Sexual Dimorphism and Retinal Mosaic Diversification following the Evolution of a Violet Receptor in Butterflies

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Abstract

Numerous animal lineages have expanded and diversified the opsin-based photoreceptors in their eyes underlying color vision behavior. However, the selective pressures giving rise to new photoreceptors and their spectral tuning remain mostly obscure. Previously, we identified a violet receptor (UV2) that is the result of a UV opsin gene duplication specific to Heliconius butterflies. At the same time the violet receptor evolved, Heliconius evolved UV-yellow coloration on their wings, due to the pigment 3-hydroxykynurenine (3-OHK) and the nanostructure architecture of the scale cells. In order to better understand the selective pressures giving rise to the violet receptor, we characterized opsin expression patterns using immunostaining (14 species) and RNA-Seq (18 species), and reconstructed evolutionary histories of visual traits in five major lineages within Heliconius and one species from the genus Eueides. Opsi expression patterns are hyperdiverse within Heliconius. We identified six unique retinal mosaics and three distinct forms of sexual dimorphism based on ommatidial types within the genus Heliconius. Additionally, phylogenetic analysis revealed independent losses of opsin expression, pseudogenization events, and relaxation of selection on UVRh2 in one lineage. Despite this diversity, the newly evolved violet receptor is retained across most species and sexes surveyed. Discriminability modeling of behaviorally preferred 3-OHK yellow wing coloration suggests that the violet receptor may facilitate Heliconius color vision in the context of conspecific recognition. Our observations give insights into the selective pressures underlying the origins of new visual receptors.

Key words: butterflies, color vision, photoreceptor cells, short-wavelength opsin, gene duplication, pseudogenes.

Introduction

How animal eyes have evolved to handle the variety of colorful stimuli they encounter in their world is a key question in visual ecology. Across the animal kingdom, the number of opsin-based photoreceptors used by different species for vision is highly variable. Some insects like honeybees have eyes containing only three opsin-based photoreceptors (Wakakuwa et al. 2005; see Friedrich et al. 2011 for an overview). Other animals have evolved a greatly expanded photoreceptor set, mediated by opsin duplication, expression differences, and filtering effects in the eye. For instance, mantis shrimp have at least 16 spectrally distinct photoreceptors, which they have achieved through multiple opsin gene duplications as well as complex patterns of filtering in the eye (Cronin and Marshall 1989; Cronin et al. 2014; Bok et al. 2015). African cichlid fishes have seven ancestrally duplicated opsin genes, and across the cichlid family the subset of these opsins that are expressed in the photoreceptors of a given species is variable. Diversity in cichlid spectral classes of photoreceptors is also produced by expression level differences (O’Quin et al. 2012; Schulte et al. 2014), coexpression of opsins (Dalton et al. 2017), and by structural changes in the opsin gene (Parry et al. 2005; Seehausen et al. 2008; Hofmann and Carleton 2009; Dalton et al. 2015). Another example is dragonflies which express up to 30 opsin mRNA transcripts, a number that varies between families within the order, although it is not yet known in which photoreceptor cells these opsins are expressed (Futahashi et al. 2015).

The family Papilionidae alone contains species with eight (Papilio xuthus) (Koshitaka et al. 2008), nine (Troides aceus) (Chen et al. 2013), and 15 (Graphium sarpedon) (Chen et al. 2016) spectral classes of photoreceptor. Other groups of butterflies, notably those in the family Nymphalidae, get by with three opsins (Briscoe et al. 2003; Macías-Muñoz et al. 2016), and some even lack the heterogeneously expressed filtering pigments that expand the number of long wavelength-
sensitive photoreceptors found in other butterflies (Briscoe and Bernard 2005). Thus, butterflies rival the mantis shrimp in sheer number of spectral types of photoreceptor but some butterfly species are more like bees, with only three types. However, even among the relatively well-studied butterflies, visual system comparisons between closely related species are rare (although see Frentiu et al. 2007a; Chen et al. 2016). Also, while high levels of photoreceptor spectral diversity have been observed across animal families such as Papilionidae, far less is known about diversification of eyes between closely related species. Given the importance of the social use of color in animals, especially butterflies, it would be surprising if photoreceptor spectral diversification did not play some role in speciation. Yet we know little about the first steps in eye evolution immediately following the fixation of duplicate opsin genes.

Moreover, while numerous kinds of photoreceptors have been discovered in different organisms, the benefits to evolving a new spectral type of photoreceptor and a visual system making use of it are less well understood. Here we begin to close this gap in our knowledge of visual system evolution by conducting an extensive investigation of the retinal mosaics and opsin sequence evolution of Heliconius species in comparison to outgroup species in the genus Eueides. Famous for their spectacular wing color pattern evolution and mimicry, Heliconius butterflies are also interesting for their eyes (Reed et al. 2011; Martin et al. 2012; Nadeau et al. 2016). Other nymphalid butterflies like the monarch butterfly Danaus plexippus or the painted lady Vanessa cardui have eyes that express three opsins which together with the chromophore 11-cis-3-hydroxyretinal encode ultraviolet (UV)-, blue (B)-, and long wavelength (LW)-sensitive rhodopsins (Briscoe et al. 2003; Sauman et al. 2005). Heliconius by contrast also express a duplicated ultraviolet (UV) opsin. The gene duplication producing UVRh1 and UVRh2 has been identified in all Heliconius species so far investigated, together with a 3-hydroxykynurenine (3-OHK) pigment producing UV-yellow coloration on their wings; both traits are shared derived characters of the genus (see below). Evidence for positive selection of UVRh2 along the branch leading to Heliconius suggested it acquired a new adaptive function; specifically, the location of positively selected codons in the chromophore-binding pocket of the rhodopsin implied its wavelength of peak absorbance had shifted (Briscoe et al. 2010). The UV1 opsin, on the other hand, retained an amino acid motif found in sexually monomorphic outgroup taxa lacking the duplicate UVRh2 gene.

Recently, we determined photoreceptor cell sensitivity and opsin spatial expression patterns in the eye of Heliconius erato and found a new spectral function for UV2, as well as maintenance of an older spectral function for UV1 (McCulloch et al. 2016a, 2016b). We found that the UV1 opsin encodes a UV-sensitive rhodopsin with a peak spectral sensitivity $\lambda_{\text{max}} = 355$ nm, similar to the UV-sensitive rhodopsin of other nymphalids ($\sim 355$ nm, Vanessa cardui; $\sim 340$ nm Danaus plexippus). The UV2 opsin, by contrast, produces a violet-sensitive rhodopsin with a peak sensitivity or $\lambda_{\text{max}} = 390$ nm. We were completely surprised to then discover that the H. erato eye is sexually dimorphic; each sex possesses a distinct set of ommatidial types based on short-wavelength opsin expression (fig. 1A–D). By contrast, other investigated nymphalid butterflies such as Danaus plexippus, Limenitis arthemis astyanax and Vanessa cardui have sexually monomorphic eyes (Briscoe et al. 2003; Sauman et al. 2005; Frentiu et al. 2015). Female H. erato have UV1, UV2, blue (B), and long wavelength (L) rhodopsin-expressing cells with peak sensitivities at 355, 390, 470, and 555 nm, respectively. Males have the same opsins and sensitivities except they lack a UV1-expressing photoreceptor cell (McCulloch et al. 2016a). The complete absence of one class of opsin in one sex is a new form of sexual dimorphism. Among butterflies with known visual system sex differences, opsin mRNA expression levels are sexually dimorphic in the dry season forms of the nymphalid Bicyclus anynana (Everett et al. 2012). Male and female pierids have the same opsin spatial expression patterns, whereas spectral sensitivity is tuned by sexually dimorphic spatial expression of filter pigments (Arikawa et al. 2005; Ogawa et al. 2012, 2013). In some lycaenid butterflies, sex differences are achieved by coexpression of two opsins in the photoreceptor cells of a female-specific ommatidial type (Sison-Mangus et al. 2006).

In light of the rapidly evolving wing signals within and between species in the genus Heliconius (for examples of wing color pattern variation in H. erato and H. melpomene see Van Belleghem et al. 2017 and Heliconius Genome Consortium 2012) and the novel pattern of UV opsin expression found in H. erato, we thought that Heliconius would be a good candidate for investigating how eye evolution and speciation might relate to one another. Other Heliconius species have two UVRh genes but it is unknown whether they have the same opsin expression as H. erato or if their eyes have diverged during speciation. We therefore characterized opsin expression patterns, relative cell abundances, and evolutionary histories of visual traits in species representing five major lineages within Heliconius (erato, sara, silvaniform, melpomene, and doris clades) and one species from the sister genus Eueides. We also used RNA-Seq data to identify changes in the intensity of selection on the opsins. We uncovered widespread diversity between clades together with different forms of sexual dimorphism. These results suggest varying levels of natural and sexual selection have shaped the Heliconius eye during speciation.

**Results and Discussion**

**Diverse Opsin Protein and mRNA Expression among Heliconius Species**

Following the fixation of duplicate genes, one scenario of early events is that males and females would each make use of both copies. Under this scenario, we might expect that after the ancestral UV opsin gene duplicated, both Heliconius sexes expressed UV1 and UV2 opsins. To better understand the origins of the violet receptor (UV2) and how it came to replace the ultraviolet receptor (UV1) in the male H. erato eye, we first asked whether the observed sexual dimorphism (McCulloch et al. 2016a) is found throughout the genus, or whether there are Heliconius species in which both UV1 and
UV2 opsins are expressed in both sexes. We used antibodies against the short-wavelength opsins (UV1, UV2, and B) to immunostain transverse sections of ommatidia in the compound eyes of males and females of 13 species in five major clades within Heliconius and one outgroup in the genus Eueides. R1 and R2 photoreceptor cell subtypes express short wavelength (SW) opsins in different combinations (fig. 1A). We discovered that the sexual dimorphism in H. erato is not conserved across the genus. In fact, there are at least six distinct retinal mosaics in Heliconius based on the different combinations of SW opsins present in the eye, with all but one incorporating the more recently derived violet-sensitive rhodopsin UV2 (fig. 1B–L).

For the 14 species examined, each sex within a clade shares the same retinal mosaic except for H. charithonia (see below); however, retinal mosaics differ considerably between clades and sexes. Other erato clade members (two species) have the same expression pattern as H. erato, where UV1 opsin expression is absent in the male eye and UV2 is present in both sexes (females, mosaic I; males, mosaic II, fig. 1C and D; supplementary fig. S1A–C, Supplementary Material online; McCulloch et al. 2016a). A second form of sexual dimorphism is found in the sara clade (five species, supplementary fig. S1D–H, Supplementary Material online). Unlike H. erato, females in this clade express at least six ommatidial types based on SW opsin expression (mosaic III, fig. 1E), whereas sara males resemble H. erato males (mosaic II, fig. 1D and F). The melpomene (two species) and silvaniform (two species) sister clades exhibit yet another retinal mosaic. In this clade, neither sex expresses the UV2 rhodopsin in the eye, resulting in mosaic IV (fig. 1G–J, supplementary fig. 1I–L, Supplementary Material online). Eueides isabella, a sister taxon to Heliconius lacking the UVRh2 duplicate, resembles melpomene and silvaniform clades with respect to sexually monomorphic UV1 expression (supplementary fig. S1M, Supplementary Material online). This expression pattern...
together with the sensitivity of the UV1 rhodopsin are similar to other nymphalid butterflies such as Vanessa cardui (355 nm), and Danaus plexippus (340 nm) suggesting maintenance of the ancestral role for this photoreceptor subtype (Briscoe et al. 2003; Stalleicken et al. 2006; Blackiston et al. 2011). A third form of sexual dimorphism is evident in H. doris. Both H. doris sexes make use of UV1 and UV2 rhodopsins, in mosaic V in females (fig. 1K) and mosaic VI in males (fig. 1L, supplementary fig. S1N, Supplementary Material online).

To complement our protein expression data, we used RNA-Seq to quantify levels of opsin mRNA expression in 18 species (n = 60 individual butterflies) (supplementary tables S1 and S2, Supplementary Material online). In the erato (four species) and sara (five species) clades, UVRh1 expression is nearly absent in males, whereas UVRh2 expression is high; females highly express both UVRh1 and UVRh2 (supplementary table S2, Supplementary Material online). Both sexes in the doris clade (three species) express both UVRh1 and UVRh2, consistent with the H. doris opsin immunostaining pattern (supplementary table S2, Supplementary Material online). In agreement with our protein expression data, all mel-pomene and silvaniform species (five species) have low expression of UVRh2 in both sexes, whereas UVRh1 remains highly expressed (supplementary table S2, Supplementary Material online). RNA sequencing together with GenBank sequences (Bybee et al. 2012; Yuan et al. 2010) also revealed that UVRh2 is pseudogenized in several silvaniform species (see below), but not in H. melpomene, H. cydno, H. pachinus, and H. timareta.

Evidence for sexually dimorphic patterns of opsin expression as seen in erato and sara clades is limited in other insects. Sexual dimorphism in other investigated butterflies is accomplished via sex differences in eye filter pigment distribution that causes shifts in photoreceptor sensitivity, or via sex-specific coexpression of opsins in a subset of photoreceptor cells (Arikawa et al. 2005; Sison-Mangus et al. 2006; Ogawa et al. 2012, 2013). Seasonally sexually dimorphic opsin mRNA expression levels are seen in Bicyclus anynana (Everett et al. 2012); the moth Manduca sexta has sexually dimorphic expression of genes involved in the deactivation of rhodopsin signaling (Smith et al. 2014). In addition to Lepidoptera, known examples of sexual dimorphism in the compound eyes of honeybees and houseflies involve differences in the domain of opsin expression rather than loss of opsin expression in one sex (Franceschini et al. 1981; Menzel et al. 1991). Some evidence for sex differences in LWRh expression has been observed in the fig wasp Ceratosolen solmsi (Wang et al. 2013), but quantification of mRNA levels alone does not reveal opsin spatial expression, or whether differences in spectral sensitivity exist. Thus, the male-specific loss of the UV1-expressing cell we document in some Heliconius lineages represents a newly described form of sexual dimorphism in insects.

Variation in Photoreceptor Cell and Ommatidial Number
If the regulatory mechanisms that control the differentiation of ommatidial types are conserved in Heliconius, then we would expect to observe fixed proportions of stochastically distributed ommatidial types shared among species within clades with the same retinal mosaic, whereas these proportions should diverge between clades (Wernet et al. 2015). Similar proportions of ommatidial types in related species is evidence of a single evolutionary origin of changes to retinal mosaic developmental pathways. We therefore calculated average percentages of ommatidial types in multiple individuals for each species and sex (n = 33,449 ommatidial total). Within each clade, the abundance of each ommatidial type is similar and sex-specific; between clades there are notable differences (supplementary table S3, Supplementary Material online). To visualize the overall pattern of the abundances for each ommatidial type, we plotted the results of a principal component analysis (PCA) using the percentages of ommatidial types from each sex from 14 species (fig. 2). This PCA reveals four distinct clusters (dotted circles, fig. 2) and one outlier, H. doris. The clusters suggest a common origin of retinal mosaic II in sara and erato clades, and of retinal mosaic IV in mel-pomene and silvaniform clades. H. doris males are distinct in that they do not obviously cluster with other clades in the PCA plot. H. doris males are the only males that express both UV1 and UV2 rhodopsins (fig. 1L). H. doris females but not males are also the only individuals where our immunohistochemistry indicates coexpression of UV1 and UV2 rhodopsins in a subset of R1 and R2 photoreceptor cells, so we did not include them in the analysis. Overall, our PCA reveals clade-specific patterns of fixed proportions of ommatidial types, providing evidence for conserved regulatory mechanisms that give rise to these differing retinal mosaics within the genus.

Stochastic spatial expression yet fixed proportions of ommatidial subtypes are found in numerous insects (Arikawa 2003; Wernet et al. 2015). In the D. melanogaster retina, stochastic expression of the transcription factor Spineless in R7 photoreceptors leads to stereotypical ratios of two ommatidial types defined by stochastic expression of opsins (Cook et al. 2003; Domingos et al. 2004; Mikeladze-Dvali et al. 2005; Wernet et al. 2006). Recently butterflies were shown to have two cells homologous to the single D. melanogaster R7 subtype (Perry et al. 2016). The decision to express either the UV or blue-sensitive rhodopsin in butterfly R1 and R2 color-sensing cells depends on spineless expression where two independent stochastic binary decisions produce the three ancestral types of ommatidia seen throughout the Lepidoptera (UV/UV, UV/B, or B/B) (Perry et al. 2016). This mechanism alone cannot account for the additional complexity found in Heliconius species that produce mosaic III. It is possible that a second stochastic decision has been added to the process of cell fate specification: initially, spineless expression could control the UV versus B decision and then a second stochastic decision may determine whether this cell is a UV1 or UV2 cell. Additional mechanisms would be required for: i) the restriction of ommatidial types observed in erato clade females—despite expression of UV1, UV2, and blue cell subtypes—and ii) the suppression of the UV1 cell subtype in erato and sara clade males in favor of UV2. This should be a rich area for future research on sexual dimorphism in visual system patterning and function.
Relaxation of Selection and Loss of UV2 Rhodopsin Expression

Despite evolving a new violet receptor at the base of the genus *Heliconius*, it appears that UV2 expression has been lost in all *melpomene* and silvaniform species so far investigated and that these species have reverted to an ancestral UV1-expressing retina. In previous work, we identified full-length *UVRh2* mRNAs in *melpomene* clade species (Briscoe et al. 2010). However, *H. melpomene* and *H. cydno* lack a UV2-expressing photoreceptor in the compound eye (fig. 1G and H, supplementary fig. S1I and J, Supplementary Material online), and in *H. melpomene* whole heads (*n* = 4) full-length *UVRh2* mRNA is present in low levels (supplementary table S2, Supplementary Material online). This suggests that *UVRh2* may be expressed in the brain in an extraretinal photoreceptor (Lampel et al. 2005). Unlike *H. melpomene*, several silvaniform species have pseudogenized *UVRh2* (fig. 3A and B, supplementary table S4, Supplementary Material online).

Specifically, *H. atthis* and *H. elevatus* have full-length *UVRh2* mRNAs, whereas *H. pardalinus* and *H. ethilla* do not. RNA-Seq shows that *H. numata* and *H. hecale* are polymorphic for full-length and truncated *UVRh2* mRNAs (fig. 3B, supplementary table S4, Supplementary Material online). Character mapping of *UVRh2* pseudogenes on a species phylogeny reveals at least four independent loss-of-function events in silvaniform *UVRh2* evolutionary history (fig. 3B).

Since *UVRh2* has lost its role in vision in the *melpomene* silvaniform clades, we expected to observe relaxation of selection on *UVRh2* in these clades. We formally tested this hypothesis using RELAX in HyPhy (Pond et al. 2005) with an expanded opsin data set consisting of sequences from 25 *Heliconius*, five *Eueides*, two *Dione* and one *Agraulis* species (*n* = 71 individuals; supplementary table S1 and fig. S2, Supplementary Material online) (Wertheim et al. 2015). We found a significant difference favoring a model where *melpomene* silvaniform *UVRh2* genes are evolving under relaxed selection compared with the null model where *melpomene* silvaniform branches have the same selection pressures as all other *UVRh2* branches (relaxation parameter, *k* = 0.6292; *k* = 1 would mean the test branch is evolving under either purifying or positive selection at the same rate as the reference branches; chi-squared test, *P* = 0.001, fig. 4A). The RELAX method builds on a random effects branch-site model (Kosakovsky Pond et al. 2011), and compares the distribution of *ω* values between the a priori defined reference branches and test branches. When selection is relaxed on the test branches, the sites on the reference branches with *ω* values undergoing either purifying selection (*ω* < 1) or positive
Evolutionary History of the UV2 Opsin and the Evolution of a New Female Retinal Mosaic

To trace the evolution of the UV2 opsin and the UV2-expressing eye of *erato* and *sara*-clade males, we reconstructed ancestral states for several traits (supplementary fig. S3, Supplementary Material online). Maximum likelihood models indicate that both sexes probably had UV1 and UV2 rhodopsins in the common ancestor of *Heliconius* (supplementary fig. S3A–D, Supplementary Material online). Down-regulation of UVRh1 in males appears to have evolved once in the ancestor to the *erato* and *sara* clade (supplementary fig. S3C, Supplementary Material online). As the *melpomene*/*silvaniform* lineage split from the rest of the genus, both sexes lost the UV2 rhodopsin in their compound eyes (supplementary fig. S3B and D, Supplementary Material online), as noted above. Further relaxation of selection of UVRh2 resulted in multiple parallel pseudogenizations as the silvaniforms diverged from the *melpomene* clade (figs. 3A, B and 4, supplementary fig. S3F, Supplementary Material online).

Although several *Heliconius* lineages have downregulated or lost the UV2 rhodopsin, other *Heliconius* lineages appear to have undergone sex-specific selection for retinal mosaics expressing UV2. It appears that the female-specific retinal mosaic III expressing both UV1 and UV2 opsin evolved after the split between *H. charithonia* and the lineage leading to the rest of the *sara*/*sapho* clade. The eyes of *H. charithonia* males resemble other *sara* and *erato* clade males (mosaic II, figs. 1D, F and 5A). However females in the *sara* and *erato* clades differ, and *H. charithonia* female eyes have mosaic I, similar to *erato*-clade females (fig. 5B, supplementary fig. S1A–H, Supplementary Material online). Evidence for the evolution of a new female retinal mosaic following speciation comes from closer phylogenetic examination of *H. charithonia* in relation to other members of the *sara* clade. In opsin gene phylogenies (266 sequences total), *H. charithonia* UVRh1, UVRh2, BRh, and LWRh opsin each fall within a monophyletic clade together with other *sara* clade opsins (supplementary fig. S2A–C, Supplementary Material online). Confirming previous phylogenetic analyses (Kozak et al. 2015), a species phylogeny using 634 orthologous genes from whole
transcriptomes of five species, also gives strong support for *H. charithonia's* inclusion within the *sara* clade (supplementary fig. S4, Supplementary Material online). Beyond the sexual dimorphisms documented above, *H. charithonia* provides further evidence that differential selection pressures may be shaping the male and female *Heliconius* eye.

**Fig. 4.** Selection on UVRh2 is relaxed in *melpomene/silvaniform* clades. (A) Gene tree showing test branches of silvaniform/melpomene UVRh2 (light green) associated with a relaxation parameter, $k < 1$, relative to reference UVRh branches (yellow) set at $k = 1$, signifying relaxed selection in the test branches. The legend represents the relative value of the selection parameter, $k$, where cooler colors show relaxed selection and warmer colors show intensified selection. (B) Comparison of $\omega$ distributions from *melpomene/silvaniform* UVRh2 branches (red) and all other UVRh branches (blue) under the alternative model of relaxed selection. Sites under positive ($\omega > 1$) or purifying ($\omega < 1$) selection in other UVRh branches move toward neutrality in the *melpomene/silvaniform* UVRh2 branches ($\omega = 1$). In the BS-REL model, $\omega$ values for each site are binned into three $\omega$ distributions corresponding to sites under strong purifying, weakly purifying/nearly neutral, and positive selection. Arrows indicate that when comparing sites in the reference branches to the corresponding sites in the test branches, all three $\omega$ distributions move toward neutrality in the test branches.

**Discriminability Modeling of 3-OHK Yellow Coloration and a Potential Benefit of the Violet Receptor**

The newly evolved UV2-expressing violet receptor appears in the eyes of butterflies in seven *Heliconius* clades ($n = 13$ species, supplementary table S2, Supplementary Material online), and has been lost in the *melpomene* and silvaniform lineages.

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*Sexual Dimorphism and Retinal Mosaic Diversification* • doi:10.1093/molbev/msx163 MBE
(n = 5 species, supplementary table S2, Supplementary Material online, see below). What might be the benefit of evolving a new violet receptor and a visual system making use of this receptor? While the violet receptor appears in retinal mosaics with varying numbers of ommatidial types in Heliconius, the biggest functional impact on color vision is expected to be due to its spectral tuning. Previously we used linear discriminant analysis to show that a tetrachromatic color vision system similar to the female Heliconius eye—and ancestral male and female Heliconius eyes—consisting of UV, V, B, and L photoreceptors would outperform both avian and butterfly visual systems lacking both duplicated UVs in discriminating Heliconius 3-hydroxykynurenine (3-OHK) UV-yellow from other yellow coloration (Bybee et al. 2012). These results suggested that the evolution of the duplicated UVs together with 3-OHK coloration (fig. 6A and B, yellow lines) at the base of the genus Heliconius (fig. 6C, arrows)—before subsequent evolution of these diverse retinal mosaics in descent lineages—may have been beneficial for conspecific communication in the context of mimicry. However, these calculations were performed before we knew that male and female Heliconius eyes were sexually dimorphic and before we had photoreceptor count data for each sex. It is worth reconsidering this argument using a more biologically realistic model, which incorporates these parameters (Vorobyev and Osorio 1998).

To understand the potential benefit of the UV2-expressing eye, we performed Heliconius erato male and female mate choice experiments with chromatic stimuli resembling 3-OHK yellows of Heliconius and non-3-OHK yellows of Heliconius conmimics from the genus Euides (fig. 6A and B, grey lines). We found that both Heliconius petiverana males and females do indeed prefer 3-OHK yellow to non-3-OHK yellows (Finkbeiner et al. 2017). To better understand this preference in relationship to male and female Heliconius erato visual performance, here we performed pairwise discriminability calculations under high light illumination comparing Heliconius erato yellow dorsal wing colors (n = 14) to Euides yellows (n = 11) for three types of visual system: a real Heliconius erato female with UV1, UV2 (V), B, and L photoreceptors (fig. 6A and B, solid and dotted black lines), a real Heliconius erato male with UV2 (V), B and L photoreceptors (fig. 6A and B, solid black lines) and a hypothetical Heliconius erato male with UV1 (fig. 6A and B, dotted black line), B and L photoreceptors (for both models, Weber fraction = 0.05, (Koshitaka et al. 2008); relative abundances of photoreceptors, UV = 0.09, V = 0.07, B = 0.17, G = 1 (female) or V (or UV) = 0.13, B = 0.2, L = 1 (male), (McCulloch et al. 2016a). We found that the actual male Heliconius erato visual system with the adaptively evolving UV2 opsin outperformed a hypothetical male Heliconius erato visual system with the ancestral UV1 opsin in discriminating Heliconius erato yellows from Euides yellows and the female eye with both UV1 and UV2. The number of pairwise comparisons (n = 144) that exceeded a threshold of one just noticeable difference (JND), meaning the colors could be discriminated, was 78.4% for the UV2 male eye, 48.6% for the UV1 male eye, and 45.1% for the female eye with both UV1 and UV2 (table 1). All three types of eyes performed similarly when discriminating ventral colors. Taken together, these results suggest that the avoidance of similar-looking but unrelated species in the context of intraspecific communication and mate choice may have been a key driving force for the evolution of the violet receptor. The deployment of a UV-yellow wing pigment for signaling at the base of the genus Heliconius helps explain the spectral tuning of the violet-sensitive rhodopsin UV2 and its use in Heliconius eyes.

The loss of the UV2 opsin in some Heliconius species nonetheless suggests it is evolving neutrally in those lineages due to other genetic and/or environmental changes. Both UV2 opsin down regulation and UVRh2 pseudogenization in the melpomene/silvaniform clades represent recent and ongoing processes. If melpomene/silvaniform species retain a

**Fig. 5.** Male and female Heliconius charithonia eyes are evolving under different selection pressures. sara and erato clade retinal mosaics mapped on to the species phylogeny. (A) Males all have the same retinal mosaic (blue circles, II), with the origin of the trait in the ancestor of both clades (blue bar). (B) sara clade females have retinal mosaic type III (purple circles) except for Heliconius charithonia, with retinal mosaic I (white circles). The most parsimonious origin of retinal mosaic III is after Heliconius charithonia split from the rest of the sara clade (purple bar). Inset shows immunostain of male and female Heliconius charithonia with UV1 (green), UV2 (magenta), and blue opsins (blue).

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**Table 1.** Percentage of pairwise comparisons that exceeded a threshold of one just noticeable difference (JND), meaning the colors could be discriminated, for different visual system models.

<table>
<thead>
<tr>
<th>Visual System</th>
<th>Heliconius erato Male</th>
<th>Heliconius erato Female</th>
<th>Euides Female</th>
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<tr>
<td>UV1</td>
<td>78.4%</td>
<td>48.6%</td>
<td>45.1%</td>
</tr>
<tr>
<td>UV2</td>
<td>48.6%</td>
<td>45.1%</td>
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<tr>
<td>B</td>
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<td>L</td>
<td>48.6%</td>
<td>45.1%</td>
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</tbody>
</table>

**Table 2.** Retinal mosaic types and origins for different species of Heliconius. (A) Males all have the same retinal mosaic (blue circles, II), with the origin of the trait in the ancestor of both clades (blue bar). (B) sara clade females have retinal mosaic type III (purple circles) except for Heliconius charithonia, with retinal mosaic I (white circles). The most parsimonious origin of retinal mosaic III is after Heliconius charithonia split from the rest of the sara clade (purple bar). Inset shows immunostain of male and female Heliconius charithonia with UV1 (green), UV2 (magenta), and blue opsins (blue).
UV1-expressing photoreceptor cell with similar sensitivity as H. erato UV1 rhodopsin, these species may be less able to discern variation in UV-yellow wing coloration than species in the erato/sara/doris clades, a possibility that would need to be confirmed with electrophysiological recordings and behavioral experiments. Interestingly, rampant hybridization exists between species in the melpomene and silvaniform clades. It is conceivable that loss of UV2 in these clades may have contributed to increased hybridization via a reduction in visual ability to recognize conspecifics (Estrada and Jiggins 2008; Heliconius Genome Consortium 2012). The multiple instances of pseudogenizing mutations in UVRh2 suggest that other kinds of cues such as olfaction (Andersson and Dobson 2003; Schulz et al. 2008; Briscoe et al. 2013; van Schooten et al. 2016), may play an increasingly critical role in mate recognition and foraging in species that have lost the duplicate UV opsin.

In summary, we have identified an unprecedented diversity of opsin expression patterns and retinal mosaics among closely related species (fig. 7). Changes in opsin expression have resulted in multiple forms of sexual dimorphism, parallel losses of the UVRh2 gene, losses of both UV1- and UV2-expressing cells, and imply that differential selection pressures have shaped the male and female eye. Nonetheless most studied Heliconius species retain the newly evolved UV2-expressing violet receptor, either alone or with the UV1-expressing ultraviolet receptor, which facilitates discrimination of 3-OHK yellow from other yellow coloration in behavioral tests. Our findings substantiate and elaborate our understanding of the origins and spectral tuning of a new opsin-based violet receptor and its relationship to 3-OHK yellow signaling in Heliconius.

Table 1. Percentage of Heliconius erato and Eueides Dorsal and Ventral Yellow Wing Colors with Chromatic JND Values >1 for Different H. erato Visual Systems.

<table>
<thead>
<tr>
<th></th>
<th>UV1 Male (%)</th>
<th>UV2 Male (%)</th>
<th>UV1 and UV2 Female (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorsal yellow</td>
<td>144</td>
<td>48.6</td>
<td>78.5</td>
</tr>
<tr>
<td>Ventral yellow</td>
<td>117</td>
<td>88.9</td>
<td>87.2</td>
</tr>
</tbody>
</table>

Note.—Three visual systems are modeled: the hypothetical UV1 male, the UV2 male and the UV1 and UV2 female H. erato eye under high light, sunny illumination. All visual systems include B and L opsins.

Materials and Methods

Animals

We obtained pupae from The Butterfly Farm—Costa Rica Entomological Supply, or from Stratford Butterfly Farm, U.K. After eclosion, butterflies were kept alive for 2–3 days in a humidified chamber and fed a diluted honey solution daily before sacrificing. Other adult butterflies used for mRNA sequencing were collected in the field in Ecuador or México and preserved in RNAlater (Life Technologies, Grand Island, NY). Only one color morph was used per species, except for H. doris where all three color morphs were sampled.

Fig. 6. Reflectance spectra of H. erato and Eueides yellow wing coloration used in the discriminability analysis. (A) Dorsal yellow wing colors and (B) Ventral yellow wing colors. Yellow indicates 3-hydroxykynurenine yellow of Heliconius erato. Grey indicates Eueides yellow. Shaded areas correspond to 95% confidence intervals. Solid yellow or light grey lines are means. Black lines indicate normalized UV2, B, and L photoreceptors’ spectral sensitivities from the male H. erato eye. Dotted lines indicate normalized UV1 photoreceptor spectral sensitivity of the H. erato female eye. Data from McCulloch et al. (2016a, b). (C) Phylogeny of Heliconini species in which presence (black) or absence (white) of the UV2 photoreceptor or presence (yellow) or absence (white) of 3OHK pigment is mapped. Modified from Briscoe et al. (2010).
The pan-UV antibody was described in Lampel et al. (2005). and also immunoaffinity purified (Thermo Fisher, MA, USA). Dry slides were placed in 100% ice-cold acetone bath for 5 min, then washed 3 × 10 min in 0.5% sodium dodecyl sulfate in 0.1 M PBS. Each slide was blocked for 30 min at room temperature using 8% (v/v) normal donkey serum and normal goat serum, and 0.3% Triton X-100 in 0.1 M PBS. Slides were incubated with 2:75 affinity-purified rabbit antipan-UV or rabbit antibleu antibody and 1:15 affinity-purified guinea pig antiUV1 antibody in blocking solution overnight at 4 °C. Slides were washed 3 × 10 min in 0.1 M PBS and then incubated with 1:1000 goat antiguinea pig Alexafluor 488 and 1:500 donkey antirabbit Cy3 or Alexafluor 555, in blocking solution for 2 h at room temperature. Slides were washed once more 3 × 10 min in 0.1 M PBS. Slides were stored for imaging by coverslipping with Aqua Poly/Mount (Polysciences, Inc. Cat. # 18606). Slides were viewed with epifluorescence microscopy using a Zeiss Axioskop 2 under a 20× lens. Images were taken using a Zeiss Axiocam HRc and associated Axiovision software, or with a Leica confocal SP700 microscope in the UC Irvine Optical Core Facility. Contrast and brightness were adjusted for clarity using Adobe Photoshop CS4 and Fiji.

For each specimen, we examined hundreds to thousands of fluorescently labeled ommatidia at multiple depths in the retina, and we noted different ommatidial types based on the combinations of UV-expressing R1 and R2 cells. For clarity, all images are presented as a small subset of the retina, where all possible ommatidial combinations can be seen in close proximity. We fluorescently labeled R1 and R2 cells expressing either BRh (blue), UVRh1 (green), or UVRh2 (magenta) (fig. 18–L, supplementary fig. S1A–N, Supplementary Material online). We did not distinguish which cell was R1 and which was R2. In double stains that did not label BRh expression, R1 and R2 cells that were not labeled were assumed to be blue opsin expressing cells, according to previous
in situ hybridization and current immunohistochemistry results (fig. 1; Zaccardi et al. 2006). We then classified ommatidial types according to their combination of R1 and R2 cells, and identified retinal mosaics by the combinations of ommatidial types present in each (fig. 1B).

**RNA-Sequencing**

Total RNA was extracted from 62 individual adult butterfly heads using Trizol (Life Technologies, Grand Island, NY). A NucleoSpin RNA II kit (Macherey-Nagel, Bethlehem, PA) was used to purify 10 μg total RNA per sample. Purified total RNA was quantified using a Qubit 2.0 Fluorometer (Life Technologies, Grand Island, NY). The quality of the RNA samples was checked using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Four micrograms of purified total RNA were used to make cDNA libraries. A TruSeq RNA sample prep kit, set A (Illumina, San Diego, CA) was used to prepare individual cDNA libraries. PCR-enriched individual cDNA libraries were quantified using the Qubit 2.0 Fluorometer and QC checked using the Agilent Bioanalyzer 2100. After being normalized according to their Qubit concentrations, the enriched individual libraries were pooled and then run on a 2% agarose gel. cDNA products ranging from 280 to 340 bp with an average of 310 bp were cut pooled and then run on a 2% agarose gel. cDNA products ranging from 280 to 340 bp with an average of 310 bp were cut and gel-purified total RNA were used to make cDNA libraries. A TruSeq RNA sample prep kit, set A (Illumina, San Diego, CA) was used to prepare individual cDNA libraries. PCR-enriched individual cDNA libraries were quantified using the Qubit 2.0 Fluorometer and QC checked using the Agilent Bioanalyzer 2100. After being normalized according to their Qubit concentrations, the enriched individual libraries were pooled and then run on a 2% agarose gel. cDNA products ranging from 280 to 340 bp with an average of 310 bp were cut and purified using a GeneClean III kit (MP Biomedicals, Solon, OH). After being repurified using Agencourt AMPure XP magnetic beads (Beckman Coulter Genomics, Danvers, MA), the cDNA pool was quantified using the Qubit 2.0 Fluorometer, and QC checked using the Agilent Bioanalyzer 2100. The cDNA pool sample was then normalized to 10 nM and run on an HiSeq 2000 (Illumina, San Diego, CA) yielding 200 million 100 bp paired-end reads per lane.

**Cell Counting and Principal Component Analysis**

Ommatidia were only counted if images captured more than 100 ommatidia, the tissue was not sheared or folded, and cell bodies were clearly labeled without a high level of background. Images were viewed at full resolution in Adobe Illustrator. The background autofluorescence found in all ommatidia was not removed, so as to see any ommatidia that might be unstained. Ommatidia were not counted if the staining was unclear or the sectioned tissue was of poor quality (e.g., folded). Total ommatidia were counted over as much area as possible for a single high quality section per individual and the percentages of each class of ommatidia were calculated. From these ommatidial classes, we could count the total number of individual R1 and R2 photoreceptor subtypes found in each section. Principal components analysis was performed in R using the prcomp function of the stats package. Ommatidial and cell count averages for each sex and species were used and log transformed for the analysis. The results were plotted using the first two principal components as the x- and y-axes.

**Ancestral State Reconstruction and Character Mapping**

Twenty-seven species of *Heliconius* and *E. isabella* were scored for the presence or absence of full-length UVRh2 transcripts. All species expressed UVRh1. Fourteen species representing each of the major *Heliconius* lineages and the outgroup *Eueides isabella* were examined using immunohistochemistry and scored for the presence or absence of the following four traits: female and male UVRh1 and UVRh2 PRcs. Each of these four characters were individually mapped onto the terminal nodes of the species tree from ref (Kozak et al. 2015) in Mesquite (Maddison and Maddison 2011) and ancestral states were then estimated using the maximum likelihood MK1 model with equal likelihood of gains and losses.
k = 1, so the ω distributions of T and R are equal. In the alternative model, k is allowed to vary, so that if k > 1 T is under stronger selection relative to R, and if k < 1, then T is under relaxed selection compared with R. The models are compared using a likelihood ratio test using a χ² distribution to test if the alternative model is a better fit.

Read-Mapping of Opsins
In order to validate the results of the immunohistochemistry and quantify the different levels of UVRh1 and UVRh2 transcript expression between major clades and sexes, we selected individual butterflies (males and females) for analysis. Our RNA-Seq reads were quality trimmed using the python script TQSFastq.py (http://genomics-pubs.princeton.edu/prv/resources/scripts/TQSFastq.py) with a quality threshold of 20 and a minimum read length of 30. Reads were paired using a second python script, paired_sequence_match.py (https://bitbucket.org/lance_parsons/paired_sequence_utils). We produced de novo assemblies of the transcriptome for each species using the programs Velvet (default settings and a kmer length of 31) (Zerbino and Birney 2008) and Oases (Schulz et al. 2012). We then used BLAT (Kent 2002) to locate the species-specific UVRh1 and UVRh2 opsin genes in our de novo transcriptomes utilizing the publically available H. mel- pomene sequences as references (NCBI Accession numbers: GU324678.1 [UVRh1] and GU324679.1 [UVRh2]). Next, we mapped the forward set of reads for each sample onto its species-specific UVRh1 and UVRh2 opsin sequences using the program Stampy (Lunter and Goodson 2011). SAMtools was then used to sort the resulting mapped reads (Li et al. 2009), and htseq-count (http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html) was employed to count the number of unique reads that mapped to each opsin sequence. We calculated the reads per kilobase of transcript per million mapped (RPKM) for each gene in each sample. We also calculated the ratio of the average log₂ UVRh2 reads over the average log₂ UVRh1 reads. Lastly, for any species and sex combination in which we had two or more samples, we determined whether there was a significant difference in the expression of UVRh1 and UVRh2 using a two-sample t-test with equal variances.

Species Phylogeny
Five representative species of the major Heliconius clades were included in this analysis; H. melpomene, H. erato, H. doris, H. sara, and H. charithonia. A de novo assembly of RNA-Seq data was performed and transcriptomic data from each of the five species was mapped back to the assembly using Velvet (Zerbino and Birney 2008) and Oases (Schulz et al. 2012). Contigs were filtered for the presence of sequence data in each of the five species, for contig lengths of >200 bp and for BLAST matches with > 90% sequence identity and >100 bp long. In total, 634 loci were obtained and alignments for each locus were produced using Clustal W (Larkin et al. 2007) and variable positions flanking indels were masked by Ns to reduce misalignment error in the data set. The 634 loci from the five representative species were concatenated and a partition annotation file denoting the coordinates of each locus was generated. This partitioned data was run in RAxML ( Stamatakis 2006 ) with rapid bootstrapping (1000 bootstraps) and a maximum likelihood search under the General Time Reversible (GTR) substitution model, with a gamma distribution. The alignment file for the 634 loci was deposited in Dryad under data identifier: doi:10.5061/dryad.1j1f3.

Discriminability Modeling
Models of color vision take into account how receptor signals contribute to chromatic (e.g., color opponent) mechanisms (Kelber et al. 2003). For H. erato males, whose yellow color preferences have been tested experimentally (Finkbeiner et al. 2014; Finkbeiner et al. 2017) and shown to prefer 3-OHK yellow models, we calculated discriminabilities for the actual H. erato male trichromatic system consisting of UV2, blue and green receptors and a hypothetical H. erato male trichromatic system in which UV2 is replaced by UV1. Equations from (Kelber et al. 2003) and (Vorobyev and Osorio 1998) were used to model discriminabilities. This model incorporates a von Kries’s transformation, that is, normalization by the illumination spectrum, which models the way in which low-level mechanisms such as photoreceptor adaptation give color constancy (Kelber et al. 2003). The sunny cage illumination spectrum from (Finkbeiner et al. 2017) was used in the model because it corresponds to the illumination used during actual Heliconius erato behavioral experiments. For H. erato photoreceptor spectral sensitivity curves with λmax values = 355 nm (UV1), 390 nm (UV2), 470 nm (B), and 555 (L) nm from (McCulloch et al. 2016a) were used. Parameters for the butterfly visual models were as follows: Weber fraction = 0.05 (Koshitaka et al. 2008), photoreceptor peak sensitivities, λmax = 355 nm (hypothetical UV1 male only) or 390 nm (actual UV2 male only), 470 and 555 nm, and relative abundances of photoreceptors, V = 0.13, B = 0.2, L = 1 (male) or UV=0.09, V=0.07, B=0.17, L=1 (females) (McCulloch et al. 2016a).

Supplementary Material
Supplementary data are available at Molecular Biology and Evolution online.

Author Contributions

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