7 Molecular and Physiological Innovations of Butterfly Eyes

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INTRODUCTION

Color vision is a complex trait that can impact the survivorship of short-lived insects like the Lepidoptera. Within this order, the color vision systems are diverse and are best known among butterflies, which are classified into five families. Several recent reviews have focused on the eyes of the basal papilionid (i.e., *Papilio xuthus*) and pierid butterfly (i.e., *Pieris rapae*) lineages (Stavenga and Arikawa 2006; Wakakuwa, Stavenga, and Arikawa 2007). Both of these groups have eyes that differ from each other and from the other butterfly families in terms of the copy number of the opsin genes that encoded the visual pigments, their spatial expression pattern, and the distribution of lateral filtering pigments. Only one study to date has examined the visual pigments in a riodinid butterfly, *Apodemia mormo* (Frentiu et al. 2007). This chapter focuses on recent advances in our understanding of the unique visual system of lycaenid butterflies, with a special emphasis on the sexually dimorphic retina of *Lycaena rubidus* (Lycaeninae) and the color vision behavior of *Polyommatus icarus* (Polyommatinae). It is clear from character mapping of opsin genes and their expression patterns on a phylogeny of butterfly families that all butterfly eyes are derived from a much simpler eye that resembles the nymphalid eye (Briscoe 2008). Hence, to put the innovations of the lycaenid butterfly visual system into an evolutionary framework, we begin by describing the much simpler visual system of nymphalid butterflies. We then trace the molecular changes in the opsin genes and their expression patterns, and the physiological changes in the visual receptors they encode. Lastly, we discuss the potential behavioral outcomes of the unique eye design of lycaenids. In the course of the review, we mentioned some fertile areas of interest for future study.

ANATOMY OF THE BUTTERFLY OMMATIDIUM

The butterfly compound eye consists of thousands of ommatidia (Yagi and Koyama 1963). Each ommatidium contains a cornea, a crystalline cone, and nine photoreceptor cells (R1–9; Figure 7.1A), along with primary and secondary pigment cells (not shown). The microvilli of each photoreceptor cell form rhabdomeres, which fuse together to form the cylindrical optical structure, a rhabdom. The rhabdom acts as an optical waveguide (Nilsson, Land, and Howard 1988), which extends from the crystalline cone to the basal lamina. The microvillar arrangement that makes up the rhabdom can vary depending on the species. In the simplest case, rhabdomeres of R1–8 have approximately the same length and contribute more or less equally to the rhabdom, and the R9 cell contributes a few microvilli at the base of the rhabdom, producing a tiered structure (Briscoe et al. 2003). At the

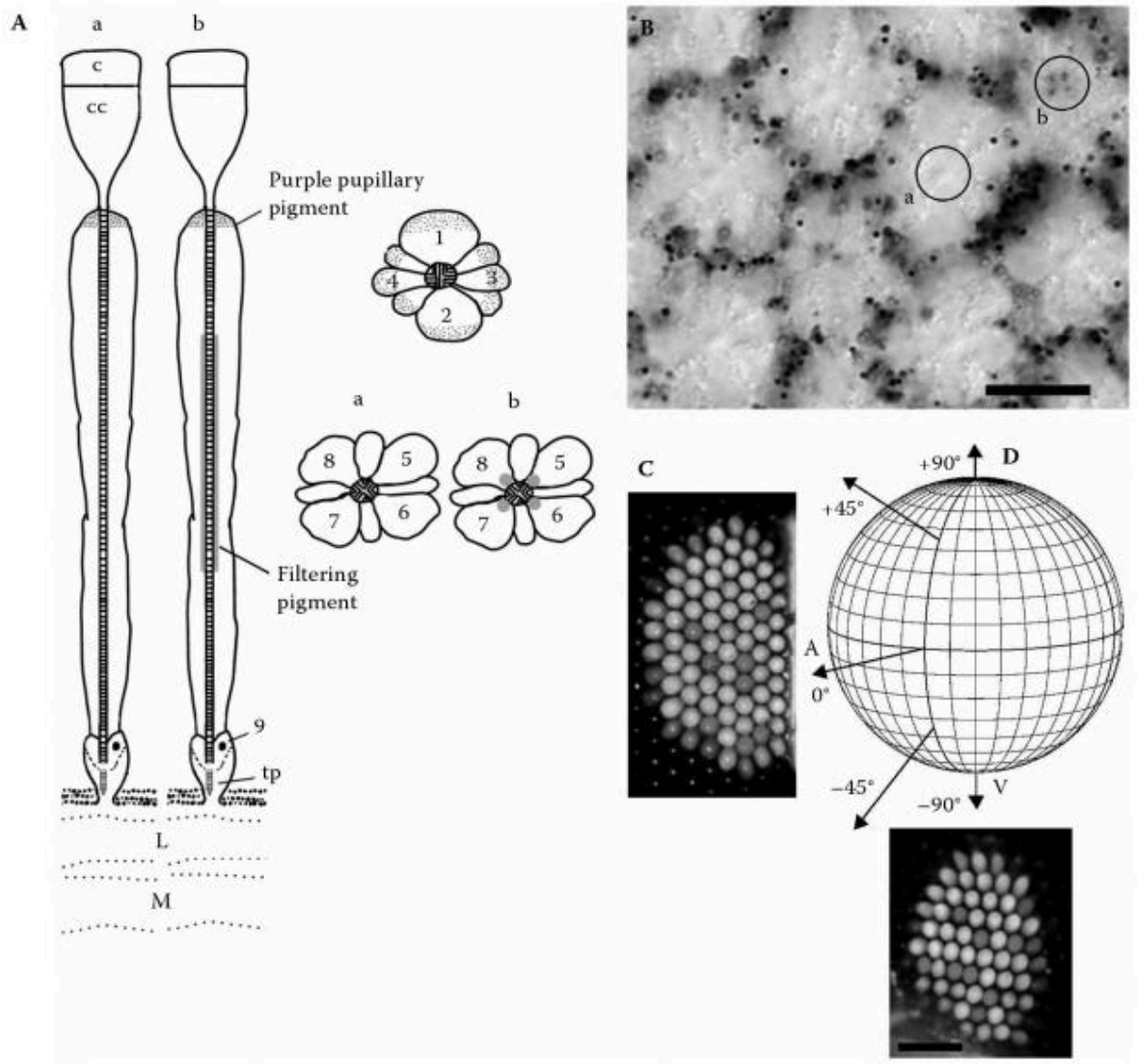

FIGURE 7.1 Ommatidia, filtering pigment distribution, and eyeshine of the lycaenid butterfly *Polyommatus icarus*. (A) Longitudinal (*left*) and tangential views (*right*) of the two types of ommatidia in the ventral eye; nonpigmented (a) and red-pigmented (b). Purple pupillary pigments are also present distally in all R1–R8 photoreceptor cells regulating the amount of light entering each ommatidium. (B) Red-filtering pigment in the lateral eye is absent in some ommatidia (a) and present in others (b). Scale bar, 10 μm. (C) Eyeshine of a female. Ommatidia looking into the anterior (A) and ventral (V) direction reflect yellow (light gray) and red (dark gray); dorsal (D) direction. Scale bar, 50 μm. c, cornea; cc, crystalline cone; 9, the ninth photoreceptor; tp, tapetum; L, lamina; M, medulla. Modified from Sison-Mangus et al. (2008).

proximal end of the rhabdom are stacks of tracheolar cells filled with air that compose the tapetum, a structure that functions as an interference mirror and can bounce unabsorbed light back through the rhabdom, allowing visual pigments to reabsorb the returning light.

The tapetum, the visual pigments, and if present, the filtering pigments (Figure 7.1B) together are responsible for the colored glow (eyeshine; Figure 7.1C) seen in most butterfly eyes (Stavenga et al. 2001). The eyeshine reflectance spectrum is of interest physiologically because it can be used as a noninvasive *in vivo* probe of the absorbance spectrum of the visual pigments found in the ommatidia, particularly in the long-wavelength part of the spectrum, in a completely intact butterfly (Bernard and Miller 1970; Vanhoutte and Stavenga 2005). It can also be used to infer the existence of heterogeneously expressed yellow, orange, and red filter pigments, which modify the wavelengths of light available to excite the visual pigments (Arikawa and Stavenga 1997; Arikawa et al. 1999; Figure 7.1B,C). The butterfly tapetum structure, however, is not universal; it is absent in papilionids (Miller 1979), and variable in its distribution within pierids. Butterflies in the genus *Pieris* have it (e.g., Figure 2 in Briscoe and Bernard 2005), whereas those in the genus *Anthocharis* lack it (Takemura, Stavenga, and Arikawa 2007). For those species that lack the tapetum, it is still possible of course to measure spectral sensitivities of photoreceptor cells using intracellular recordings, and for those species with and without tapeta, histological sections provide the most direct evidence of filtering pigment distributions.

Visual Pigments of the Butterfly Eye

Visual pigments are the light-absorbing molecules located in the rhabdomeric microvilli of each photoreceptor cell. Butterfly visual pigments are composed of a rhabdomeric opsin protein (Briscoe 1998; Kitamoto et al. 1998), a member of the G protein–coupled receptor (GPCR) subfamily similar to vertebrate melanopsin (Provencio et al. 1998), covalently linked to a light-sensitive chromophore, 11-*cis*-3-hydroxyretinal (Smith and Goldsmith 1990). Because butterflies use only one type of chromophore, the absorbance spectrum maximum (λ_{max}) of the visual pigment depends on the amino acid residues of its opsin. Thus, it is the opsin protein that allows animals to see different wavelengths of light and is responsible for photosensory responses. Opsins are ubiquitous in all animals and have mediated the phototransduction cascades prior to the evolution of Metazoa (Terakita 2005; Plachetzki, Degnan, and Oakley 2007). Animal opsins have been classified according to the type of photoreceptor cell in which they are found (e.g., rhabdomeric or ciliary-type; Arendt 2003) and are also based on the specific G protein subtype that links proper GPCRs (Santillo et al. 2006).

Butterfly photoreceptor cells may also be roughly classified according to their sensitivity to ultraviolet (UV; 300–400 nm), blue (B; 400–500 nm), and long-wavelength (LW; 500–600 nm) light. Surveys of spectral sensitivity measurements using electroretinogram, intracellular, or epi-microspectrophotometric recordings suggest that most moth and nymphalid butterfly eyes contain at least one class of UV-, B-, and LW-sensitive photoreceptor cell (Briscoe and Chittka 2001). The nymphalid, *Vanessa cardui* (Nymphalinae), has photoreceptor cells with peak sensitivities at 360, 470, and 530 nm; the monarch, *Danaus plexippus* (Danaiinae), has photoreceptors cells with peak sensitivities at 340 nm, 435 nm, and 545 nm (Figure 7.2A; Stalleicken, Labhart, and Mouritsen 2006; Frentiu et al. 2007); and the sphingid moth, *Manduca sexta*, has photoreceptor cells with peak sensitivities at 357 nm, 450 nm, and 520 nm (Bennett and Brown 1985). Physiological data for the photoreceptor cells of the lycaenid eye are rare but are consistent with this general pattern. The adult eye of the thecline, *Narathura japonica*, for instance, has at least three classes of photoreceptor with peak sensitivities to 380 nm, 460 nm, and 560 nm light (Imafuku et al. 2007); the polyommatine, *Celastrina argiolus*, has at least three photoreceptors with peak sensitivities at 380 nm, 440 nm, and 560 nm (Eguchi et al. 1982); and the polyommatine, *Pseudozizeeria maho*, has at least three photoreceptors with peak sensitivities at 400 nm, 520 nm, and 560 nm (Eguchi et al. 1982). Using epi-microspectrophotometry, however, four photoreceptor spectral types were identified in the retina of butterflies in the genus *Lycaena* (Lycaeninae): *L. rubidus*, *L. heteronea*, *L. dorcas*, and

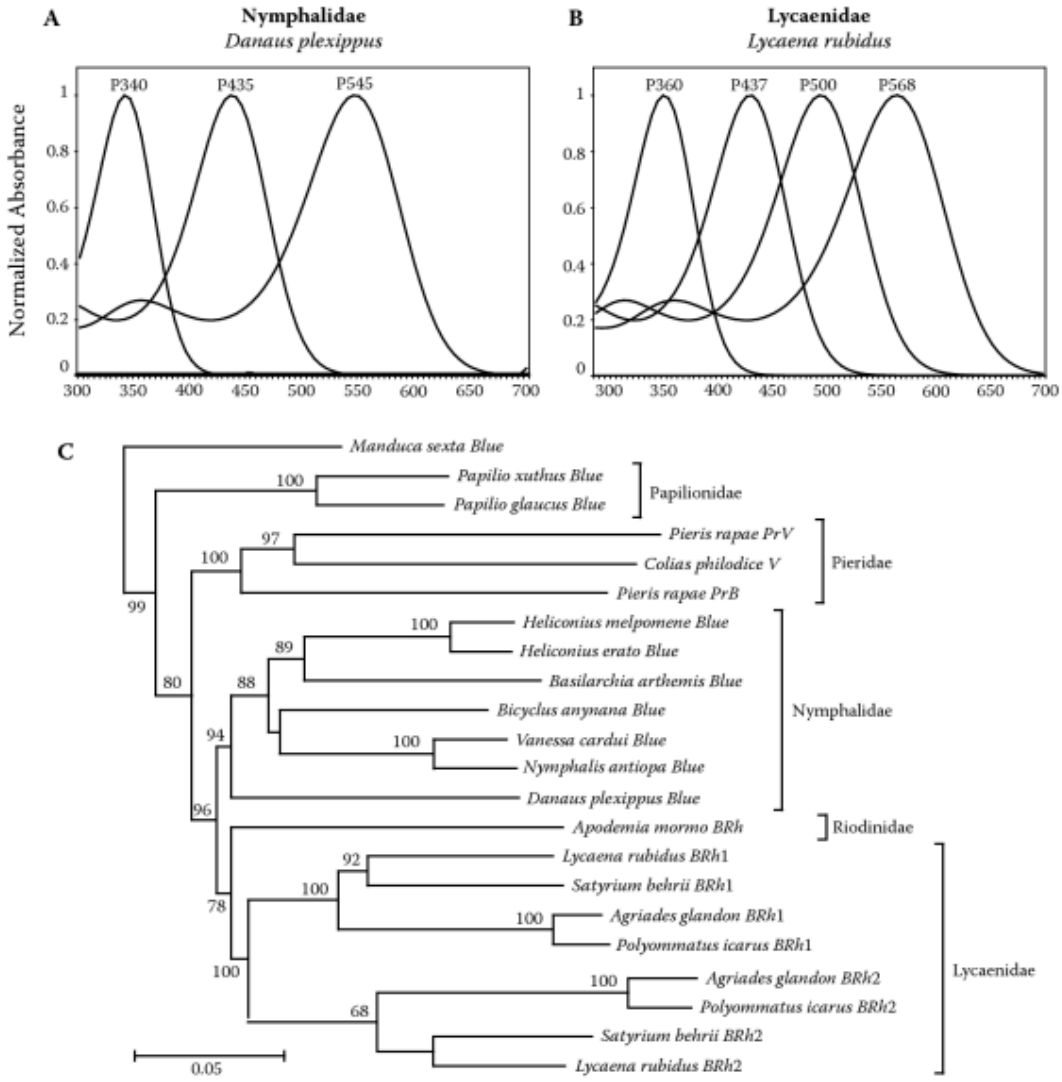

FIGURE 7.2 Representative nymphalid and lycaenid visual pigment absorption spectra and lepidopteran blue opsin phylogeny. (A) The monarch *Danaus plexippus* eye contains three visual pigments. P denotes maximum peak absorption (λ_{max}) of the visual pigment. (B) The ruddy-winged copper *Lycaena rubidus* eye contains four visual pigments. (C) Phylogeny of blue lepidopteran opsin genes based upon neighbor-joining analysis of 1,077 nucleotide sites, using Tamura-Nei distance and heterogeneous pattern of nucleotide substitution among lineages. Bootstrap values shown are based upon 500 maximum likelihood (ML) bootstrap replicates determined using the GTR+Γ+I model with estimated gamma shape parameter = 0.574 and proportion of invariant sites = 0.1474. GenBank accession numbers are as follows: *Manduca sexta* (Sphingidae; *Manop3*, AD001674); *Papilio xuthus* (*PxRh4*, AB028217); *Papilio glaucus* (*PglRh6*, AF077192); *Pieris rapae* (*PrB*, AB208675; *PrV*, AB208674); *Colias philodice* (*V*, AY918899); *Danaus plexippus* (*Blue*, AY605544); *Bicyclus anynana* (*BlueRh*, AY918894); *Heliconius erato* (*BlueRh*, AY918906); *Heliconius melpomene* (*BlueRh*, AY918897); *Basilarchia arthemis astyanax* (*BlueRh*, AY918902); *Nymphalis antiopa* (*BlueRh*, AY918893); *Vanessa cardui* (*BRh*, AY613987); *Apodemia mormo* (*BRh*, AY587906); *Polyommatus icarus* (*BRh1*, DQ402500; *BRh2*, DQ402501); *Agriades glandon* (*BRh1*, DQ402502; *BRh2*, DQ402503); *Satyrium behrii* (*BRh1*, DQ402498; *BRh2*, DQ402499); *Lycaena rubidus* (*BRh1*, AY587902; *BRh2*, AY587903). Phylogeny modified from Sison-Mangus et al. (2006).

L. nivalis have visual pigments with peak absorbances at 360 nm, 437 nm, 500 nm, and 568 nm (or 575 nm in *L. nivalis*), respectively (Figure 7.2B; Bernard and Remington 1991). Whereas these early physiological studies seem to suggest a similar number (3) of opsins in some nymphalids and lycaenids, it is now clear from molecular studies that the number of opsin genes and spectral receptors inferred from their spatial expression patterns in the eye differ strikingly between these groups of butterflies (see below).

THE BLUE OPSIN GENE HAS DUPLICATED IN LYCAENID BUTTERFLIES

The nymphalid butterflies *V. cardui* and *D. plexippus* have the least number of opsins, with eyes containing only a single copy of the UV, B, and LW opsin genes (Briscoe et al. 2003; Sauman et al. 2005). Although these cDNAs were originally cloned from eye-specific cDNA pools, BLAST searches of an EST library consisting of 9,484 unique cDNA sequences derived from monarch brain yielded the same result (Zhu, Casselman, and Reppert 2008). Deviating from nymphalids, *L. rubidus* (Lycaenidae) and *Apodemia mormo* (Riodinidae), considered to be sister taxa to the nymphalids (Campbell, Brower, and Pierce 2000), have four opsins each. However, *L. rubidus* has two copies of the B opsin gene (*BRh1*, encoding a P437 nm pigment, and *BRh2*, encoding a P500 nm pigment), a single copy of UV (*UVRh*, encoding a P360 pigment) and LW (*LWRh*, encoding a P568 pigment) opsin genes; whereas *A. mormo* has two LW copies (*LWRh1*, P505 and *LWRh2*, P600) and only a single copy of B (*BRh*, P450) and UV (*UVRh*, P340) genes (Sison-Mangus et al. 2006; Frentiu et al. 2007; Briscoe 2008). Strikingly, both butterflies have acquired visual pigments of similar λ_{max} in the blue-green range (500–505 nm), which are encoded by two different opsin gene family members, a *BRh2* (P500) opsin in *L. rubidus* and an *LWRh1* (P505) opsin in *A. mormo*. This suggests that these closely related butterflies, which diverged more than 70 million years ago (Wahlberg 2007), have co-opted different ancestral genes to achieve the same visual pigment physiology (i.e., wavelength of peak absorbance).

The blue-absorbing visual pigments of *P. rapae* are also noteworthy in this regard because they have λ_{max} of 425 nm and 453 nm, and are encoded by duplicate B opsin genes, *PrV* and *PrB* (Arikawa et al. 2005). Because the handful of papilionid and nymphalid eyes that had been investigated contained only one B opsin-encoding cDNA, we decided to investigate the evolutionary origins of the lycaenid and pierid gene duplications to see if they were independent of each other. To do this, we screened eye-specific cDNA libraries from ten additional butterfly taxa including lycaenids from the three largest (out of seven) lycaenid subfamilies (Lycaeninae, Polyommatinae, and Theclinae) and from three other butterfly families (Pieridae, Nymphalidae, and Riodinidae). We cloned a total of fourteen full-length blue opsin-encoding cDNAs from the ten taxa, including homologues of both *BRh1* and *BRh2* in all surveyed lycaenid subfamilies (Figure 7.2C). We detected only one blue opsin cDNA in each of the seven species of nymphalid surveyed. Phylogenetic analyses unambiguously indicated that the blue opsins of *L. rubidus* evolved independently from that of *P. rapae crucivora*, which is consistent with the very different λ_{max} of the pierid blue visual pigments compared with those of the lycaenid. Our results also indicated that the *L. rubidus* blue opsin gene duplication event occurred before the radiation of the coppers, hairstreaks, and blues (Lycaeninae+Theclinae+Polyommatinae; Sison-Mangus et al. 2006). We subsequently investigated the remaining opsins of a lycaenid in the subfamily Polyommatinae, *P. icarus,* and found that like *L. rubidus*, besides the duplicate blue opsins, its eye contains one UV opsin mRNA and one LW opsin mRNA (Sison-Mangus et al. 2008). Although the electroretinogram studies of individual species from the Theclinae and Polyommatinae mentioned previously detected only three major spectral peaks in the eye, it is important to note that our molecular studies made it clear that the eyes of butterflies in all three lycaenid subfamilies contain four visual pigments and not three.

Why have duplicate blue opsins evolved in butterflies? The molecular evolution of a blue-green–absorbing visual pigment (P500) in *L. rubidus*, which is encoded by a blue opsin gene (*BRh2*) and red-shifted by 63 nm compared with the visual pigment (P437) encoded by its paralogue (*BRh1*)

along with the green-absorbing visual pigment (P568 nm) encoded by the LW opsin gene *LWRh* (Sison-Mangus et al. 2006), might enhance color vision in the blue-green part of the light spectrum. We attempted to examine this hypothesis in the lycaenid *P. icarus* (see below). The duplication of blue opsins in *P. rapae crucivora*, on the other hand, may allow the animal to gain a violet receptor of blue opsin origin to discriminate better in the short wavelength light spectra, a hypothesis that remains to be tested behaviorally.

OPSIN SPATIAL EXPRESSION PATTERN IN THE NYMPHALID EYE

To appreciate how different the lycaenid eye is as a result of these blue opsin gene duplications, it is necessary first to describe the simpler nymphalid eye. The butterfly compound eye is subdivided into three domains, the dorsal rim area (DRA), and the dorsal and the ventral domains of the main retina. The DRA of butterflies, like that of many insects, is specialized for detecting polarized light (Labhart and Meyer 1999; Stalleicken, Labhart, and Mouritsen 2006). This area is typically composed of a few rows of ommatidia on the dorsal edge of the eye that have microvilli that differ in structure and orientation compared with those in the rest of the eye. The microvillar membranes or rhabdomeres of the ommatidia in the DRA are arranged at right angles to each other, in contrast to the more random orientation of the microvilli in the rhabdomeres of the main dorsal and ventral retina (Figure 7.3A,B; Briscoe et al. 2003; Reppert, Zhu, and White 2004). Like the main retina, the ommatidia of the DRA each contain nine photoreceptor cells (R1–9). Photoreceptor

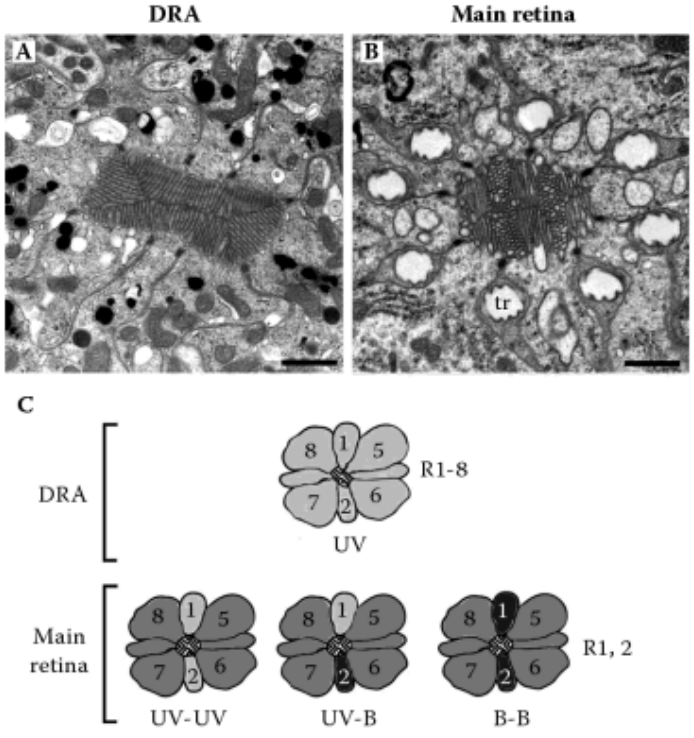

FIGURE 7.3 Rhabdomeric microvilli in the DRA and main retina of the butterfly and opsin expression patterns in the typical nymphalid eye. (A) DRA ommatidia of the lycaenid *Lycaena rubidus* are square shaped and have microvilli organized for polarized light detection. (B) Main retina ommatidia of *L. rubidus* are round shaped and have microvilli that are not organized for polarized light detection. Modified from Sison-Mangus et al. (2006). (C) Opsin expression in the DRA ommatidia of the nymphalid butterfly *Danaus plexippus* consists entirely of UV opsin in R1–R8 photoreceptor cells. By contrast, opsin expression in the main retina of *D. plexippus* consists of LW opsin in all R3–R8 cells, and either UV-UV, UV-B or B-B in the R1 and R2 photoreceptor cells.

subtype-specific patterns of opsin expression vary dramatically, however, between the main retina and DRA of the nymphalid eye (see below).

The opsin expression pattern among the ommatidia of the main retina of nymphalids is similar to that of the bee worker wherein the short-wavelength–sensitive opsins, UV and B, are expressed in the R1 and/or R2 cells, while the LW opsin is expressed in the six receptor cells R3–R8 (Spaethe and Briscoe 2005; Wakakuwa, Stavenga, and Arikawa 2007). Because UV and B opsin expression is restricted to R1 and R2 cells, three ommatidial subtypes based on the expression of these opsins in R1 and R2 cells have been identified (Figure 7.3C): UV-UV (type I ommatidia), UV-B (type II), B-B (type III). These patterns of opsin expression are exemplified by the sphingid moth *M. sexta* (White et al. 2003) and nymphalid butterflies *D. plexippus*, *V. cardui*, and *Heliconius erato* (Briscoe et al. 2003; Sauman et al. 2005; Zaccardi et al. 2006). The pattern of opsin expressed in the DRA is different from that of the main retina. In *D. plexippus*, using an antibody generated against a short peptide in the C-terminal region of the *Papilio glaucus* UV opsin, we found that only UV opsin is found in R1–R8 photoreceptor cells in the dorsal rim, whereas in the main retina, UV opsin is only expressed in R1 and/or R2 cells (Sauman et al. 2005). Input from the ultraviolet polarized light–sensitive DRA photoreceptor cells is important for directional orientation by migratory monarchs (Reppert et al. 2004; Sauman et al. 2005).

SEXUALLY DIMORPHIC RETINA OF *LYCAENA RUBIDUS*

In all nymphalids studied so far, the pattern of opsin mRNA expression in the eye does not vary between the sexes. By contrast, we found that the pattern of opsin mRNA expression in the main retina of the lycaenid *L. rubidus* eye is sexually dimorphic (the DRA has not yet been investigated), a pattern that confirmed an earlier epi-microspectrophotometric report of sexually dimorphic distribution of visual pigments in the eye of the same species (Bernard and Remington 1991). After cloning the opsin cDNAs from *L. rubidus* (*UVRh*, *BRh1*, *BRh2*, and *LWRh*), we found that all four opsin mRNAs are not only expressed in the eyes in a sex-specific manner, but are also distributed differentially in a dorso-ventral manner. The male dorsal retina expresses only *UVRh* and *BRh1*. New ommatidial subtypes dominate the male dorsal retina, because *BRh1* is expressed in the R3–R8 cells instead of the *LWRh* opsin that is commonly seen in the nymphalid eye (and all butterfly eyes examined to date). Moreover, the *UVRh-UVRh* (R1 and R2 cell) ommatidial type is dominant dorsally with a small number of *UVRh-BRh1* and *BRh1-BRh1* ommatidia in this part of the eye. The dorsal eye of the male is therefore likely color-blind in the red range and can only see short-wavelength light. The female dorsal retina is a different story, with *UVRh*, *BRh1*, and *LWRh* mRNAs expressed in this region (Figure 7.4A–H). Most strikingly, the two opsin genes *BRh1* and *LWRh* are coexpressed in the R3–R8 photoreceptor cells, making *L. rubidus* sexually dimorphic in this eye region (Figure 7.5E). To our knowledge, *L. rubidus* is the first insect species that has two visual pigments, one short-wavelength absorbing and one long-wavelength absorbing, coexpressed in the same photoreceptor cells (Sison-Mangus et al. 2006). Assuming that both visual pigments are involved in phototransduction, their coexpression in a single photoreceptor cell suggests that the receptors will have a broad sensitivity from blue to yellow spectral range. Intracellular recordings of these coexpressing opsins are needed to confirm this idea. Together with the UV receptors in R1 and R2 cells, this ommatidial type dominates the dorsal eye and implies that the *L. rubidus* female will be able to detect light spanning from UV to the long-wavelength region, in sharp contrast to the male dorsal eye, which is specialized for detection of UV to blue light. Interestingly, it is likely that the female dorsal eye is not specifically adaptive in itself, but rather reflects an intermediate step along the evolutionary pathway to developing the unique male dorsal eye (see discussion in Sison-Mangus et al. 2006 and below).

The ventral eye of both sexes, on the other hand, has a more typical opsin expression pattern; *LWRh* is expressed in R3–R8 cells, while the short-wavelength sensitive opsin mRNAs (*UVRh*, *BRh1*, *BRh2*) are expressed in the R1–R2 cells in a nonoverlapping fashion. However, because of

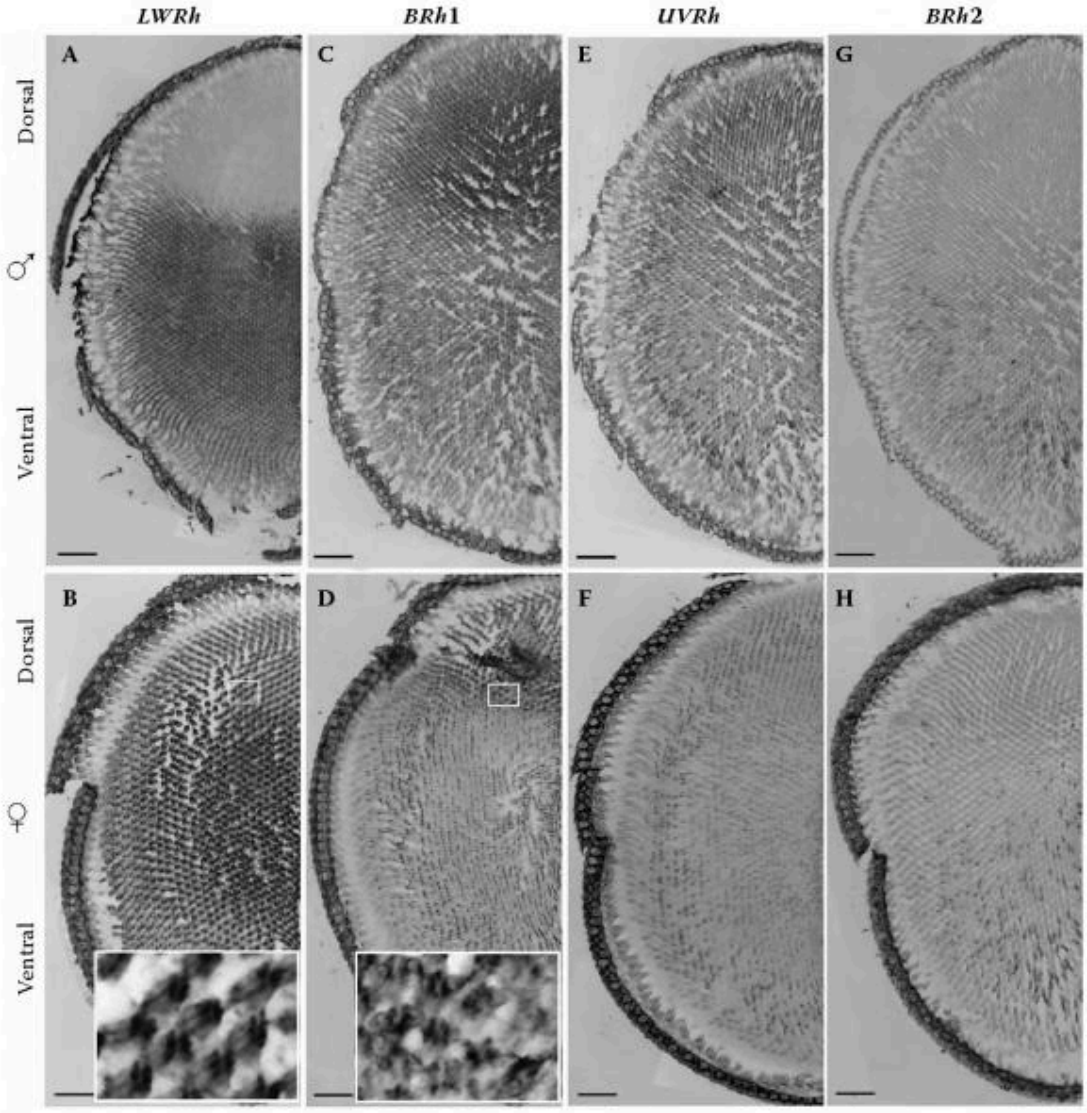

FIGURE 7.4 Sexually dimorphic *LWRh* opsin mRNA expression, coexpression of *LWRh* and *BRh1* opsin mRNAs in female dorsal eye, and dorso-ventral differences in *UVRh* and *BRh2* opsin mRNA expression in the adult retina of *Lycaena rubidus*. (A) *LWRh* is only expressed ventrally in males. (B) By contrast, *LWRh* is expressed uniformly across the retina in females. Inset: magnified view of *LWRh* mRNA expression in R3–R8 photoreceptor cells of female dorsal eye. (C) *BRh1* is expressed abundantly in the dorsal eye and less abundantly in the ventral eye in males. (D) Similarly, *BRh1* expression is more abundant in the dorsal area than in the ventral area in females. However, *BRh1* is coexpressed with *LWRh* in the dorsal eye in females. Inset: magnified view of *BRh1* mRNA expression in R3–R8 photoreceptor cells. *UVRh* expression is more abundant in the dorsal area than in the ventral area in both males (E) and females (F). *BRh2* mRNA expression is absent in the dorsal area and only seen in the ventral part of the retina in both males (G) and females (H). Scale bar = 100 μm.

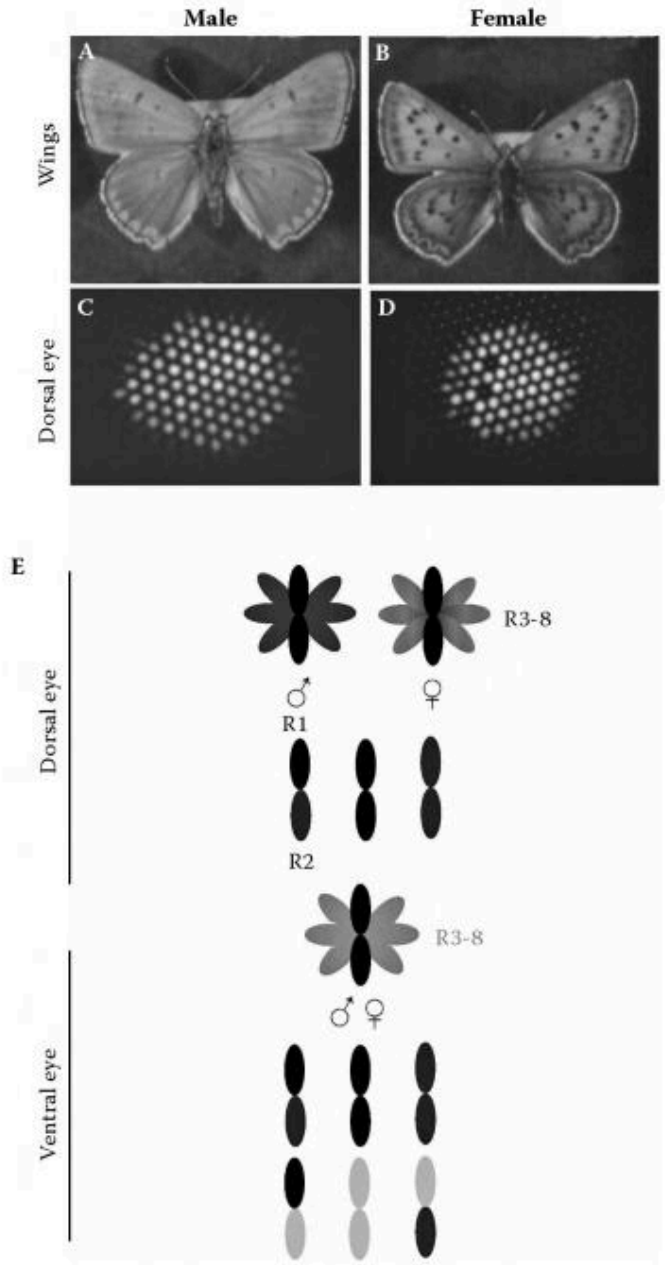

FIGURE 7.5 ***A color version of this figure follows page 176.*** Sex differences in wing color pattern, eyeshine, and opsin expression patterns in *Lycaena rubidus*. (A) UV-reflecting scales (iridescent purple) on the lower forewing and outer hind wing margins of males. (B) Non-UV-reflecting scales on wings of females. Eyeshine from the dorsal eye of a male (C) and a female (D) showing strongly sexually dimorphic coloration. (E) Diagram summarizing the pattern of opsin expression in the *L. rubidus* eye. Dark blue indicates *BRh1* opsin mRNA expression. Orange indicates *LWRh* opsin mRNA expression. Dark blue and orange indicate coexpression of *BRh1* and *LWRh* opsin mRNAs. Black indicates *UVRh* opsin mRNA expression. Light blue indicates *BRh2* opsin mRNA expression. Adapted from Sison-Mangus et al. (2006).

the presence of the duplicate blue opsin, *BRh2*, in the ventral retina, the ventral eye is equipped with six ommatidial types (UV-UV, UV-B1, UV-B2, B1-B1, B1-B2, B2-B2) in contrast to the typical three ommatidial types found in nymphalid butterfly eyes (UV-UV, UV-B, and B-B). Moreover, a pink filtering pigment is always found in the ommatidia in which *BRh2* is expressed. Presumably, this could result in an additional receptor, but behavioral experiments are needed to determine if it actually participates in color vision.

Are Sexually Dimorphic Retinas Correlated with Sexually Dimorphic Wings?

Many butterfly wing color patterns serve a function in the context of crypsis or mimicry. But in butterflies like the lycaenids, whose coloration varies between sexes, the difference in wing color suggests sexual selection. For example, the male *L. rubidus* displays bright, intense red-copper coloration, whereas the female appears dull brown (Figure 7.5A,B). Measurement of the dorsal wing reflectance spectrum of males indicates that they reflect light in the UV and red spectra, whereas the female reflects only in the red (Bernard and Remington 1991). Similar wing reflectance was also observed in the New Zealand species *L. salustris* (Meyer-Rochow 1991). Male lycaenids surveyed from three subfamilies, Polyommatinae, Theclinae, and Lycaeninae, reflect in the UV as well (Vertesy et al. 2006). UV is an important component of sexual signaling among butterflies (Silberglied 1979), and it has been demonstrated for blue lycaenids in mate choice (Burghardt et al. 2000; Knüttel and Fiedler 2001). In addition, male lycaenids are well known for displaying territoriality (Clark and Dickson 1971; McCubbin 1971; Atsatt 1981), a behavior that suggests strong male-male competition. The pattern of exclusively *BRh1* opsin mRNA expression in the R3–R8 photoreceptor cells, along with predominantly *UVRh* expression in the R1 and R2 cells (Figure 7.4 and Figure 7.5E), suggests that the male *Lycaena* dorsal eye is specialized for the detection of flickering moving objects, such as other airborne males. So far, there is no direct behavioral proof of the utility of the male dorsal eye of lycaenids. Identifying the circumstances under which the male dorsal eye outperforms the female eye is crucial for providing a connection to male-male competition.

The eye of the pierid *P. rapae crucivora* is also sexually dimorphic (Arikawa et al. 2005). However, its sexually dimorphic eye is not achieved through modification of the opsin expression but is achieved through a filter pigment strategy. Male and female *P. rapae crucivora* are indistinguishable by color in full-spectrum light, but when photographed with a UV filter, the wing reflectance varies between sexes. The female wing strongly reflects UV, whereas the male barely reflects UV; moreover, UV reflectance (more evident in female wings) is the main cue that would elicit sexual behavior in the male (Obara 1970). In light of these differences in wing patterns, it is interesting to note that *P. rapae crucivora* has three short-wavelength sensitive opsins, one UV and a pair of blue opsins (PrV and PrB); but one of the copies, PrV, is sensitive to violet. These short-wavelength opsin mRNAs are expressed independently in R1 and R2 cells, but only the usual three types of ommatidia are found, with PrV restricted to type II ommatidia. It is in these ommatidia that the fluorescing pigment is found in males but not in females. Because the pigment acts as a violet-absorbing spectral filter, the violet receptor sensitivity has been modified into a double-peak blue, with a small peak in the violet range and a high peak in the blue range. Given the animal's spectral set, the male may be able to acutely discriminate a conspecific female on the basis of her UV wing reflectance, corroborating the observations made on the mating behavior in this *Pieris* subspecies (Obara and Hidaka 1968; Obara 1970).

These pigment modifications strongly suggest that sexually dimorphic eyes evolved to accommodate communication via UV signaling by the opposite sex in *P. rapae crucivora* and by the same sex in *L. rubidus*. It would be interesting to evaluate the visual systems of other sexually dimorphic butterflies to determine whether similar patterns are observed. Butterflies from the genus *Colias*, for instance, have been studied extensively for UV signaling in the context of sexual selection (Silberglied and Taylor 1978; Silberglied 1979; Rutowski 1985). A violet sensitive opsin has been cloned from *Colias philodice,* which is homologous to that of *PrV* in *Pieris*

(Sison-Mangus et al. 2006), a good indication that this animal likely has three short-wavelength opsins. The presence of a violet sensitive opsin in *C. philodice* corroborates the finding of a violet receptor (λ_{max} = 400 nm) reported in another species, *C. erate* (Eguchi et al. 1982), suggesting that this receptor is also encoded by a violet-sensitive opsin. Another good candidate for examination is the sexually dimorphic nymphalid butterfly, *Hypolimnas bolina*, in which males posses bright, iridescent UV-reflecting markings in the dorsal wing (Kemp and Macedonia 2006) and females show preference for males with bright, iridescent markings that highly reflect in the UV (Kemp 2007).

COLOR VISION STUDIES IN BUTTERFLIES

The spectral diversity of visual pigments and their arrangement in the butterfly eye have the potential to be utilized for color vision. However, the mere occurrence of multiple receptors in the compound eye does not demonstrate color vision. To have color vision, a butterfly needs to discriminate objects of different colors irrespective of the intensity (Goldsmith 1990). Color vision requires the following: two visual pigments with distinct spectral sensitivity located in different photoreceptor cells, the presence of interneurons in the optic lobe with antagonistic input from these receptors, proper wiring in the brain to compare the signals from different stimuli, and the behavioral response of the animal being tested in color choice experiments (Goldsmith 1990; Kelber, Vorobyev, and Osorio 2003; Kelber 2006). Receptors produced by a visual pigment and a filtering pigment can participate in color discrimination because the presence of a filtering pigment can modify the absorption spectrum of a receptor.

Our knowledge of how the brains of nymphalid or lycaenid butterflies process color information is scant (Swihart 1968), and this is a fertile area for further study. The clearest demonstration of color vision in nymphalid and lycaenid butterflies has been shown through behavioral tests. The empirical testing and solid demonstration of true color vision in Lepidoptera involves training a naive animal to associate color with a food reward. Training or the ability of the animal to learn the rewarding color is an essential aspect of the behavioral test. This gives the experimenter a handle on the sensory competence of the animal, because learning indicates perception of color information. Once the animal has "learned" the rewarding color, it is then given a series of choices between the rewarding and unrewarding colors, while the light intensity of the color stimuli is manipulated (Kelber 1999; Kelber and Pfaff 1999; Zaccardi et al. 2006; Sison-Mangus et al. 2008) or the colors are compared with varying shades of gray (Kinoshita, Shimada, and Arikawa 1999). The animal has true color vision if it chooses the rewarding color independent of intensity.

ALTERNATIVE STRATEGIES FOR RED-GREEN COLOR VISION

Behavioral experiments that utilize this rigorous approach have shown that nymphalids and lycaenids, like other butterflies and moths, have true color vision; they can discriminate between pairs of colors of different wavelengths over intensity ratios ranging from 0.01 to 100 or when compared with gray (Kelber, Balkenius, and Warrant 2002; Table 7.1). The total range of colors that nymphalid and lycaenid butterflies can discriminate has not been exhaustively studied. From the experiments that have been performed, however, it is clear that different butterfly species apply different strategies to achieve color vision in the green-red range. For example, the nymphalid *V. atalanta*, whose eye contains the typical three visual pigments, UV-, B-, and LW-absorbing, can discriminate blue from orange light (440 vs. 620 nm) but is unable to discriminate yellow from orange light (590 vs. 620 nm; Zaccardi et al. 2006). In contrast, the nymphalid *H. erato*, whose eye also contains UV-, B-, and LW-absorbing visual pigments, can see in the red range (620 vs. 640 nm) despite having only a single LW opsin. It is with the aid of a heterogeneously distributed red filtering pigment that the animal produces a fourth, red-sensitive receptor (Zaccardi et al. 2006). The lycaenid *P. icarus*, on the other hand, can

TABLE 7.1
Species and Wavelengths Tested in Lepidopteran Color Vision Experiments

Family	Species	Wavelength (nm)/ Color Tested	Color Vision	Sources
Sphingidae	*Macroglossum stellatarum*	380 vs. 360	yes	Kelber and Henique 1999
		380 vs. 420	yes	
		380 vs. 470	yes	
		470 vs. 500	yes	
		500 vs. 420	yes	
		620 vs. 470	yes	
		470 vs. 620	yes	
		595 vs. 620	no	
Sphingidae	*Deilephila elpenor*	blue vs. shades of gray	yes	Kelber et al. 2002
		blue vs. shades of blue	no	
		yellow vs. shades of gray	yes	
		yellow vs. shades of yellow	yes	
Papilionidae	*Papilio aegeus*	430 vs. 590 and 640	yes	Kelber and Pfaff 1999
		640 vs. 430 and 590	yes	
Papilionidae	*Papilio xuthus*	red vs. yellow, green, blue	yes	Kinoshita, Shimada, and Arikawa 1999
		yellow vs. red, green, blue	yes	
		green vs. red, yellow, blue	yes	
		blue vs. red, yellow, green	no*	
Nymphalidae	*Heliconius erato*	590 vs. 440	yes	Zaccardi et al. 2006
		620 vs. 590	yes	
		620 vs. 640	yes	
Nymphalidae	*Vanessa atalanta*	620 vs. 440	yes	Zaccardi et al. 2006
		620 vs. 590	no	
Lycaenidae	*Polyommatus icarus*	450 vs. 590	yes	Sison-Mangus et al. 2008
		560 vs. 590	yes	
		570 vs. 590	no	

The color or wavelength first listed was used as the training and rewarding color.
* No preference for blue.

discriminate colors in the green range up to 560 nm when feeding (Sison-Mangus et al. 2008). It cannot discriminate colors in the red range (590 vs. 640 nm), however, despite having a red-reflecting ommatidium produced by the LW receptor and a red-filtering pigment (Figure 7.1; Sison-Mangus et al. 2008). The photoreceptors being used for this task are most likely the B2- and LW-absorbing visual pigments, which by homology are most similar to the P500 and P568 visual pigments of *L. rubidus*. Nevertheless, physiological data for *P. icarus* are needed to demonstrate more fully that it is, indeed, the duplicate blue opsin, in conjunction with the LW opsin, that is mediating this behavior.

The contrasting results between *P. icarus* and *H. erato* suggest that the impact of filtering pigments on butterfly color discrimination should be evaluated on a species-specific basis. Further behavioral studies, in conjunction with physiological and molecular studies on these and other butterfly species known to have lateral filtering pigments such as *P. rapae crucivora* (Wakakuwa et al. 2004), *D. plexippus* (Sauman et al. 2005), *Bicyclus anynana*, *Zizeeria maha* (Stavenga 2002), *Sasakia charonda*, and *Polygonium c-aureum* (Kinoshita, Sato, and Arikawa 1997), are needed to

determine whether filtering pigments play a role in their color vision. Such studies should identify the number of opsins in the eye and establish whether lateral filtering pigments have an impact on the spectral sensitivity of individual photoreceptor cells. Direct measurement of the λ_{max} values of the reconstituted visual pigments, via transgenic expression of the opsins in *Drosophila* or cultured cells, together with *in vivo* measurements, would also provide crucial experimental evidence demonstrating the effects of the lateral filtering pigment on color vision.

CONCLUSION

The butterfly eye has been elegantly molded by evolution. The fact that lycaenid and nymphalid butterflies bear modifications of their visual systems through different mechanisms that lead to similar traits (e.g., red-green color vision) is highly suggestive of adaptive evolution. So far, gene duplication of the B and LW opsins and the addition of filter pigments are the most common strategies utilized by butterflies to achieve similar physiological and behavioral ends. The ease with which some butterflies have changed opsin expression patterns in a particular domain of their eye, as occurs among some sexually dimorphic butterflies in which such eye modifications are most pronounced, also suggests a lack of developmental constraints. It will be interesting to determine in the future whether or not some of the unique characteristics of the nymphalid and lycaenid eye described here turn out to be defining features of each of these families as has been the case for classical morphological characters.

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