UV Photoreceptors and UV-Yellow Wing Pigments in *Heliconius* Butterflies Allow a Color Signal to Serve both Mimicry and Intraspecific Communication

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abstract: Mimetic wing coloration evolves in butterflies in the context of predator confusion. Unless butterfly eyes have adaptations for discriminating mimetic color variation, mimicry also carries a risk of confusion for the butterflies themselves. *Heliconius* butterfly eyes, which express recently duplicated ultraviolet (UV) opsins, have such an adaptation. To examine bird and butterfly color vision as sources of selection on butterfly coloration, we studied yellow wing pigmentation in the tribe Heliconiini. We confirmed, using reflectance and mass spectrometry, that only *Heliconius* use 3-hydroxy-DL-kynurenine (3-OHK), which looks yellow to humans but reflects both UV- and long-wavelength light, whereas butterflies in related genera have chemically unknown yellow pigments mostly lacking UV reflectance. Modeling of these color signals reveals that the two UV photoreceptors of *Heliconius* are better suited to separating 3-OHK from non-3-OHK spectra compared with the photoreceptors of related genera or birds. The co-occurrence of potentially enhanced UV vision and a UV-reflecting yellow wing pigment could allow unpalatable *Heliconius* private intraspecific communication in the presence of mimics. Our results are the best available evidence for the correlated evolution of a color signal and color vision. They also suggest that predator visual systems are error prone in the context of mimicry.

Keywords: 3-hydroxy-kynurenine, opsin, vision, UV coloration, adaptation.

Introduction

The bright colors of animal courtship displays and aposematic signals are well known, but for the majority of

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species their function and evolution remain controversial. Allen (1879) proposed that color vision is adapted primarily for finding food. This, he argued, can lead secondarily to color preferences that are then exploited by colorful displays. An alternative scenario is that courtship signals and sensory mechanisms evolve in a correlated manner as specialized communication systems. Determining the extent to which visual displays exploit fixed sensory capacities or preferences, perhaps associated with feeding (as opposed to a correlated evolutionary scenario), is central to understanding biological communication (Endler and Basolo 1998; Seehausen et al. 2008).

In work on animal defenses, it is similarly unclear why aposematic signals involve colorful displays and strong contrasts. It may be that they are easily detected at the level of the predator's photoreceptors, thereby also exploiting fixed sensory capacities. Alternatively, bright coloration may be the outcome of an arms race where defended models evolve to differentiate themselves in the eyes of potential predators from undefended mimics (Ruxton et al. 2004). For co-occurring mimics, a related question involves the extent to which a predator's sensory perception is error prone (Speed and Ruxton 2010), perhaps because of limitations of the photoreceptor cells themselves. Evolutionary interactions between mimicry and predator perception are, however, little studied. Indeed, few studies of mimicry and aposematism consider the differences between human color vision and that of natural predators (Stevens 2007).

Heliconius butterflies are a useful system for investigating the relationship between a mimetic color signal—yellow wing pigmentation—and their own color vision. They are also a useful system for investigating the potential role of predator (bird) vision in shaping the evolution of mi-

metic wing coloration. In the genus *Heliconius*, as for other butterflies, wing coloration serves as both defense and intraspecific communication (Oliver et al. 2009; Allen et al. 2011). Many *Heliconius* are distasteful to birds (Chai 1986, 1996; Langham 2004), and thus the wing patterns of these defended species are considered aposematic. *Heliconius* are involved in mimicry rings throughout the Neotropics, where several defended species in a given area share a common warning signal (Mallet and Joron 1999) and hence benefit from looking alike. Conversely, *Heliconius* warning coloration may be under selection to make them discriminable by predators from poorly defended (Batesian) mimics, such as various Dismorphiini (Pieridae) and Melitaeini (Nymphalidae; Brower et al. 1963; Brower and Brower 1964; Brown and Benson 1974; J. Llorente-Bousquets, personal observation). Evidently, multiple selective factors might affect the evolution of butterfly wing coloration and color vision: defended species and undefended mimics are communicating with their own species and with predators such as birds, because both butterflies and predators need to identify or discriminate these signals according to their interests in mating or feeding.

In this context, it is noteworthy that several *Heliconius* species share duplicated ultraviolet (UV) opsin genes, *UVRh1* and *UVRh2*, allowing their compound eyes to express two UV-absorbing visual pigments, in addition to blue- and long-wavelength-absorbing visual pigments (Briscoe et al. 2010*a*). *UVRh2* evolved under positive selection, and the gene duplication of this spectral class of rhodopsin is so far known only from *Heliconius* and not from related genera in the subfamily Heliconiinae (Zaccardi et al. 2006*b*; Briscoe et al. 2010*a*, 2010*b*; Yuan et al. 2010; see also Nozawa et al. 2010). The duplicate genes encode spectrally distinct visual pigments, with sensitivity peaks estimated at ∼355 nm (UV) and ∼398 nm (violet), which offer the potential for enhanced spectral discrimination at short wavelengths (Briscoe et al. 2010*a*).

The study by Briscoe et al. (2010*a*) also showed that in two *Heliconius* species, the wing colors that are yellow to human eyes have higher UV reflectance than do other butterfly yellows. This is due to the light scattering from wing scale elements and the presence of the pigment 3-hydroxy-L-kynurenine (3-OHK), which does not absorb UV as strongly as other pigments. We refer to spectra with maxima in the UV (<400 nm) and at long (>450 nm) wavelengths, such as that of 3-OHK, as UV yellows. Ancestral state reconstruction from a limited set of taxa ($n = 14$ species) suggested that the UV opsin gene duplication occurred at about the same time that UV-yellow wing colors appeared (i.e., they originated at the same node; Briscoe et al. 2010*a*).

Yellow or UV-yellow wing coloration is likely to be important in visual signaling to both the butterflies and their predators. In *Heliconius* species that are polymorphic for yellow or white colors, mate preferences are correlated with wing coloration (Chamberlain et al. 2009), while many insects use yellow in aposematic signals (Kauppinen and Mappes 2003). However, to our knowledge, the role of yellow as a warning/mimetic color for *Heliconius* has not been studied with predator behavioral tests or models of predator vision.

To begin to investigate how these factors interact, we combine a detailed phylogenetic examination of a signaling trait—yellow wing pigmentation—with modeling of color signals available to different species of butterflies and birds. We measure yellow reflectance spectra from the wings of 49 species of butterfly in eight genera in the tribe Heliconiini. We then look at the history of yellow pigmentation in these same heliconiine butterflies based on phylogenetic reconstruction. Next, we model color signals for the eyes of *Heliconius erato*, for a butterfly species in a related genus, and for birds that are representative of the UV- or violet-type avian visual system. This modeling permits us to ask whether birds' use of four types of cone pigments for color vision (i.e., tetrachromacy) provides adequate information for discrimination of UV yellow from other kinds of yellow pigmentation. It also suggests how the different types of yellow pigmentation work as visual signals for different types of bird or butterfly eyes. The evidence supports the hypothesis that in the genus *Heliconius*, 3-OHK pigmentation and the UV opsin duplication evolved in a correlated manner as a complex trait that allows these unpalatable butterflies to distinguish themselves from mimics that do not use 3-OHK pigmentation. By comparison, the photoreceptors of other butterflies in the tribe (outside the genus *Heliconius*) and birds are less well suited to making this distinction, thus affording some *Heliconius* butterflies a kind of private UV communication channel. This is the best available evidence that correlated evolution of color vision and a color signal resulted in a specialized communication system.

Material and Methods

Taxon Sampling

To determine the origins of yellow wing colors within the tribe Heliconiini, we obtained specimens of 49 of the 59 species (table A1, available online) that are represented in a recent phylogeny (Mallet et al. 2007). Specimens were kindly provided by L. Gilbert and M. Kronforst; gathered from the field by J.L.B., A.D.B., and R.D.R.; measured while pinned at the Natural History Museum of Los Angeles County; or purchased from insect dealers (Ianni Butterfly Enterprises, Parma, OH; Butterflies and Things, Spencer, OH; Insectes Mondiaux, Lac-Beauport, Canada;

Tropical Butterflies and Insects of America, Tampa, FL). To minimize variation due to natural aging, every attempt was made to measure well-preserved specimens with little to no wing wear or scale loss. The 49 taxa broadly represent the major yellow color patterns displayed by the tribe.

Reflectance Spectrometry, UV-VIS Spectrophotometry, and Mass Spectrometry

Reflectance spectra of yellow wing pigments from the dorsal right/ventral left fore- and hindwings of each specimen were measured by first aligning each measured wing in the same orientation as shown in appendix B (available online). If the viewer were looking directly from above at the oriented wings shown in the appendix B figures, the probe holder (Ocean Optics RPH-1) was placed precisely horizontally on top of the wing such that the axis of the illuminating and detecting fiber (Ocean Optics R400-7-UV/VIS) was at an elevation of 45° to the plane of the wing and pointed left with respect to the body axis. Illumination was by a DH-2000 deuterium-halogen lamp, and reflectance spectra were measured with an Ocean Optics USB2000 spectrometer. Data were processed in MATLAB. We measured all distinct wing color patches with a diameter exceeding about 2 mm. At least three individuals per taxon were measured for each color patch for all but eight of the 49 taxa (table A1). In total, we examined 185 specimens and measured 507 yellow reflectance spectra.

To investigate whether reflectance spectra can signify 3-OHK pigmentation, we extracted wing pigments from one to four specimens from each of 15 species (table A2, available online) representing all closely related outgroup taxa, as well as from *Heliconius* species that have been screened for opsins. For each specimen, all yellow color patches from one or two wings were dissected before pigment extraction to avoid possible contamination with orange and red pigments. The pigments were extracted by immersing the wing patches in 300 μL of acidified methanol (0.5% HCl) for 2–3 min. The extracted pigments were then dried down completely in a vacuum centrifuge for 45 minutes, resuspended in 100 μL of 100% methanol, dried down again, and then resuspended in 100 μL of 100% methanol. The absorbance spectrum of a 25-μL aliquot was measured in a UV-VIS spectrophotometer (Hitachi U-3310). The molecular mass profile of a 100-μL 1 : 20 dilution in methanol (Optima, Fisher catalog A454-1) was analyzed on a Micromass LCT mass spectrometer. The results from these species were compared with a 3-OHK standard (Sigma-Aldrich, St. Louis).

Ancestral State Reconstruction of Yellow Wing Colors

Where complex pigment mixtures generate colors, reflectance spectra such as carotenoid-based wing colors of birds have been mapped on phylogenies as continuous characters (Hofmann et al. 2006). For pierid butterflies, the presence or absence of UV or melanic dorsal marking were mapped as discrete characters (Kemp et al. 2005). We treat color as a discrete character according to whether the reflectance spectrum is characteristic of pigment scales containing 3-OHK. Such scales have a steplike reflectance spectrum that increases sharply above 450 nm and includes a UV component with a peak between 300 and 350 nm that generally exceeds 10% reflectance (fig. 1*A*). The yellows of species in genera closely related to *Heliconius* lack an abrupt reflectance increase at 450 nm but have a more gradual slope starting at ∼425 nm, and they generally lack a UV component (*Eueides heliconioides* is an exception). The topology of Mallet et al. (2007) was used to trace the evolution of yellow coloration. Species entirely missing any yellow coloration were coded as 0, species with 3-OHK yellow reflectance spectra were coded as 1, and species with non-3-OHK yellow reflectance spectra were coded as 2. Ancestral state reconstruction was performed in Mesquite (ver. 2.72; Maddison and Maddison 2009) using the parsimony option with default settings.

Sequencing and Phylogenetic Analysis of UV Opsins

To increase our sampling of the UV opsins of outgroup taxa, we collected *Eueides aliphera*, *Eueides lineata*, and *Dione juno* from Oaxaca, Mexico. We performed reverse transcriptase polymerase chain reaction (RT-PCR; AffinityScript multiple temperature cDNA synthesis kit [Stratagene, La Jolla, CA] and BD *Taq* polymerase [BD Biosciences, San Jose, CA]) from total RNA extracted from eyes using the primers 5′-CACGCTACCGAGGACTGC-3′/5′-TCGTCTAGTTTTCATTATTTGTTC-3′ for *Eueides* and 5′-CAMGCTACCGAGGACTGCTCMC-3′/5′-AGTCGT-TGCTTTTCTCGTTCTAC-3′ for *Dione*. These RT-PCR products were directly sequenced in both directions.

The opsin sequences were aligned in MEGA 4.0 and then back translated, resulting in an alignment of 1,137 base pairs for the *UVRh* gene. Table A3 (available online) lists GenBank accession numbers. All analyses used the maximum likelihood algorithm as implemented in RAxML (ver. 7.0.4; Stamatakis 2006) under the GTR+Γ model and were rooted with a *Danaus plexippus* UV opsin sequence. Support values were based on 1,000 bootstrap pseudoreplicates in RAxML.
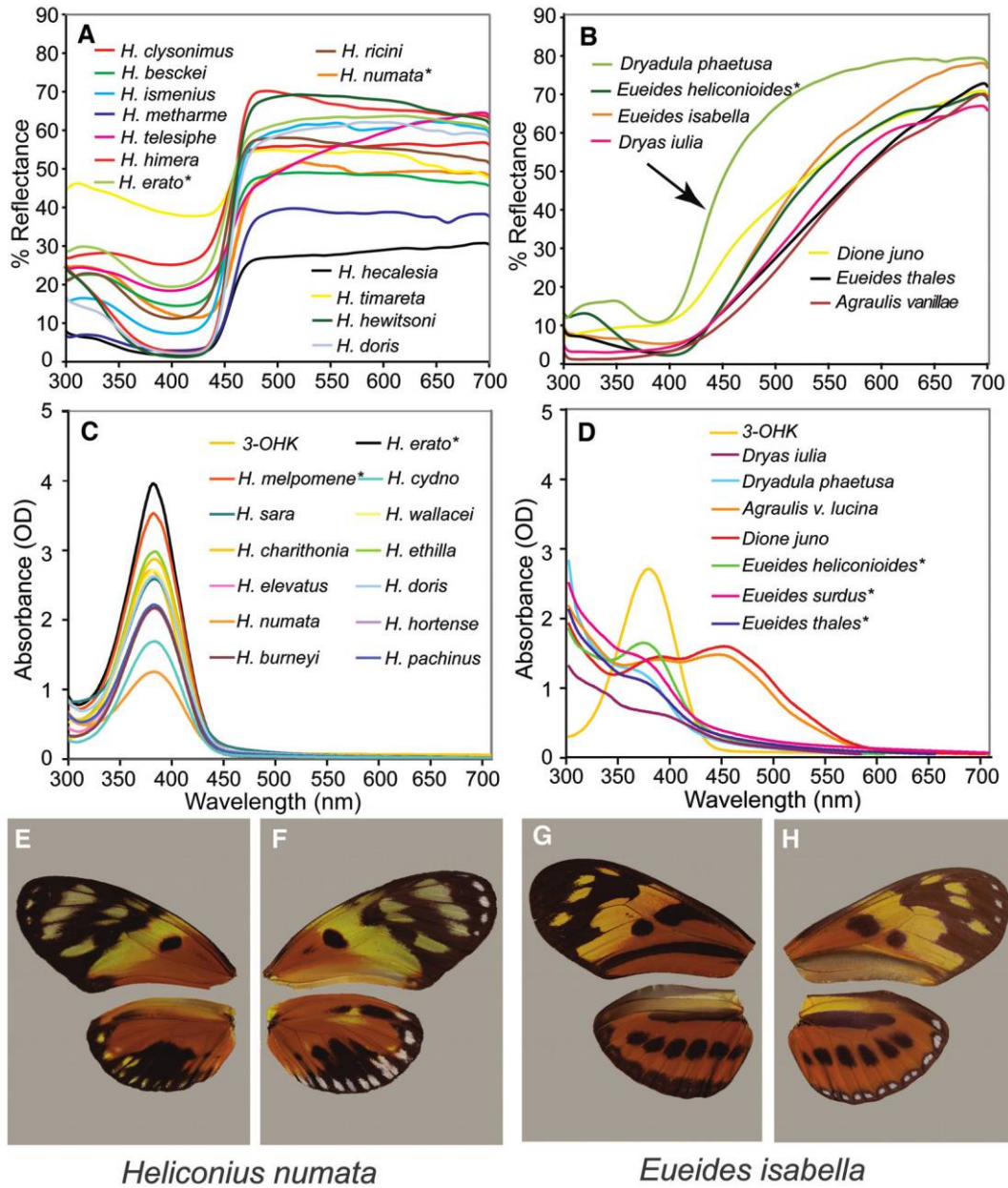
Figure 1: Reflectance and absorbance spectra of representative yellow wing pigments from butterflies in the tribe Heliconiini, and photographs of mimics whose wings contain 3-hydroxy-L-kynurenine (3-OHK; *left*) and non-3-OHK (*right*) yellow pigments. *A*, Spectra from butterflies in the genus *Heliconius*. *B*, Spectra from butterflies in related genera in the same tribe. In *B*, arrow indicates *Dryadula phaetusa* yellow with the UV component. *C*, Absorbance spectra of extracted yellow pigments from wings of butterflies in the genus *Heliconius*. *D*, Absorbance spectra of extracted yellow pigments from wings of butterflies in related genera. The 3-OHK corresponds to a chemically pure control standard (Sigma-Aldrich, St. Louis). All extracted pigments from *Heliconius* species have absorbance spectra that appear to be nearly pure 3-OHK. All pigments extracted from species in related genera have absorbance spectra that appear to be different from 3-OHK. Asterisks indicate spectra reported by Briscoe et al. (2010*a*) and redrawn here. See appendix B (available online) for species photographs from which yellows were extracted. Dorsal (*E*) and ventral (*F*) views of *Heliconius numata*, and dorsal (*G*) and ventral (*H*) views of *Eueides isabella*, which belong to the tiger-stripe mimicry ring (Moulton 1909).

Color Space Calculations

We estimated photoreceptor excitations for the 507 yellow pigment reflectance spectra and mapped them in color spaces of butterflies and birds (Wyszecki and Stiles 1982; Goldsmith 1990; Vorobyev 2003). We considered two related butterflies in the tribe Heliconiini that differ in the number of visual pigments in the eye—namely, *Dryas iulia*, with three opsins (known as UV, blue, and long), and *Heliconius erato*, with four (UV, violet, blue, and long; Hsu et al. 2001; Zaccardi et al. 2006*a*; Briscoe et al. 2010*a*). We also modeled two birds that differ primarily in the spectral sensitivities of their short-wavelength-sensitive cone visual pigments: blue tit *Parus caeruleus*, which is a typical passerid (oscine passerine) with a UV-type visual system (Hart et al. 2000), and chicken *Gallus gallus*, representing the violet-type visual system. The latter system is found in tyrannid passerines such as the New World flycatchers (Ödeen and Håstad 2003), which are common Neotropical insectivores (Pinheiro 2003).

Models of color vision need to take account of how receptor signals contribute to chromatic (e.g., color opponent) mechanisms (Kelber et al. 2003). Experimental evidence suggests that birds use their four single cones for tetrachromatic color vision (Osorio et al. 1999; Goldsmith and Butler 2003, 2005). We know also that honeybees are trichromatic (Vorobyev et al. 2001). There is evidence, however, that butterfly behaviors are based on a subset of spectral receptor types (Scherer and Kolb 1987; Koshitaka et al. 2008). We therefore modeled performance for subsets of butterfly receptors as well as full tetrachromacy. Similar comparisons between different combinations of butterfly (*Pieris rapae*) and bird photoreceptors have been made to evaluate sexually dimorphic wing signals (Morehouse and Rutowski 2010).

Accordingly, for *H. erato*, we calculated (1) achromatic contrast and the chromaticity[1] loci for (2) the six possible dichromatic receptor combinations, (3) the four trichromatic receptor combinations, and (4) the full tetrachromatic eye. For *D. iulia*, we calculated (1) achromatic contrast, (2) the three possible dichromatic receptor combinations, and (3) the single trichromatic system. In birds, the achromatic (or luminance) mechanism has a spectral sensitivity close to that of the double cones (Goldsmith and Butler 2005; Osorio and Vorobyev 2005). Ac-

cordingly, we calculated achromatic contrast of their double cones and loci for the tetrahedral color space of their single cones. Equations of Kelber et al. (2003; their eqq. [A1]–[A5], [A8]–[A12]) were used to model the tri- and tetrachromatic color spaces, respectively. 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). Equations (1)–(4) below were used to calculate achromatic contrast (q_L) and the coordinates of the dichromatic color loci, where the quantum catch of a photoreceptor viewing a stimulus (Q_i) or background (Q_i^0) is defined by

$$Q_i = \int I(\lambda)R(\lambda)S_i(\lambda)d\lambda, \qquad (1)$$

where $S_i(\lambda)$ is the photoreceptor spectral sensitivity, $R(\lambda)$ is the reflectance spectrum of the stimulus or the adapting background, and $I(\lambda)$ is the illuminating light spectrum.

Achromatic contrast is given by

$$q_L = \frac{Q_L - Q_L^0}{Q_L + Q_L^0}. \qquad (2)$$

For a dichromat, with a mechanism based on receptors A and B, the coordinates of the color loci in the dichromatic space are given by

$$q_i = \frac{Q_i}{Q_i^0}, \qquad (3)$$

$$x = \frac{q_A - q_B}{q_A + q_B}, \qquad (4)$$

where the quantum catch for a particular receptor for a stimulus Q_i is first divided by those for the background stimulus, Q_i^0.

For *H. erato* and *D. iulia*, photoreceptor absorbance spectra were based on λ_{max} value estimates (*H. erato*: 355, 398, 470, and 555 nm; *D. iulia*: 385, 470, and 555 nm; Struwe 1972*a*, 1972*b*; Frentiu et al. 2007*b*; Briscoe et al. 2010*a*) and a visual pigment template (Palacios et al. 1996). The rhodopsin templates do not incorporate effects of intraocular filtering but, for our purposes, are reasonable estimates of the spectral sensitivity curves (see "Discussion"). For the birds, the model's spectral sensitivity curves do take into account the effects of oil droplets and corneal transmission. The λ_{max} values of the four blue tit single cones were 372, 451, 537, and 605 nm, and the λ_{max} values of chicken single cones were 420, 470, 540, and 600 nm. Our model does not consider the effects of photoreceptor noise (Vorobyev and Osorio 1998) and hence makes no assumption about the relative abundances of each of the

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[1] Color is often considered to have two main components (Wyszecki and Stiles 1982; Osorio and Vorobyev 2008; Kelber and Osorio 2010): an achromatic component that is related to overall reflectance (roughly brightness) and chromatic components that are related to spectral shape (roughly hue and saturation). For a wide range of animals, there is evidence that chromaticity serves different behaviors from achromatic signals, so it is convenient and biologically appropriate to consider them separately as we do here. In practice, overall reflectance is unlikely to be useful for separating *Heliconius* from other heliconiine yellow wing pigmentation.

spectral classes of photoreceptor in the eye, merely that they are present in the same part of the eye at physiologically significant levels. This assumption is reasonable in light of available physiological and anatomical data for butterflies (Struwe 1972*a*, 1972*b*; Briscoe et al. 2010*a*; A. D. Briscoe, unpublished data).

The irradiance spectrum used for all calculations was measured in a sunny open habitat where several *Heliconius* species and their close relatives were most often observed to fly—in a cloud forest in Oaxaca, México (Briscoe et al. 2010*a*). The model also assumes that the photoreceptors were adapted to a background spectrum of the *H. erato* dorsal forewing brown pigment. The choice of natural illuminant and of the background reflectance, however, is very unlikely to have any consequence for our main conclusions. This is because the effects of changes in either illuminant or background are to translate all color loci along a nearly fixed vector in the color space, which has within broad limits a negligible effect on the relative locations of color loci within the color space (Foster and Nascimento 1994; Vorobyev et al. 1998).

To evaluate the potential of the different types of color vision (i.e., avian tetrachromacy in comparison with simpler combinations of butterfly receptors) to recognize *Heliconius* yellows separately from yellows found on the wings of species in closely related genera, we modeled the information available from the photoreceptors for visual processing. Stimulus classification is seldom evaluated in models of animal color vision, which tend to focus on discrimination between pairs of spectra, but the approach is broadly similar to that of Regan et al. (2001), who investigated the ability of primate color vision to separate fruit from leaves. All that is required is to determine whether a sensory signal exceeds some threshold value, which may be innate or learned. There is ample behavioral evidence that animals do this when using sensory information (Scherer and Kolb 1987; Giurfa et al. 1995).

Specifically, we use linear discriminant analysis to find a point, a line, or a plane in the di-, tri-, or tetrachromatic color spaces that best separates the *Heliconius* yellows from other yellows found in closely related genera. Linear discriminant analysis maximizes variance between the classes while minimizing variance within each class. The analysis assumes that the classes are normally distributed and the covariance between classes is equal. Performance is specified by the error rate, or the proportion of pigments that would be incorrectly classified from their reflectance spectra as 3-OHK or non-3-OHK by the best linear discriminator available to a given eye. The model also assumes equal prior probabilities for each type of pigmentation. Error rates associated with the probability of misclassifying a color were calculated in MATLAB. We explored the possibility of using nonlinear discriminators, but effects on

performance were small and did not affect the overall conclusions.

Results

Wing Reflectance Characterizes a Distinct Heliconius *Yellow*

Reflectance measurements of yellow wing pigmentation ($n = 507$) showed that *Heliconius* species have a distinct spectrum that is absent from the other major lineages in the tribe (fig. 1*A*, 1*B*). The spectra from *Heliconius* species have a distinct step increase at ∼450 nm and a variable UV component (fig. 1*A*), whereas those of species in related genera consistently have more gradual slopes starting at ∼425 nm and low UV reflectance (fig. 1*B*). The forewing yellow patches of *Eueides heliconioides* and hindwing spots of *Dryadula phaetusa* were the only exceptions. In particular, the *Dryadula* spectrum has a significant UV peak (fig. 1*B*, arrow), but the increase from 425 nm lacks the steplike character of the 3-OHK spectrum.

Phylogenetic Distribution of Yellow Pigments in the Tribe Heliconiini

While the reflectance spectra of the yellow *Heliconius* wing patterns were consistent with 3-OHK, as previously described for *Heliconius erato* and *Heliconius melpomene* (Briscoe et al. 2010*a*), we verified their identities by analyzing the UV-visible absorbance spectra of the extracted yellow pigments from 15 additional taxa (fig. 1*C*, 1*D*). Absorbance spectra of the *Heliconius* yellow pigment extracts matched the spectrum of chemically pure 3-OHK (fig. 1*C*). *Eueides heliconioides* had a similar but separable UV peak to that of 3-OHK; otherwise, no yellow pigment from species in sister genera had an absorbance spectrum resembling 3-OHK (fig. 1*D*).

Mass spectrometry confirmed the identities of the extracted yellow pigments. All of the *Heliconius* yellow pigment extracts measured had a single major mass spectrometric peak of 225 m/z that matched the absorbance spectrum of hydrogenated $[M + H^+]$ 3-OHK (fig. A1*A*–A1*L*, available online). None of the pigment extracts from sister genera and outgroups had the 225 m/z peak, demonstrating the absence of 3-OHK (fig. A1*M*–A1*P*), including the yellow wing pigment extracts from *E. heliconioides* as previously reported (Briscoe et al. 2010*a*).

Ancestral state reconstruction of 3-OHK yellow, as inferred from spectral reflectance data and a phylogeny of Heliconiini, strongly indicates a single origin of 3-OHK-type yellow at the base of the genus *Heliconius* (fig. 2). There is a single potential secondary loss in the species
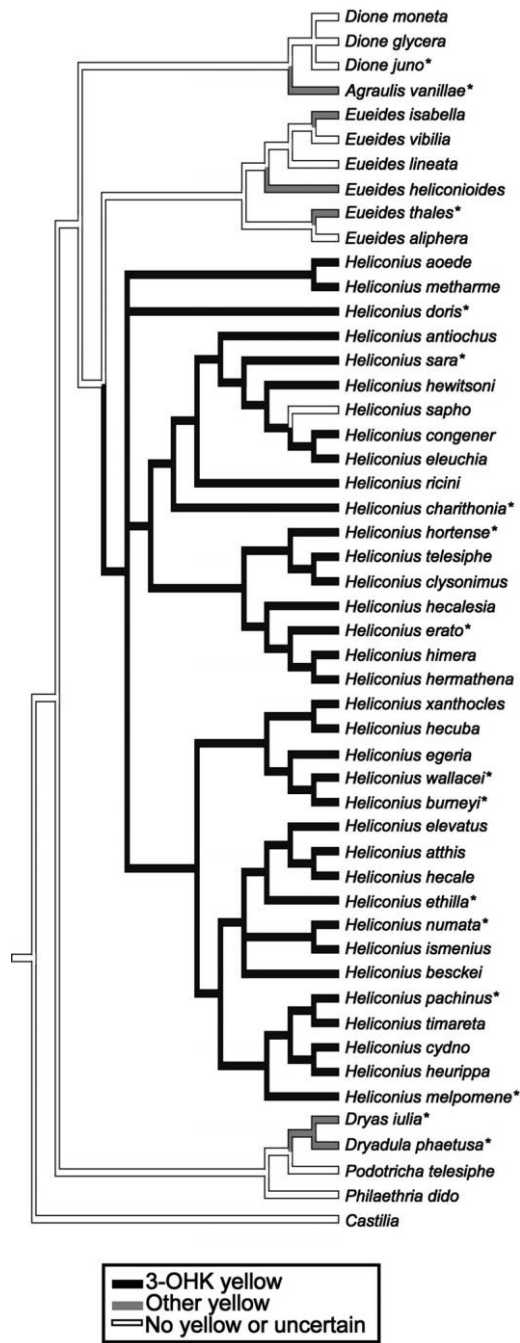
Figure 2: Character mapping of yellow wing colors from *Heliconius* species and from sister taxa and outgroups on the topology of Mallet et al. (2007). Since members of the genus *Heliconius* can be polymorphic for yellow, this character mapping simply reflects whether yellow is found in the species. Black indicates the presence of reflectance spectra characteristic of 3-hydroxy-L-kynurenine (3-OHK), gray indicates reflectance spectra of non-3-OHK yellow, and white indicates an absence of yellow pigment on the wings. Asterisks correspond to specimens where yellow wing pigments were analyzed using UV-visible spectrophotometry and mass spectrometry.

Heliconius sapho, where we were unable to find any variants with yellow wing pigment.

Phylogenetic Origins of a UV Opsin Duplication in Heliconius

Previously, cDNA sequences from 10 *Heliconius* butterfly species revealed two UV opsins in each species (Briscoe et al. 2010*a*; Yuan et al. 2010). In contrast, we found only one UV opsin cDNA in each of the newly screened species belonging to the same tribe: *Dione juno* (HM366553), *Eueides aliphera* (HM366554), and *Eueides lineata* (HM36655; fig. 3). The branching pattern of the UV opsin gene tree and high bootstrap support for the clade joining *Heliconius UVRh1* and *UVRh2*, together with the sequence data mentioned above, imply that seven close *Heliconius* relatives—in the genera *Agraulis*, *Dryas*, *Dione*, and *Eueides*—lack the duplicate UV opsin (fig. 3).

Predictions from Models of Color Vision

Although yellow pigmentation from species within the genus *Heliconius* and close relatives outside the genus look similar to humans, the reflectance spectra differ in shape (fig. 1*A*, 1*B*; app. B). Thus, a question arises as to whether they appear similar to the eyes of animals whose visual pigments differ from ours, such as birds. Strikingly, for the task of separating *Heliconius* yellows from those of species in closely related genera, the best performing model di-, tri-, and tetrachromatic photoreceptor combinations were those of *H. erato* (fig. 4), and all involve the two UV photopigments (table 1). The linear discriminant analysis error rates for birds were four to six times higher than for *H. erato* using its four photopigments in a tetrachromatic mechanism (error rates: chicken, 0.119; blue tit, 0.081; *Heliconius*, 0.019; fig. 4*E*–4*G*), while the predicted error rate for the *Dryas iulia* trichromatic eye was 0.181 (fig. 4*C*). Achromatic (or luminance) mechanisms would be unable to discriminate 3-OHK from other yellow wing pigmentation: the *Heliconius* long wavelength (green) signal error rate exceeded 0.50 (fig. 4*A*), while the avian double cone (Osorio and Vorobyev 2008) error rates were 0.48–0.50. Among possible dichromatic signals based on hypothetical opponent interactions between pairs of receptors for *H. erato* (table 1), the best by far was that comparing UV versus violet receptor signals, which had a predicted error rate of 0.078 and hence matched avian tetrachromacy (fig. 4*B*). The best *H. erato* trichromatic mechanism (UV, violet, blue) had an even better predicted error rate of 0.066 (fig. 4*D*), while as noted above, the error rate of 0.019 for the fully tetrachromatic *H. erato* eye outperformed them all.
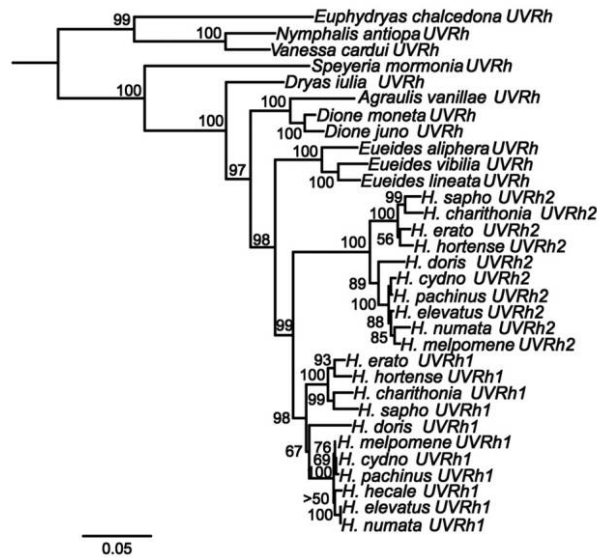
Figure 3: Gene tree based on maximum likelihood analysis of UV opsin nucleotide sequences. The topology and bootstrap values were generated in RAxML, using an alignment consisting of 1,137 base pairs. The branching pattern of the UV opsin gene tree indicates that the duplication of the UV opsin occurred at the base of the genus *Heliconius*.

Discussion

Heliconius *Replaced Other Yellow Wing Pigments with 3-OHK*

This study traces in detail the phylogenetic origin of a butterfly wing pigment. A body of biochemical work that peaked in the mid-twentieth century describes numerous butterfly wing pigments with varying rigor (Nijhout 1991). This literature shows a broad trend for butterfly families to specialize in different pigment classes: papiliochromes in Papilionidae, pterins in Pieridae, and ommochromes in Nymphalidae (summarized in Nijhout 1991). Generally speaking, there is a paucity of comparative data illustrating the phylogenetic distribution of any specific wing pigment.

It has long been suspected that the yellow wing pigment of *Heliconius erato* is 3-hydroxy-L-kynurenine (3-OHK; Tokuyama et al. 1967). Earlier studies stated, without showing experimental evidence or even the names of the species investigated, that this pigment occurs throughout the genus, while close relatives use non-3-OHK yellow pigments (Brown 1967, 1981). For *H. erato* and *Heliconius melpomene*, we previously confirmed that the pigment is indeed 3-OHK and that this pigment is chemically distinct from yellow wing pigments from two species in the sister genus *Eueides* (Gilbert et al. 1988; Koch 1993; Reed et al. 2008; Briscoe et al. 2010*a*).

Wing reflectance spectra of nearly all species included in recent phylogenies of the tribe Heliconiini ($n = 49$ taxa; Beltran et al. 2007; Mallet et al. 2007) together with mass spectrometry of the yellow wing pigments of 20 species now verify the presence of 3-OHK throughout the genus *Heliconius* (figs. 1, 2, A1*A*–A1*L*). This uniformity contrasts with the chemical variation in yellow pigments found in related genera (fig. A1*M*–A1*P*; fig. S4*D*–S4*F* in Briscoe et al. 2010*a*) and is indicative of a developmental constraint or purifying selection in *Heliconius*.

Interpretation of Models of Color Vision

Color vision is among the most tractable of sensory mechanisms to model because it depends on a small number of spectral receptor signals that are encoded by photoreceptor excitations, which can be calculated for a given spectral stimulus (Kelber et al. 2003). Photoreceptor signals specify the sensory information that is available to the brain. Theory suggests that animals should make optimal use of receptor signals in behavioral tasks; if they do not, natural selection would simply favor a more economical eye, in terms of the number of receptors and neural processing (Snyder et al. 1986; Laughlin 2001). For example, one might expect fewer spectral receptors for color vision. Thus, for birds, insects, and other animals, the psychophysical color discrimination threshold is related directly to photoreceptor signals and photoreceptor noise. Noise is uncertainty in these signals caused by random fluctuations in the number of light quanta absorbed and other physiological mechanisms (Vorobyev et al. 2001; Kelber et al. 2003; Goldsmith and Butler 2005).

The fundamental finding from our color modeling is that the acquisition of two separate UV rhodopsins offers *Heliconius* butterflies the potential to discriminate 3-OHK from non-3-OHK yellow wing pigments much more effectively than butterflies in related genera or birds that lack the duplication. This is consistent with 3-OHK pigmentation and with the UV opsin gene duplicating to form a complex trait adapted for intraspecific communication. The conclusion is strikingly illustrated by the fact that— if we consider only the information available from the photoreceptors—a simple dichromatic signal comparing the outputs of two UV receptors would equal or outperform avian tetrachromacy in distinguishing the two types of yellow. The model does assume that animals have a physiological mechanism for distinguishing between classes of color that is approximated by a linear discriminator, for example, that a parameter such as the ratio of receptor excitations falls above or below a certain threshold (Scherer and Kolb 1987; Giurfa et al. 1995; Regan et al. 2001).

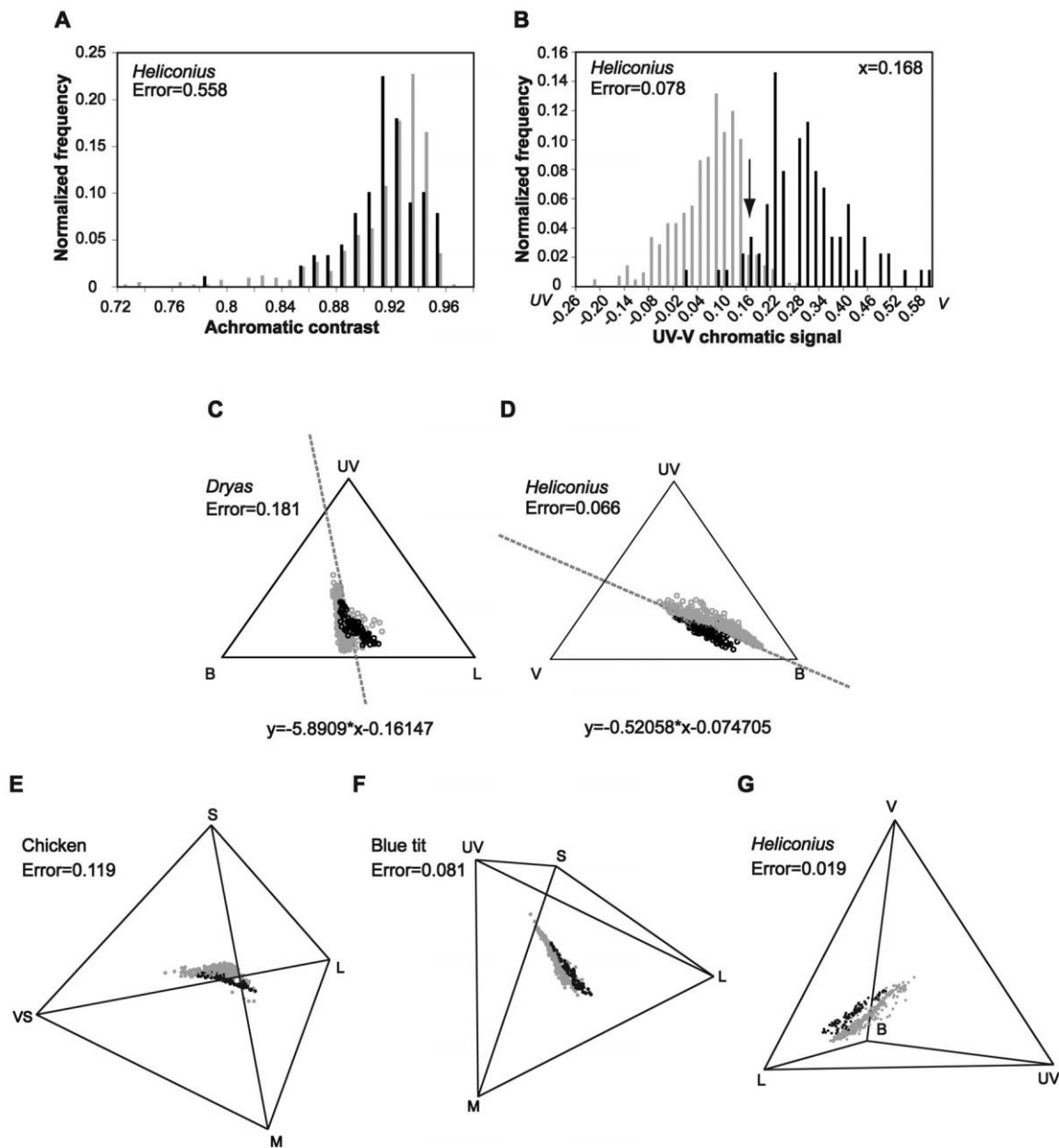Unlike Briscoe et al. (2010*a*), our model does not con-

Figure 4: Locations of yellow wing colors in chromaticity diagrams based on receptor signals for butterflies and birds. *A,* Achromatic contrast of yellow wing colors of species in the genus *Heliconius* (gray) and species in related genera (black) modeled using the *Heliconius erato* long wavelength (green) receptor calculated using equation (2). *B,* Dichromatic plot corresponding to the best pair of *H. erato* photoreceptors for classifying yellow colors, namely, UV and violet, calculated using equation (3). Trichromatic plots for the three receptors in the *Dryas iulia* eye (*C*) and for the best possible triplet of *H. erato* receptors (*D*). Tetrachromatic tetrahedral plots for different bird species' receptors (chicken *Gallus gallus,* blue tit *Parus caeruleus; E, F*) and for *H. erato* (*G*) if all four receptors are used in vision; the model uses formulas from Kelber et al. 2003 (see "Material and Methods"). Gray circles represent coordinates—(*x, y*) for trichromatic plots, (*x, y, z*) for tetrachromatic plots for individual *Heliconius* yellow spectra—and black circles represent *Heliconius* outgroup spectra. For *C* and *D,* a two-dimensional linear discriminant analysis (LDA) was run, and for *E–G,* a three-dimensional LDA was run in order to classify yellows from species in the genus *Heliconius* compared with species in related genera. The discriminant line is shown for the color triangle (*C, D*), while the tetrahedra are rotated so that the plane that best separates the two types of pigmentation is in view. Errors indicate the likelihood of misclassifying any given point in the plot when using the line or plane produced by the LDA as a guideline.

Table 1: Error rates of discriminating 3-hydroxy-L-kynurenine (3-OHK) yellows from non-3-OHK yellows

Color space and species	Receptor combination	Error rate
Achromatic contrast:		
Dryas	L	.558
Heliconius	L	.558
Blue tit	Double cones	.501
Chicken	Double cones	.480
Dichromatic:		
Dryas	UV, B	.459
Dryas	UV, L	.483
Dryas	B, L	.209
Heliconius	UV, L	.365
Heliconius	V, L	.456
Heliconius	B, L	.209
Heliconius	UV, B	.404
Heliconius	V, B	.373
Heliconius	UV, V	.078
Trichromatic:		
Dryas	UV, B, L	.188
Heliconius	UV, B, L	.211
Heliconius	V, B, L	.136
Heliconius	UV, V, L	.075
Heliconius	UV, V, B	.066
Tetrachromatic:		
Blue tit	UV, S, M, L	.081
Chicken	VS, S, M, L	.119
Heliconius	UV, V, B, L	.019

Note: B, blue; L, long; M, medium; S, short; UV, ultraviolet; V or VS, violet.

sider the effects of photoreceptor noise (Vorobyev and Osorio 1998) and hence makes no assumption about the noise levels in each receptor mechanism. Noise, or uncertainty, in receptor signals due to receptor density, photon catch, and other factors limits discrimination thresholds. It is an essential factor in any model that seeks to estimate the number of discriminable colors or the likelihood that any two colors from a given population of stimuli will be discriminable from each other (Vorobyev and Osorio 1998; Vorobyev et al. 2001). Were noise to be incorporated into the models used in this study, the effect would be to blur the boundary between the two types of color, possibly degrading discrimination of colors close to the boundary. In this context, it is noteworthy that for the *Heliconius* tetrachromatic eye model (fig. 4*G*), a clear space separates the two types of yellow. This means that few colors lie at the boundary so that degradation of performance due to receptor noise will be negligible. By comparison, the absence of a clear separation of the two types of yellow in avian color space means that the classification would be degraded by receptor noise.

The model—in the absence of experimental evidence—does not prove that birds are worse than *Heliconius* at discriminating *Heliconius* species from other genera. The existence of mimetic butterflies found in the same geographical location whose wings deploy either 3-OHK or other yellow pigmentation suggests, however, that in natural conditions the mimicry between UV-yellow and yellow coloration is effective for birds. Indeed, a common assumption in the application of signal detection theory to mimicry is that predators' perceptual recognition is error prone (Speed and Ruxton 2010). Our prediction of higher avian error rates in classifying *Heliconius* yellow colors from those of closely related genera lends theoretical support to this assumption and shows how errors may originate with the photoreceptors of predators themselves, as opposed to high-level neural mechanisms (table 1).

Our model does of course assume that *Heliconius* species can compare the outputs of the UV and violet receptors. There is no direct evidence for such a mechanism yet, but there is clear evidence that in *H. erato* the two UV rhodopsins are present in separate photoreceptors in the same region of the eye and in sufficient abundance for color vision (A. D. Briscoe, unpublished data). In addition, while more work is needed, prior intracellular, electroretinographic, and retinal densitometric results are consistent with the presence of two distinct UV receptors in *H. erato* and *Heliconius numata* eyes (Struwe 1972*a*, 1972*b*; Briscoe et al. 2010*a*). Last, while butterfly receptor sensitivities are often modified by filtering or screening pigments, any such filtering would be unlikely to affect the main conclusions above (see, e.g., the measured spectral sensitivity curves of the monarch butterfly compared with the unmodified photoreceptor absorbance spectra shown in fig. 1 of Blackiston et al. 2011); that is, they would be most unlikely to substantially reduce the great advantage of having two UV receptors for the task in question.

We note that the spectral sensitivities of the photoreceptor cells of actual *Heliconius* predators are unknown. Nonetheless, our conclusions are likely to be highly robust to minor differences in spectral sensitivity between actual predators and the bird species modeled here, unless *Heliconius* predators have an eye that is unlike any presently known bird species. Put simply, we predict that an eye adapted to discriminate the two types of yellow would have two short-wavelength spectral receptors, much like *Heliconius*. Discovery of a specialized *Heliconius* predator with multiple photoreceptors in the UV/violet part of the spectrum would provide strong evidence for the correlated evolution of a prey signal and a predator's color vision. So far, no bird is known to have such a visual system. There is evidence, however, that the bobolink *Dolichonyx oryzivorous* has a 403-nm pigment in the accessory member of its double cones—along with the usual long-wavelength pigment in the primary member—in addition to

the typical 372-nm single cone receptor (Beason and Loew 2008).

Color Vision and Communication

This study provides an example of how a color signal can be tailored into separate and otherwise incompatible roles according to a receiver's ability to discriminate and classify mimetic colors (fig. 4) and suggests roles for both natural and sexual selection in shaping the evolution of a color signal. Our results can be compared with the proposal by Håstad et al. (2005), which states that because of differences in the spectral sensitivity of the UV/violet photoreceptor in birds, UV-reflecting colors of passerine birds are relatively inconspicuous to raptors and hence cryptic compared with conspecifics, thus providing a kind of private UV communication channel (Cummings et al. 2003).

To our knowledge, the co-occurrence of 3-OHK pigments and the duplication of UV rhodopsins in *Heliconius* is the clearest evidence yet for correlated evolution of color vision and a color signal as a complex trait for biological communication. This stands against much evidence accumulated over the past 2 decades that within major terrestrial groups that have good color vision—such as birds, hymenopteran insects, and catarrhine primates—there is little variation in the spectral sensitivities of photoreceptors in relation to behavioral ecology (Briscoe and Chittka 2001; Frentiu and Briscoe 2008; Osorio and Vorobyev 2008). As Allen (1879) proposed, there is little evidence for adaptation of color vision in response to specific color signals, especially sexual signals (Briscoe and Chittka 2001; Osorio and Vorobyev 2008). Even in fish, such as East African Great Lakes cichlids, where there is considerable diversity and rapid evolutionary change in photoreceptor spectral sensitivities, variation can be attributed to general environmental factors such as the illumination spectrum, with the fishes' coloration then evolving in response to changes in visual ability (Seehausen et al. 2008).

It may well be that butterflies will offer further examples of correlated evolution of color signals and color vision. They have marked evolutionary diversity of photoreceptor spectral sensitivities, which is achieved by visual pigment gene duplication, spectral tuning, and filtering pigments (Arikawa et al. 2005; Briscoe and Bernard 2005; Frentiu et al. 2007*a*; Sison-Mangus et al. 2008). The reason why butterflies should differ from other taxa in this way is unclear. It is reasonable to speculate that there is a relationship between color vision and specific types of color signals, but so far evidence is very limited. One example is Arikawa et al.'s (2005) finding that in the butterfly *Pieris rapae*, males of the Japanese race (*Pieris rapae crucivora*) have sexually dimorphic eyes due to the presence of a UV-absorbing photostable filter pigment and sexually dimor-

phic UV wing coloration, perhaps allowing the males to discriminate between the sexes. By comparison, the European race (*Pieris rapae rapae*) lacks sexual dimorphism in both coloration and eye filter pigments. Another recent study found that *P. rapae* males that were more conspicuous to female eyes were more preferred by females but also more conspicuous to avian predator eyes, highlighting the trade-offs between natural and sexual selection (Morehouse and Rutowski 2010).

Conclusion

This study shows how detailed phylogenetic examination of wing pigmentation and visual pigments combined with modeling of color signals can inform our understanding of the evolution of visual communication in a closely related group of butterfly species. Our previous findings that one of the UV opsin genes of several *Heliconius* species had evolved under positive selection suggested that color signaling and vision may have evolved in a correlated manner in *Heliconius* species as a suite of adaptations to facilitate communication specifically within and between conspecifics (Briscoe et al. 2010*a*). Our greatly increased evidence for the presence of the 3-OHK yellow pigment as well as the presence of the duplicate UV opsin gene in divergent *Heliconius* lineages clearly support this scenario. More importantly, our newly presented modeling of avian and butterfly color space shows that full avian tetrachromacy—that is, using the four photoreceptor types for color vision—is not as effective as simply having two UV-sensitive photoreceptors when the task is to discriminate UV-yellow from other yellows. Our results predict that if there were a specialized *Heliconius* predator adapted to discriminating between mimics that differ in the display of these yellow colors, its eyes would contain two UV-sensitive photoreceptors, similar to *Heliconius* itself.

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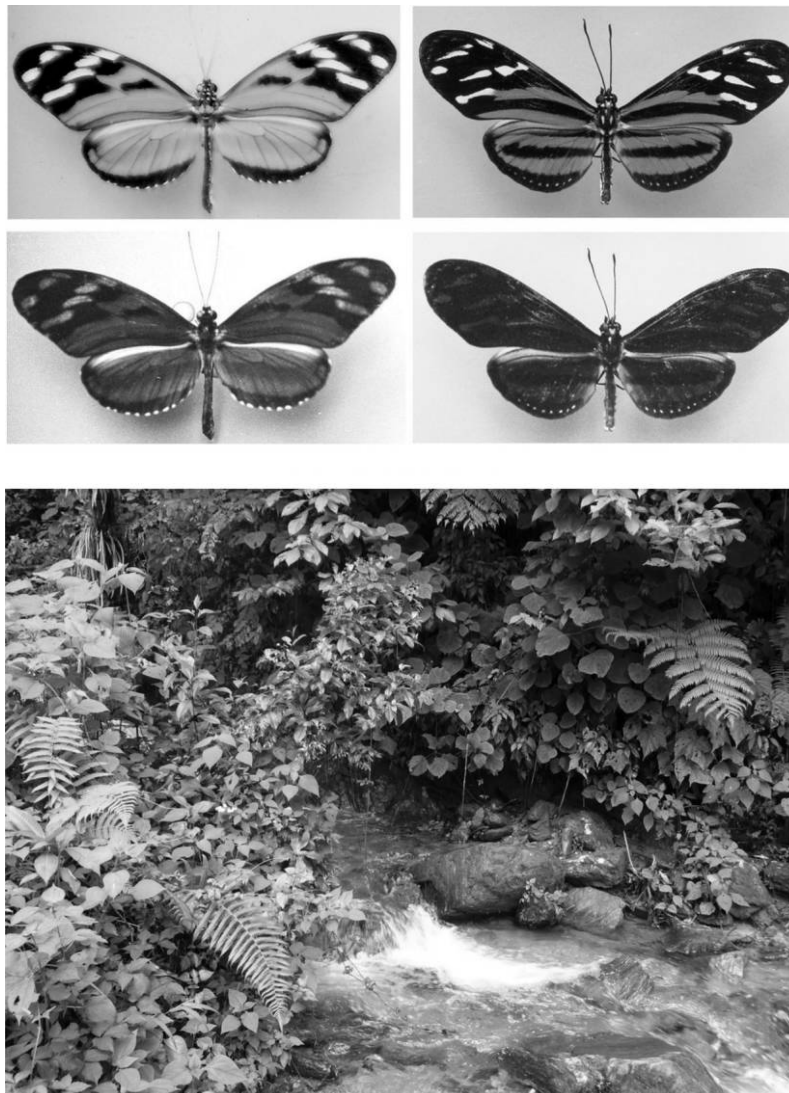
Top, mimetic butterflies photographed in daylight (*first row*) and through an ultraviolet filter (*second row*). *Heliconius* (*first column*) are colorful in the ultraviolet, while those of mimetic *Eueides* (*second column*) are colorful only in the visible light. *Bottom*, cloud forest habitat for mimetic butterflies in Oaxaca, Mexico. Photographs by Adriana Briscoe/UCI.