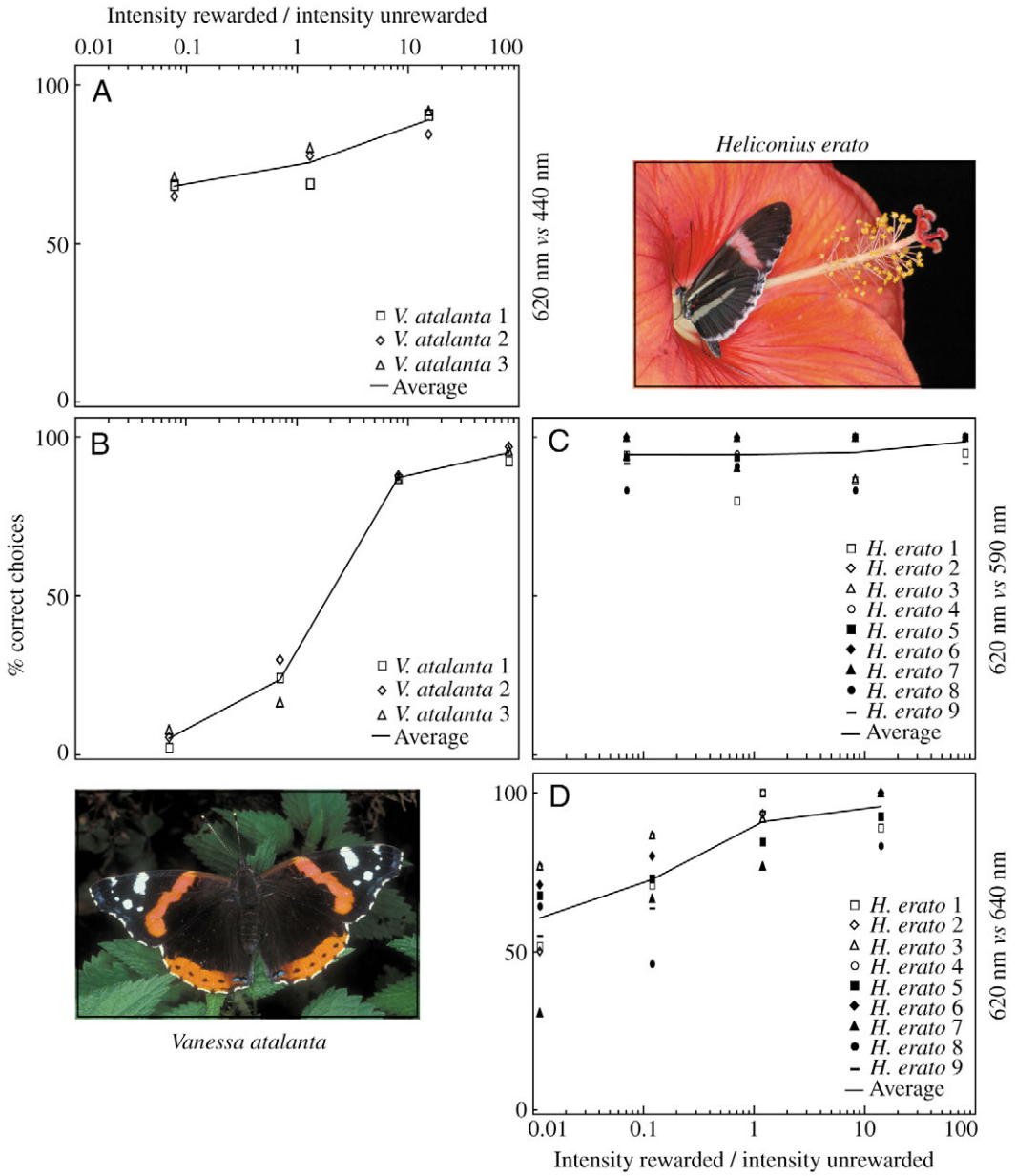

Fig. 4. Choice frequencies of *H. erato* and *V. atalanta* for the four colors after training, as a function of the ratio between the intensities of the rewarded color and the unrewarded color. The symbols represent the individual performance and the line the average. (A) Three *V. atalanta* trained to 620 nm as the rewarded color and 440 nm as unrewarded. All choices differ significantly from chance (P<0.05). (B) The same three butterflies as in A but this time 620 nm as the rewarded color and 590 as unrewarded. All choices differ significantly from chance (P<0.05). (C) Nine *H. erato* trained to 620 nm as the rewarded color and 590 nm as the unrewarded. The choices of every single animal differ significantly from chance (P<0.05). (D) The same nine animals from the previous experiment trained to 620 nm rewarded and 640 nm unrewarded. The choices of butterflies nos. 3, 4, 5 and 6 differ significantly from chance (P<0.05) at the intensity ratio 0.01.

Different kind of opsins and their expression in the retina

In order to see if the capability of *H. erato* to discriminate between stimuli of long wavelengths is based on the expression of more than one L opsin we screened a cDNA library synthesized from adult eyes. More than 200 clones were screened and only one L opsin-encoding mRNA was found in the compound eyes of *H. erato*, along with blue and ultraviolet opsin-encoding mRNA transcripts. The GenBank accession numbers for these genes are as follows: *UVRh*, AY918904; *BlueRh*, AY918906; *LWRh*, AY918907. To see how these three opsins are expressed, at least 50 eyes were analyzed after performing *in situ* hybridization in the frontal and fronto-lateral part of the compound eye. Fig. 5 shows an example of the hybridization pattern obtained after using *UVRh*, *BlueRh* and *LWRh* digoxigenin-labeled antisense riboprobes. Fig. 5A shows the pattern produced by the *UVRh* riboprobe. Some

ommatidia show two cells stained, others show only one cell stained and some no staining. The same configuration can be observed when the *BlueRh* riboprobe was used (Fig. 5B). Since the sections in Fig. 5A,B are consecutive, the position of the cells in both pictures can be followed (black circles in the insets mark the same ommatidia). We observed three different types of ommatidia. The ommatidia that express *UVRh* in two cells do not express *BlueRh*. Reciprocally, the ommatidia that express *BlueRh* in two cells do not express *UVRh*. The third type of ommatidia expresses each opsin mRNA in one cell. This is the same situation as in *V. cardui* (Briscoe et al., 2003), *P. xuthus* (Arikawa, 2003), *Pieris rapae* (Arikawa et al., 2005; Wakakuwa et al., 2004), *Manduca sexta* (White et al., 2003), and in *Danaus plexippus* (Sauman et al., 2005). Fig 5C,D show the *LWRh* pattern, in which six cells are stained in all ommatidia across the whole section. Each cell expresses only one of the three opsins, and we found no evidence of co-expression. This pattern was seen in all the eyes examined. We, therefore, conclude that the difference between *H. erato* and *V. atalanta* in their ability to discriminate light in the long wavelength part of the spectrum cannot be attributed to the presence of a second L opsin.

Eye glow and histology: Lateral filtering pigment heterogeneity in H. erato

Since *H. erato* can extend their color vision range into the long wavelengths using only one L opsin which is homogeneously distributed, we next studied whether the ommatidia differ in aspects other than the S and M opsin expression. We first examined the light that is reflected by the tapetum and emerges from the compound eye (eye glow) of *H. erato*, using an ophthalmoscope, and found two classes of ommatidia (Fig. 6A). One class of ommatidia reflects yellow light and the other reflects red. By contrast, all ommatidia of *V. atalanta* reflect an homogeneous orange (Fig. 6D) in the same fashion as in *V. cardui*, a close relative (Briscoe and Bernard, 2005).

When we applied lights of different wavelengths by placing

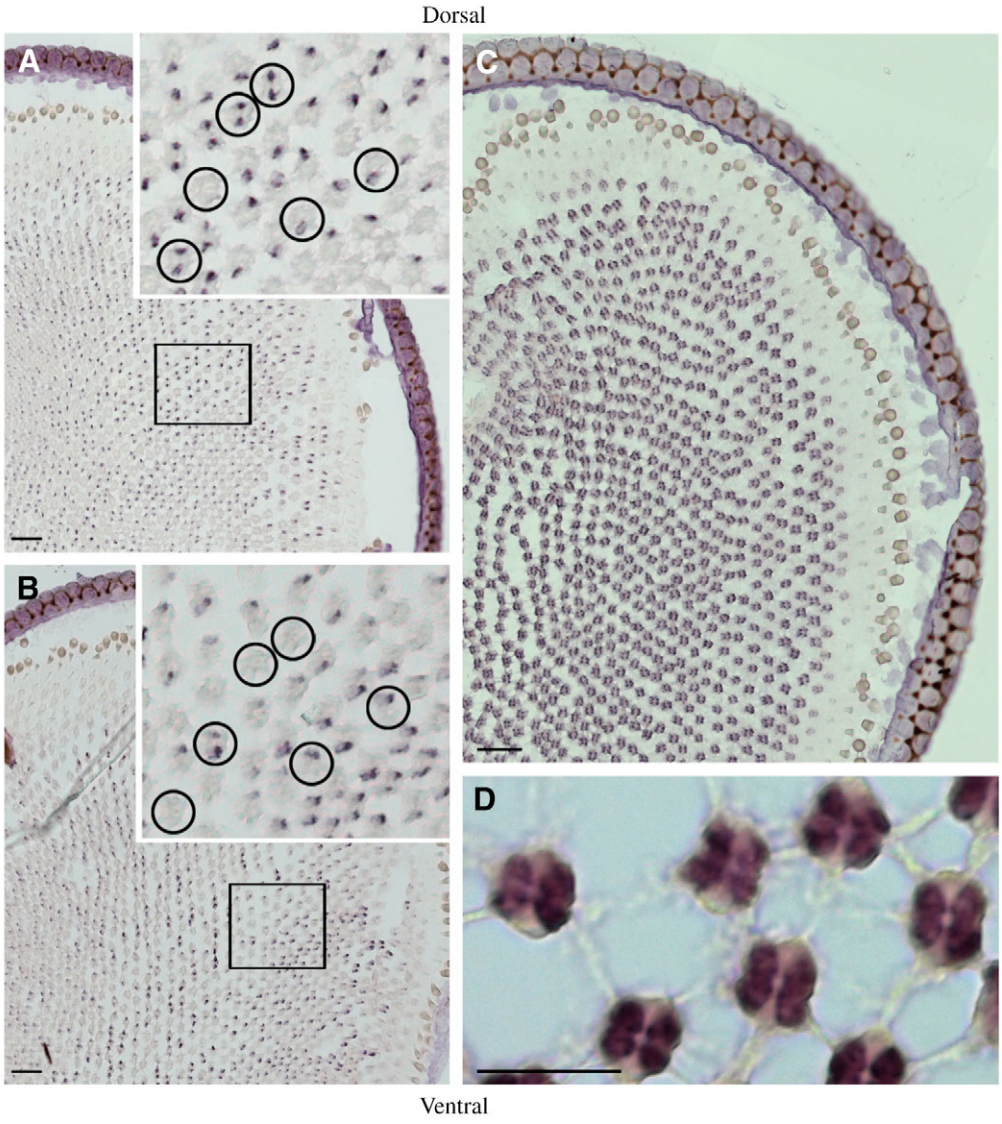

Fig. 5. *In situ* hybridizations of cryostat sections of the compound eye of *H. erato* using *UVRh*, *BlueRh* and *LWRh* digoxigenin-labeled antisense riboprobes. (A) *UVRh* and (B) *BlueRh* opsin mRNAs, respectively, detected using an alkaline phosphatase-conjugated anti-digoxigenin antibody. The insets show the magnification of the boxed region. Since the sections are consecutive, the position of the same ommatidium in A can be identified in B, as indicated by aligning the black circles. The butterflies express these two opsin mRNAs in three different ways in different ommatidia: S-S, S-M and M-M in the R1 and R2 photoreceptor cells. (C) Expression pattern of the *LWRh* opsin mRNA transcript. In every ommatidium in the main retina, six photoreceptor cells express the L opsin mRNA. (D) Close up view of the L opsin mRNA expression pattern in the R3-8 photoreceptor cells. Sections are at around 160 μm from the cornea. Scale bars, 50 μm (A–C); 20 μm (D).

interference filters (680 nm, 650 nm, 620 nm, 590 nm) between the light source and the eye of *H. erato*, we observed that the red and the yellow ommatidia closed to different degrees (data not shown). Since the pupil closure is under the control of the photoreceptors in each ommatidium (Stavenga, 1979), the different degrees of closure are a result of different sensitivities of the receptors in both ommatidial classes to these wavelengths. Considering the wavelengths of the test lights, we conclude that the L receptors must differ in their spectral sensitivity between ommatidial classes, and this could provide the basis for color vision in the L range. In *V. atalanta* all ommatidia close completely and at the same speed when illuminated with 620 nm and 590 nm. When illuminated with 650 nm a partial closure was observed indicating that the photoreceptors are slightly sensitive to this wavelength.

We hypothesized that, as has been shown in *Pieris rapae* (Wakakuwa et al., 2004) the occurrence of the two physiologically distinct *H. erato* ommatidial classes might result from different populations of lateral filter pigments. Because of the waveguide properties of the narrow butterfly rhabdom (Nilsson et al., 1988), lateral filter pigments can affect the wavelengths of light to which the receptors are sensitive (Miller and Bernard, 1968; Ribi, 1979; Stavenga, 2002a; Stavenga, 2002b).

We tested this hypothesis by inspecting serial sections through the compound eye, and found that the difference between the heterogeneous eye glow of *H. erato* and the homogeneous eye glow of *V. atalanta* may be due to differences in the presence or absence, and distribution of lateral filter pigments. In a tangential section of the fronto-lateral region of the compound eye of *H. erato* (190 μm from the cornea), the presence of pupillary pigments can be observed (Fig. 6B). In dark-adapted eyes these pigments are further away from the rhabdoms than in light adapted eyes (picture not shown). These pigments disappear completely around 220 μm and after a gap, a second type of red pigmentation appears around 320 μm from the cornea. The pigments at this depth (Fig. 6C, 370 μm from the cornea) are closer to the rhabdom than the pupillary pigments (distance between opposite pigment spots 0.68±0.20 μm, mean ± s.d.; N=10) and do not move as a function of light adaptation. Their coating of the rhabdom means that they can filter the short wavelength light traveling in the waveguide, and therefore change the spectral sensitivity. The color of this pigment is heterogeneous among different ommatidia (see circles marked a and b, in Fig. 6C). This pigment heterogeneity is also evident at sections taken more proximal in the eye (480 μm, picture not shown). We propose that this heterogeneity of pigmentation may be the cause of the heterogeneous eye glow and the difference in speed of the pupil closure (i.e. different sensitivities).

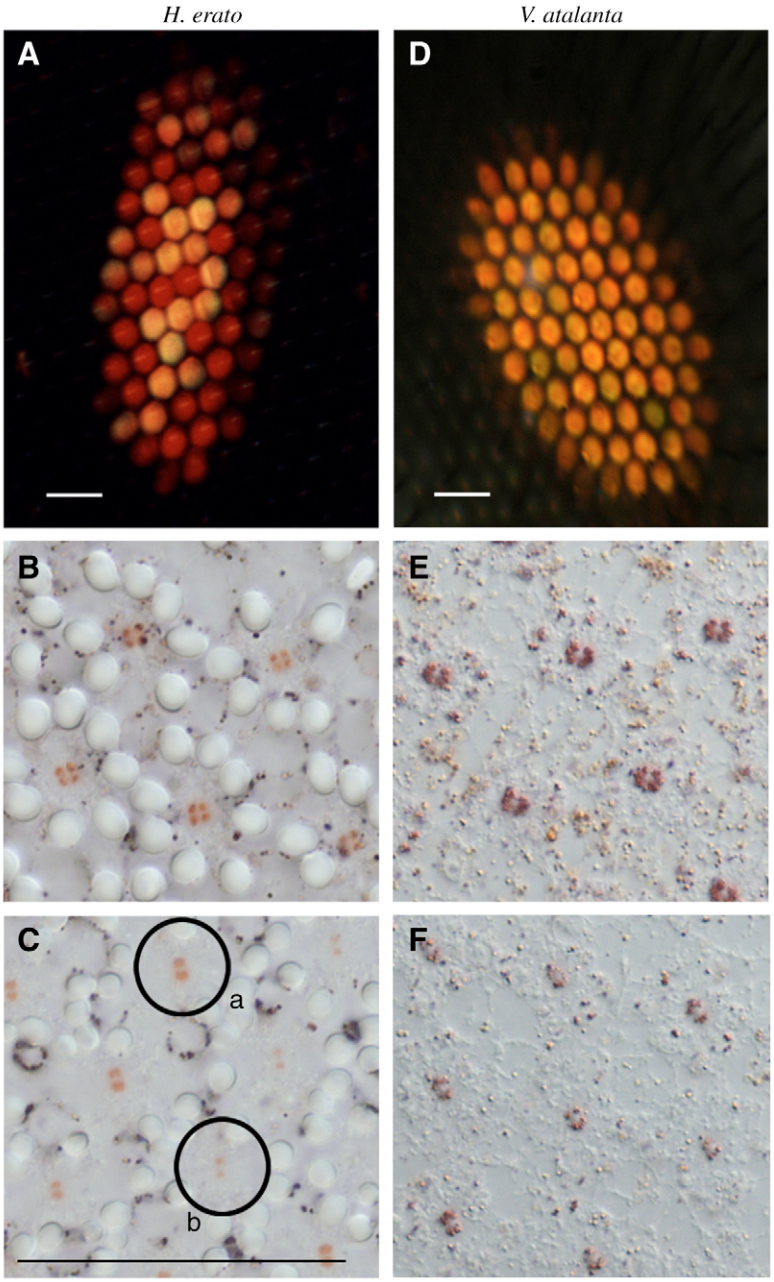

Fig. 6. Eye glow, pupillary pigments and lateral filtering pigments of *H. erato* (A–C) and *V. atalanta* (D–F) from the fronto-lateral eye region. (A) Eye glow of *H. erato*. Two classes of ommatidia can be observed, one of them reflects yellow and the other red. (B) Pupillary pigments at 190 μm from the cornea. (C) Lateral filtering pigments at 370 μm from the cornea vary between ommatidia (circles a and b) compared to the uniformly distributed pupillary pigments. (D) Eye glow of *V. atalanta*. Only one orange reflecting class exists. (E) Pupillary pigments at 180 μm from the cornea. (F). Pupillary pigments at 250 μm from the cornea. Uniform pupillary pigment density between ommatidia can be observed in all photoreceptor cells. Sections are 10 μm thick. Scale bars, 50 μm.

In the eye of *V. atalanta* (Fig. 6E,F), the only pigments detected were the pupillary pigments (see also Stavenga, 1979). We found no histological evidence of a lateral filtering pigment or pigment heterogeneity between ommatidia, which is consistent with the homogeneous eye glow, the pupil closure, experimental reflectance spectra (G. Bernard, personal communication), and with the data from the other species of this genus that was recently examined, *V. cardui* (Briscoe et al., 2003).

Discussion

Color discrimination in the long-wavelength range with one opsin

We conclude that *H. erato* has color vision in the red range, based on two photoreceptor types containing the same opsin. The difference in the spectral sensitivities necessary to accomplish color vision must result from other mechanisms than a new opsin, and is probably the result of the expression of different lateral filtering pigments in different ommatidia.

We have compared the color vision abilities of two butterfly species that possess three opsin genes coding for opsins in the S, M and L range. One of the species, *V. atalanta*, was able to discriminate between blue (440 nm) and yellow (590 nm) with high accuracy but not between yellow (590 nm) and red (620 nm; Fig. 4A,B). This indicates that only one receptor type is sensitive to both long wavelengths. The blue-sensitive receptor is insensitive to these long wavelengths making a comparison of different receptor signals (and thus color vision) impossible. In the absence of a color difference, *V. atalanta* used the intensity of the stimulus as a choice criterion. This result is similar to that obtained with a sphingid that also possesses three spectral types of receptors leaving it unable to discriminate yellow from red (Kelber and Hénique, 1999).

In contrast, *H. erato* is able to discriminate 620 nm not only from 590 nm but also from 640 nm (Fig. 4C,D). Two spectral types of receptors must therefore be sensitive to light of 620 nm wavelength. In *P. aegeus*, the ability to discriminate 590 nm from 630 nm is mediated by a separate red receptor containing a separate red opsin (Kelber and Pfaff, 1999; Matic et al., 1983). This second L opsin evolved as a result of a gene duplication event, which occurred after the divergence of the nymphalid and papilionid lineages (Briscoe, 2001). In *H. erato*, our extensive search for an additional L opsin gene was unsuccessful. Six of eight proximal photoreceptors in each ommatidium, and probably the ninth basal receptor express the same known green-sensitive opsin (Fig. 5C,D). The remaining two receptors are two UV receptors, two blue receptors or one of each (Fig. 5A,B). This situation is the same in *V. cardui*, a close relative of *V. atalanta* (Briscoe et al., 2003). The blue receptors of both species have a peak sensitivity at longer wavelengths than other insect blue receptors (470 nm) (Struwe, 1972) [for comparison see Briscoe and Chittka (Briscoe and Chittka, 2001)] but this cannot explain our results (Fig. 2). We conclude that *H. erato* has color vision in the red range, without having two opsins sensitive in this spectral range.

Do lateral filtering pigments create a second long-wavelength receptor?

The ophthalmoscope studies of *H. erato* revealed two classes of ommatidia differing in eye glow color (Fig. 6A). This is similar to many butterfly eyes studied by Stavenga (Stavenga, 2002a; Stavenga, 2002b), including another species of the large genus *Heliconius*, *H. melpomene*. By contrast, a uniform eye glow was seen in *V. atalanta* (Fig. 6D), similar to that of *V. cardui* and other nymphalids including *Nymphalis antiopa, Siproeta steneles, Inachis io* and *Polygonia c-album* (Briscoe and Bernard, 2005; Stavenga, 2002a). Possible mechanisms underlying the different eye glow colors include lateral filtering pigments, different opsin densities and tapetal reflection. Different opsin densities are hard to prove; nonetheless, we cannot exclude them as a possible cause for the difference in the ommatidial reflection seen in *H. erato*. Differences in tapetal reflection have little relevance since they only affect light absorbed on the way out of the receptor. This is a very small amount as most light is absorbed on the way into the eye. The most likely candidate causing differences between receptor spectra are the lateral filtering pigments found in *H. erato* but not in *V. atalanta* (Fig. 6). These pigments are close enough to the rhabdom to act as lateral filters. Different pigments in different ommatidia (Fig. 6C) can result in two kinds of ommatidia with different spectral sensitivities as has been shown for *P. rapae* (Wakakuwa et al., 2004). Further studies including electrophysiological measurements are needed to reveal the exact receptor sensitivities.

Color vision with filter pigments?

To our knowledge, *H. erato* is the first animal shown to use two photoreceptors containing the same opsin for color vision. We hypothesize that lateral filtering pigments may be the basis of the difference between the two receptor types in *Heliconius* as well. Filtering pigments can extend the range of color vision by shifting the L receptor sensitivity in one class of ommatidia. The comparison between different receptors created in this way allows the animal to discriminate colors at longer wavelengths than those expected by the opsins alone. The total range of vision, which is set by the sensitivity of the opsins, is unaltered. The fact that *Pieris* and *Papilio* also have lateral filtering pigments (Arikawa, 2003; Wakakuwa et al., 2004) makes it probable that this mechanism is older than the evolution of a separate red opsin in *Papilio,* and common in butterflies. There is a high probability that butterflies such as *Sasakia charonda*, *Polygonium c-aureum* (Kinoshita et al., 1997) and *Pieris rapae* are also able to discriminate colors in the red range. Given the receptor curves measured (Wakakuwa et al., 2004), the color discrimination ability of *P. rapae* might extend even further into the red range than color vision of *H. erato*.

Red filtering pigments that may change receptor sensitivity have earlier been found in a hymenopteran insect (Ribi, 1978). We expect that careful studies will reveal more such cases and conclude that the study of opsin genes and their expression is not sufficient to understand the evolution of color vision systems in animals.

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References

Arikawa, K. (2003). Spectral organization of the eye of a butterfly, *Papilio*. *J. Comp. Physiol. A* **189**, 791-800.

Arikawa, K., Scholten, D. G. W., Kinoshita, M. and Stavenga, D. G. (1999). Tuning of photoreceptor spectral sensitivities by red and yellow pigments in the butterfly *Papilio xuthus*. *Zool. Sci*. **16**, 17-24.

Arikawa, K., Wakakuwa, M., Qiu, X., Kurasawa, M. and Stavenga, D. G. (2005). Sexual dimorphism of short-wavelength photoreceptors in the small white butterfly, *Pieris rapae crucivora*. *J. Neurosci*. **25**, 5935-5942.

Bernard, G. D. (1979). Red-absorbing visual pigment of butterflies. *Science* **203**, 1125-1127.

Bernhard, C. G., Boethius, J., Gemme, G. and Struwe, G. (1970). Eye ultrastructure, colour and behaviour. *Nature* **226**, 865-866.

Briscoe, A. D. (2001). Functional diversification of lepidopteran opsins following gene duplication. *Mol. Biol. Evol*. **18**, 2270-2279.

Briscoe, A. D. and Bernard, G. D. (2005). Eyeshine and spectral tuning of long wavelength-sensitive rhodopsins: no evidence for red-sensitive photoreceptors among five Nymphalini butterfly species. *J. Exp. Biol.* **208**, 687-696.

Briscoe, A. D. and Chittka, L. (2001). The evolution of color vision in insects. *Annu. Rev. Entomol*. **46**, 471-510.

Briscoe, A. D., Bernard, G. D., Szeto, A. S., Nagy, L. M. and White, R. H. (2003). Not all butterfly eyes are created equal: rhodopsin absorption spectra, molecular identification, and localization of ultraviolet-, blue-, and green-sensitive rhodopsin-encoding mRNAs in the retina of *Vanessa cardui*. *J. Comp. Neurol*. **458**, 334-349.

Douglas, R. H. and Marshall, N. J. (1999). A review of vertebrate and invertebrate ocular filters. In *Adaptive Mechanisms in the Ecology of Vision* (ed. S. N. Archer, M. B. A. Djamgoz, E. R. Loew and S. Vallerga), pp. 95-162. Dordrecht, Boston, London: Kluwer.

Goldsmith, T. H. and Bernard, G. D. (1974). The visual system of insects. In *The Physiology of Insecta*. 2nd edn (ed. M. Rockstein), pp. 165-272. New York: Academic Press.

Hill, C. A., Fox, A. N., Pitts, R. J., Kent, L. B., Tan, P. L., Chrystal, M. A., Cravchik, A., Collins, F. H., Robertson, H. M. and Zweibel, L. J. (2002). G protein-coupled receptors in *Anopheles gambiae*. *Science* **298**, 176-178.

Hsu, R., Briscoe, A. D., Chang, B. S. W. and Pierce, N. E. (2001). Molecular evolution of a long-wavelength-sensitive opsin in mimetic *Heliconius* butterflies (Lepidoptera: Nymphalidae). *Biol. J. Linn. Soc. Lond.* **72**, 435-449.

Jiggins, C. D., Naisbit, R. E., Coe, R. L. and Mallet, J. (2001). Reproductive isolation caused by colour pattern mimicry. *Nature* **411**, 302-305.

Kelber, A. (1999). Ovipositing butterflies use a red receptor to see green. *J. Exp. Biol.* **202**, 2619-2630.

Kelber, A. (2001). Receptor based models for spontaneous colour choices in flies and butterflies. *Entomol. Exp. Appl*. **99**, 231-244.

Kelber, A. and Hénique, U. (1999). Trichromatic colour vision in the hummingbird hawkmoth, *Macroglossum stellatarum* L. *J. Comp. Physiol. A* **184**, 535-541.

Kelber, A. and Pfaff, M. (1999). True colour vision in the orchard butterfly, *Papilio aegeus*. *Naturwissenschaften* **86**, 221-224.

Kelber, A., Vorobyev, M. and Osorio, D. (2003). Animal colour vision-behavioural tests and physiological concepts. *Biol. Rev. Camb. Philos. Soc*. **78**, 81-118.

King-Smith, P. E. (1969). Absorption spectra and function of the coloured oil drops in the pigeon retina. *Vision Res*. **9**, 1391-1399.

Kinoshita, M., Sato, M. and Arikawa, K. (1997). Spectral receptors of nymphalid butterflies. *Naturwissenschaften* **84**, 199-201.

Kinoshita, M., Shimada, N. and Arikawa, K. (1999). Colour vision of the foraging swallowtail butterfly *Papilio xuthus*. *J. Exp. Biol.* **202**, 95-102.

Kong, K. L., Fung, Y. M. and Wasserman, G. S. (1980). Filter-mediated color vision with one visual pigment. *Science* **207**, 783-786.

Kumar, S., Tamura, K. and Nei, M. (2004). MEGA3, an integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinformatics* **5**, 150-163.

Land, M. F. (1984). The resolving power of diurnal superposition eyes measured with an ophthalmoscope. *J. Comp. Physiol. A* **154**, 515-533.

Lunau, K. and Knüttel, H. (1995). Vision through colored eyes. *Naturwissenschaften* **82**, 432-434.

Matic, T. (1983). Electrical inhibition in the retina of the butterfly *Papilio* I Four types of photoreceptors. *J. Comp. Physiol*. **152**, 169-182.

Menzel, R. (1979). Spectral sensitivity and color vision in invertebrates. In *Handbook of Sensory Physiology.* Vol. VII/6A (ed. H. Autrum), pp. 503-580. Berlin, Heidelberg, New York: Springer.

Miller, W. H. and Bernard, G. D. (1968). Butterfly glow. *J. Ultrastruct. Res*. **24**, 286-294.

Nilsson, D.-E., Land, M. F. and Howard, J. (1988). Optics of the butterfly eye. *J. Comp. Physiol. A* **162**, 341-366.

Peitsch, D., Fietz, A., Hertel, H., de Souza, J., Ventura, D. F. and Menzel, R. (1992). The spectral input systems of hymenopteran insects and their receptor-based colour vision. *J. Comp. Physiol. A* **170**, 23-40.

Qiu, X. and Arikawa, K. (2003). Polymorphism of red receptors: sensitivity spectra of proximal photoreceptors in the small white butterfly *Pieris rapae crucivora*. *J. Exp. Biol.* **206**, 2787-2793.

Qiu, X. D., Vanhoutte, K. A. J., Stavenga, D. G. and Arikawa, K. (2002). Ommatidial heterogeneity in the compound eye of the male small white butterfly, *Pieris rapae crucivora*. *Cell Tissue Res*. **307**, 371-379.

Ribi, W. A. (1978). Colour receptors in the eye of the digger wasp, *Sphex cognatus* Smith: evaluation by selective adaptation. *Cell Tissue Res*. **195**, 471-483.

Ribi, W. A. (1979). Coloured screening pigments cause red eye glow hue in pierid butterflies. *J. Comp. Physiol. A* **132**, 1-9.

Rohlf, F. J. and Sokal, R. R. (1995). *Statistical Tables*. New York: Freeman and Co.

Sakamoto, K., Hisatomi, O., Tokunaga, F. and Eguchi, E. (1996). Two opsins from the compound eye of the crab *Hemigrapsus sanguineus*. *J. Exp. Biol.* **199**, 441-450.

Sauman, I., Briscoe, A. D., Zhu, H., Shi, D., Froy, O., Stalleicken, J., Yuan, Q., Casselman, A. and Reppert, S. M. (2005). Connecting the navigational clock to sun compass input in monarch butterfly brain. *Neuron* **46**, 457-467.

Shimizu, I., Yamakawa, Y., Minamoto, T. and Sakamoto, K. (1998). Cloning of gene encoding the visual pigments in the silkworm, *Bombyx mori*. *Appl. Entomol. Zool*. **33**, 199-204.

Scherer, C. and Kolb, G. (1987). Behavioural experiments on the visual processing of color stimuli in *Pieris brassicae* L (Lepidoptera). *J. Comp. Physiol. A* **160**, 645-656.

Spaethe, J. and Briscoe, A. D. (2005). Molecular characterization and expression of the UV opsin in bumblebees: three ommatidial subtypes in the retina and a new photoreceptor organ in the lamina. *J. Exp. Biol.* **208**, 2347-2361.

Stavenga, D. G. (1979). Pseudopupils of compound eyes. In *Handbook of Sensory Physiology.* Vol. VII/6A (ed. H. Autrum), pp. 357-439. Berlin, Heidelberg, New York: Springer.

Stavenga, D. G. (2002a). Colour in the eyes of insects. *J. Comp. Physiol. A* **188**, 337-348.

Stavenga, D. G. (2002b). Reflections on colourful ommatidia of butterfly eyes. *J. Exp. Biol.* **205**, 1077-1085.

Stavenga, D. G., Smits, R. P. and Hoenders, B. J. (1993). Simple exponential functions describing the absorbency bands of visual pigment spectra. *Vision Res*. **33**, 1011-1017.

Stavenga, D. G., Kinoshita, M., Yang, E. C. and Arikawa, K. (2001). Retinal regionalization and heterogeneity of butterfly eyes. *Naturwissenschaften* **88**, 477-481.

Struwe, G. (1972). Spectral sensitivity of the compound eye in butterflies (*Heliconius*). *J. Comp. Physiol.* **79**, 191-196.

Sweeney, A., Jiggins, C. and Johnsen, S. (2003). Insect communication: polarized light as a butterfly mating signal. *Nature* **423**, 31-32.

Swihart, C. A. (1971). Colour discrimination by the butterfly, *Heliconius charitonius* Linn. *Anim. Behav*. **19**, 156-164.

Swihart, S. L. (1972). The neural basis of color vision in the butterfly, *Heliconius erato*. *J. Insect Physiol*. **18**, 1015-1025.

Swihart, S. L. and Gordon, W. C. (1971). Red photoreceptors in butterflies. *Nature* **231**, 126-127.

Vorobyev, M. (2003). Coloured oil droplets enhance colour discrimination. *Proc. R. Soc. Lond. B Biol. Sci.* **270**, 1255-1261.

Wakakuwa, M., Stavenga, D. G., Kurasawa, M. and Arikawa, K. (2004). A unique visual pigment expressed in green, red and deep-red receptors in the eye of the small white butterfly, *Pieris rapae crucivora*. *J. Exp. Biol.* **207**, 2803-2810.

Wakakuwa, M., Kurasawa, M., Giurfa, M. and Arikawa, K. (2005). Spectral heterogeneity of honeybee ommatidia. *Naturwissenschaften* **93**, 464-467.

Walls, G. L. (1942). *The Vertebrate Eye and its Adaptive Radiation*. Bloomfield Hills, MI: The Cranbrook Press.

Weiss, M. R. and Papaj, D. R. (2003). Colour learning in two behavioural contexts: how much can a butterfly keep in mind? *Anim. Behav*. **65**, 425-434.

White, R. H., Xu, H. H., Munch, T. A., Bennett, R. R. and Grable, E. A. (2003). The retina of *Manduca sexta*: rhodopsin expression, the mosaic of green-, blue- and UV-sensitive photoreceptors, and regional specialization. *J. Exp. Biol.* **206**, 3337-3348.