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Journal

Journal of Experimental Biology, 224(18)

ISSN

0022-0949

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Publication Date

2021-09-15

DOI

10.1242/jeb.242802

Copyright Information

Peer reviewed

RESEARCH ARTICLE

True UV color vision in a female butterfly with two UV opsins

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ABSTRACT

In true color vision, animals discriminate between light wavelengths, regardless of intensity, using at least two photoreceptors with different spectral sensitivity peaks. *Heliconius* butterflies have duplicate UV opsin genes, which encode ultraviolet and violet photoreceptors, respectively. In *Heliconius erato*, only females express the ultraviolet photoreceptor, suggesting females (but not males) can discriminate between UV wavelengths. We tested the ability of *H. erato*, and two species lacking the violet receptor, *Heliconius melpomene* and *Eueides isabella*, to discriminate between 380 and 390 nm, and between 400 and 436 nm, after being trained to associate each stimulus with a sugar reward. We found that only *H. erato* females have color vision in the UV range. Across species, both sexes show color vision in the blue range. Models of *H. erato* color vision suggest that females have an advantage over males in discriminating the inner UV-yellow corollas of *Psiguria* flowers from their outer orange petals. Moreover, previous models (McCulloch et al., 2017) suggested that *H. erato* males have an advantage over females in discriminating *Heliconius* 3-hydroxykynurenine (3-OHK) yellow wing coloration from non-3-OHK yellow wing coloration found in other heliconiines. These results provide some of the first behavioral evidence for female *H. erato* UV color discrimination in the context of foraging, lending support to the hypothesis (Briscoe et al., 2010) that the duplicated UV opsin genes function together in UV color vision. Taken together, the sexually dimorphic visual system of *H. erato* appears to have been shaped by both sexual selection and sex-specific natural selection.

KEY WORDS: Visual system, Wavelength discrimination, Ultraviolet, Insect vision, Behavior

INTRODUCTION

Color vision in animals is characterized by wavelength discrimination based on the spectral composition of the stimuli, independent of intensity (Kelber and Pfaff, 1999). Animals that have true color vision must use at least two types of photoreceptor, with different spectral sensitivities, to successfully discriminate between wavelengths where their sensitivity curves overlap. Insects use color vision for multiple tasks including foraging (Spaethe et al., 2001; Muth et al., 2015), host plant detection (Scherer and Kolb, 1987) and conspecific recognition (Kemp and Rutowski, 2011). Most insects have at least one ultraviolet, one blue and one green photoreceptor, but many insects lack red receptors (Briscoe and Chittka, 2001) and some have lost their blue receptors (Sharkey

et al., 2017). Numerous butterflies, however, have visual systems with more than three photoreceptor classes (van der Kooi et al., 2021).

While butterflies typically have only one kind of UV opsin (Briscoe et al., 2003; Koshitaka et al., 2008), and variable numbers of blue and green opsins, *Heliconius* have single-copy blue and green opsins and duplicated UV opsins (Briscoe et al., 2010). The two UV opsin-encoded photoreceptors of *H. erato* have peak sensitivities or λ_{max} values at 355 and 390 nm as measured by intracellular recordings (McCulloch et al., 2016a,b). Although the gene encoding UVRh2, which together with the chromophore produces a violet receptor, is present throughout the genus, the UVRh2 protein, is only expressed at detectable levels in the eye in certain *Heliconius* clades (specifically *doris*, *sara*, *charithonia* and *erato* clades) (McCulloch et al., 2017). In *H. erato*, besides the BRh and LWRh opsins, adult females express both UVRh1 and UVRh2 opsins but males only express the violet opsin, UVRh2, with sensitivity at 390 nm (McCulloch et al., 2017).

Heliconius butterflies also express a specific pigment, 3-hydroxy-DL-kynurenine (3-OHK), genus-wide in the yellow scales of their wings (Brown, 1967). Together, the pigment and the wing ultrastructure reflect UV light in the 300–400 nm range and have a distinctive step-like reflectance starting about 440 nm. This wing pigment has evolved in *Heliconius* along with their duplicated UV opsins (Briscoe et al., 2010), and close relatives to this genus lack both the opsin duplication and the 3-OHK wing pigment (Yuan et al., 2010). It has been proposed that the second UV opsin might allow for better discrimination of yellow-winged *Heliconius* conspecifics from yellow-winged non-*Heliconius* mimics (Bybee et al., 2012); recent experiments lend some support to this hypothesis (Finkbeiner et al., 2017; Dell'Aglio et al., 2018) but more behavioral experiments examining the functional significance of the duplicate UV opsins are needed.

In *Heliconius* or passion-vine butterflies, adults have large heads relative to body mass (compared with other butterflies) with notable investment in the visual neuropile (Jiggins, 2017), implying selective pressures for increased visual function. *Heliconius* vision has been investigated using a variety of broad and narrow-band stimuli such as colored paper flowers (Crane, 1955), narrow-spectrum color fibers (Swihart, 1972) and narrow band interference filters (Swihart, 1967; Zaccardi et al., 2006). Available evidence demonstrates that *Heliconius* have true color vision in the long wavelength range (590–640 nm) (Zaccardi et al., 2006), but so far, investigations in the short wavelength range have been limited.

Here, we test whether *H. erato* is capable of discriminating between narrow band wavelengths within the UV range in the context of foraging. We use male and female *H. erato* butterflies, and as controls, male and female *Heliconius melpomene* and *Eueides isabella* butterflies. Both *H. melpomene* and *E. isabella* lack a second UV opsin protein expressed in the eye but for different reasons: protein expression of UVRh2 was lost in *H. melpomene*

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(McCulloch et al., 2017) and *E. isabella* – a closely-related outgroup – never evolved a second UV opsin (Yuan et al., 2010). By confirming UV color discrimination in female *H. erato* butterflies, and ruling it out in both sexes of *H. melpomene* and *E. isabella*, we demonstrate the functional significance of UV opsin duplication in *Heliconius*.

MATERIALS AND METHODS
Animals
Heliconius erato (Linnaeus 1758), *Heliconius melpomene* (Linnaeus 1758) and *Eueides isabella* (Stoll 1781) butterflies were purchased as pupae from the Costa Rica Entomological Supply (La Guácima, Costa Rica). The pupae were kept in a humidified chamber until they eclosed, then they were sexed and marked with a unique number. The butterflies were fed using a 10% honey solution with one bee pollen granule dissolved per 2 ml of solution. Butterflies were only allowed to feed on the positive stimulus during the training and testing. A total of 362 butterflies were used in the study, of which 200 were successfully trained and used in complete trials: 80 *H. erato* (40 females, 40 males), 80 *H. melpomene* (40 females, 40 males) and 40 *E. isabella* (20 females, 20 males).

Behavioral experiments and apparatus
The experiments and training took place indoors in a mesh enclosure constructed from PVC pipes, measuring 1 m×75 cm×75 cm, and the room temperature was 24°C. The top of the enclosure was lined with 8 fluorescent tubes (Philips TLD 965 18 W; Eindhoven, The Netherlands). Spectra for these illuminating lights have been previously published (see fig. 1B of Nahon et al., 2010). Our apparatus for training and experiments was based on a design described in Zaccardi et al. (2006) and has been used to test color vision in the monarch butterfly (Blackiston et al., 2011; see also Swihart and Swihart, 1970; Weiss and Papaj, 2003; Takeuchi et al., 2006; Rodrigues et al., 2010; Kinoshita and Arikawa, 2014; and Drewniak et al., 2020 for other apparatus used in butterfly visual learning). It consists of two 2.5 cm diameter stimuli presented side by side, separated by 6 cm on two black platforms set on a larger black plate, measuring 20×10 cm (see Fig. 2 and Movie 1). The apparatus was positioned vertically at the far end of the enclosure. Two wavelength stimuli were presented to the butterflies at a time. Light was emitted from two KL2500 Schott cold light sources (Mainx, Germany) into light guides held stable with a light guide holder. The light from each guide passed through a diffusor, a 10 nm narrow band-pass filter, and then through a transparent sapphire glass feeder disk (Edmund Optics; Barrington, NJ, USA) (see fig. 3 in Zaccardi et al., 2006 for a diagram). For our experiments, we used four narrow band-pass filters in paired choice tests: 380 nm versus 390 nm and 400 nm versus 436 nm. We use 380 and 390 nm as the UV stimuli because the sensitivity curves of the two UV photoreceptors overlap in this range (McCulloch et al., 2016a) (Fig. 1). If the butterflies have UV color discrimination using the UV and the violet photoreceptors together then we would expect that they would be able to discriminate between these two wavelengths. We also chose 400 nm and 436 nm as a control for color vision in all three species using the UV (or violet) and blue photoreceptors in combination. The light intensities for each wavelength were adjusted so that between these four wavelengths of light, the intensities for the experiments ranged from 9.56×10^{15} to 1.71×10^{17} quanta s^{-1} steradian^{-1} cm^{-2}. Irradiance spectra of the filtered lights under each of the intensity ratios are given in Fig. S1.
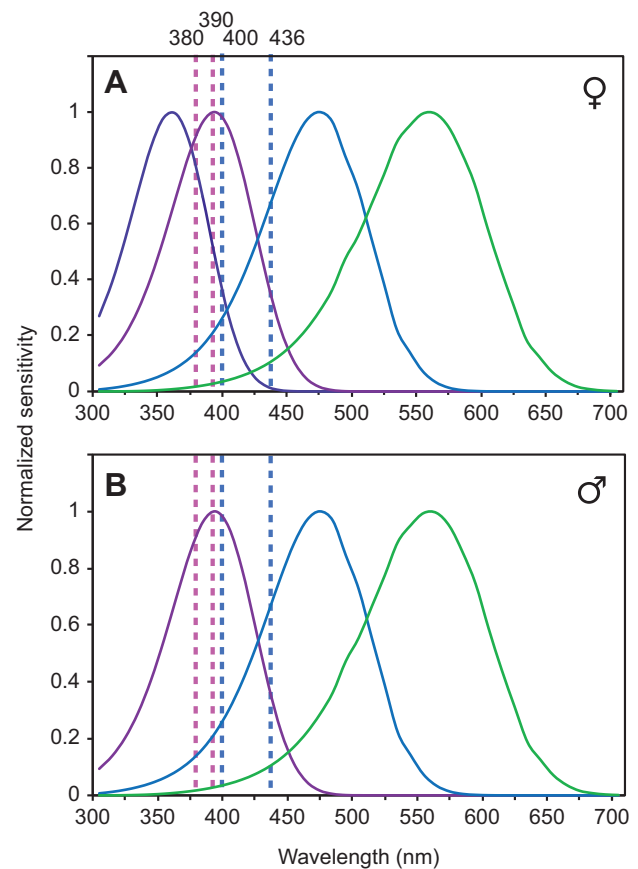

Fig. 1. Normalized spectral sensitivities of *Heliconius erato* photoreceptors. Adult (A) female and (B) male *H. erato* photoreceptor spectral sensitivities based on recorded intracellular spectral sensitivity maxima (McCulloch et al., 2016a,b). The UV photoreceptor (dark purple), encoded by *UVRh1*, has a peak sensitivity at 355 nm, the violet photoreceptor (light purple), encoded by *UVRh2*, has a peak sensitivity at 390 nm, the blue photoreceptor (blue), encoded by *BRh* has a peak sensitivity of 470 nm and the green photoreceptor (green), encoded by *LWRh*, has a peak sensitivity of 555 nm. A fifth receptor class, with a peak at ~600 nm due to filtering of the green rhodopsin by a red filtering pigment is not shown. Dotted lines represent the wavelength of peak transmission of the narrow bandpass fibers, 380 nm, 390 nm, 400 nm and 436 nm, used in color discrimination tests. Male *H. erato* (B) lacking the UV photoreceptor (dark purple) are unable to discriminate between 380 and 390 nm light. *Eueides isabella* express mRNA for only one UV opsin in their eyes (encoding a receptor of unknown peak sensitivity) while *H. melpomene* lack UVRh2 (light purple) opsin protein in their eyes entirely. Both species have a blue and a green receptor.

Butterfly training
Butterflies were trained and fed for the first time within 15 h of eclosion. Before training, they were allowed to acclimate to the experimental cage for up to 1 min, and only one butterfly was trained at a time. A droplet of food was placed in a small trough attached to the front of the feeder disk for the rewarded stimulus (+). The unrewarded stimulus (−) feeder trough remained empty. Each butterfly was trained by having its wings held together with forceps, and then slowly moved from the rear of the enclosure toward the apparatus to simulate a flying motion. The butterfly was then slowly waved in front of both the rewarded and unrewarded stimuli, and finally held in front of the rewarded stimulus where its proboscis was uncoiled with an insect pin until it came into contact with the food solution. At this point the butterfly would begin to drink. After the proboscis was manually uncoiled 2–3 times, the butterfly was
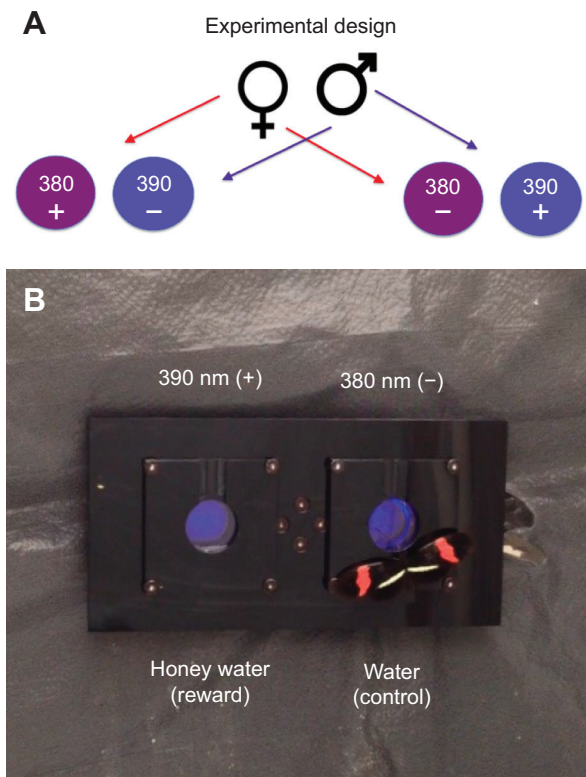
Fig. 2. Experimental design of behavioral trials and experimental apparatus. (A) Female and male butterflies of three species, *Heliconius erato*, *H. melpomene* and *Eueides isabella* were reciprocally trained to associate honey water with a rewarded UV light (+) and tested using an apparatus (B) consisting of a rewarded light (+) and an unrewarded light (−). Butterflies were trained and tested on their ability to discriminate 380 nm (right) from 390 nm (left) and 400 nm from 436 nm lights (not shown). Shown is a male *H. erato* butterfly that has just landed on the light source apparatus during a trial.

able to uncoil the proboscis on its own in response to the stimulus. The procedure of carrying the butterfly with forceps from the rear of the cage to the light sources to feed was repeated 5 times per training session, with two training sessions per day separated by approximately 6 h. Each time the butterfly fed from the rewarded stimulus, it was allowed to drink for 10 s, except for the very last training segment of the day where it was allowed to drink for several minutes. During training and between training sessions, the placement of the rewarded and unrewarded stimuli was randomly switched so that the butterfly did not learn to associate the left or right light with a food source. The apparatus was also cleaned thoroughly after each training session to minimize the association of chemical cues to the stimulus. After about 4–5 days of training, butterflies were capable of independently flying toward the apparatus when released from the rear of the cage and making a choice to fly to one of the two light stimuli (Movie 1). At this point, the trained butterflies were starved for 20–24 h then their choice trials began.

Experimental trials
A separate cohort of butterflies was trained with each wavelength pair because the butterflies did not survive long enough to be trained multiple times. Both sexes of each species were first trained to 390 nm (+) (both 390 nm and 380 nm lights were on during training at 1:1 intensity but only 390 nm light was rewarded), and then tested for UV discrimination ability between 390 nm (+) and 380 nm (−)

(10 per sex for *H. erato* and *H. melpomene* and 5 per sex for *E. isabella*). The same number of individuals was trained to 380 nm (+) and given the choice between the two UV stimuli. Two new cohorts of butterflies were used for reciprocal training to 400 nm and to 436 nm. Three different approximate ratios of the peak physical intensities or absolute brightnesses of the rewarded/unrewarded stimuli were used: 0.067, 1.0 and 15.0 (or 1:15, 1:1, and 15:1). The calculated ratios are 0.062, 1.0, 16.213 for 380 versus 390 nm; and 0.0635, 1.0, 15.741 for 400 versus 436 nm. These intensity ratios are described throughout the rest of this study as 1:15, 1:1, and 15:1, i.e. the rewarded stimulus (+) at 15 times less bright than the unrewarded stimulus (−), equal intensities for both stimuli, and the rewarded stimulus (+) at 15 times brighter than unrewarded stimulus (−). Butterflies first completed trials at an intensity combination of 1:1 (15 choices each). Following this test they were given random choices between intensities of 1:15 or 15:1 (rewarded:unrewarded) until they had completed 15 choices with each intensity combination.

The number of correct versus incorrect choices each butterfly made at different intensity combinations was modeled as dependent upon wavelength using general linear models in R statistical software (https://www.r-project.org/). We compared the ability of each category of butterfly to discriminate between the wavelength combinations at the different intensities. We also examined how discrimination abilities differed between all three butterfly species used in the study.

Reflectance spectrometry
Live tissue was collected by accessing the butterfly and plant collection of Dr Lawrence Gilbert at the Brackenridge Field Laboratory at the University of Texas, Austin on 20 July 2010. Reflectance spectra of *Heliconius erato petiverana* eggs, *Passiflora biflora* egg mimics, *Psychotria tomentosa* yellow inflorescences, red bracts and green leaves, and *Psiguria warscewiczii* yellow and orange inflorescences and green leaves were measured by placing a probe holder (Ocean Optics RPH-1) over the specimen such that the axis of the illuminating and detecting fiber (Ocean Optics R400-7-UV/VIS) was at an elevation of 45 deg to the plane of the tissue surface. 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. Four to nine biological replicates per taxon were measured for each tissue type.

Discriminability modeling
To examine whether male or female *H. erato* eyes perform differently when viewing ecologically relevant objects, we constructed visual models. 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, we calculated discriminabilities for a trichromatic system consisting of UV2, blue and green receptors. For *H. erato* females, we calculated discriminabilities for a tetrachromatic system consisting of UV1, UV2, blue and green receptors. We excluded the red receptor from our calculations for both sexes because we do not have count data for this receptor class. 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). Endler's daylight illumination spectrum (Endler, 1993) was used in the model. *H. erato* photoreceptor spectral sensitivity curves with λ_{max}

values=355 nm (UV1) (female only), 390 nm (UV2), 470 nm (B), and 555 nm (L) from (McCulloch et al., 2016a) were used. Parameters for the butterfly visual models were as follows: Weber fraction=0.05 (Koshitaka et al., 2008) 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). Data from spectral measurements and behavioral trials are available from Dryad (https://doi.org/10.7280/D1ZD6D).

RESULTS
Ultraviolet discrimination
At the intensity of 1:1 for 390 and 380 nm light, female *H. erato* chose the rewarded light stimulus, 390 nm (+), significantly more than the unrewarded stimulus, 380 nm (−) (z-value=6.791, $P<0.0001$, Fig. 3A). This indicates the ability of female *H. erato* to distinguish between the two UV wavelengths. The females continued to choose the correct, rewarded color stimulus under varying light intensity combinations. At an intensity ratio of 1:15 (rewarded:unrewarded), females significantly chose 390 nm (+) over 380 nm (−) (z-value=5.19, $P<0.0001$); and at an intensity of 15:1 (rewarded:unrewarded), females also chose 390 nm (+) over 380 nm (−) (z-value=7.35, $P<0.0001$). There was no difference between female preference for the correct stimulus with a 1:1 and 1:15 light ratio (z-value=−0.794, $P=0.427$), or with a 1:1 and 15:1 light ratio (z-value=0.319, $P=0.749$), showing that females chose the correct light stimulus (390 nm) equally across all tested light intensity combinations.

With respect to male behavior, at the intensity of 1:1 for 390 (+) and 380 nm (−), male *H. erato* chose both the rewarded and unrewarded light stimuli equally (z-value=−0.49, $P=0.624$,

Fig. 3B). This suggests they cannot distinguish between the two UV wavelengths. However, the males significantly preferred the correct, rewarded stimulus (390 nm) (+) when it was presented 15 times brighter than the unrewarded stimulus (ratio 15:1 for rewarded:unrewarded; z-value=6.421, $P<0.0001$); and they significantly preferred the incorrect, unrewarded stimulus, 380 nm (−), at the intensity of 1:15 (rewarded:unrewarded; z-value=−6.671, $P<0.0001$). These results imply that males prefer the brighter stimulus regardless of light wavelength, and further support their inability to discriminate between 390 and 380 nm. Comparing male and female performance, females significantly prefer the correct stimulus (390 nm) (+) more than males when 390 versus 380 nm are at intensities of 1:1 (z-value=−3.427, $P=0.0006$) and at intensities of 1:15 (z-value=−6.126, $P<0.0001$), respectively. However, males and females equally chose the correct stimulus, 390 nm (+), when the rewarded:unrewarded intensity ratio was at 15:1 (z-value=−0.514, $P=0.607$, Fig. 3A,B).

With *H. melpomene* and *E. isabella*, at the intensity of 1:1 for 390 and 380 nm, both sexes had similar wavelength discrimination behavior to male *H. erato* in that they chose both the rewarded (390 nm) (+) and unrewarded (380 nm) (−) light stimuli equally (z-value=0.923, $P=0.356$ for *H. melpomene*, Fig. 3C,D; z-value=0.327, $P=0.744$ for *E. isabella*, Fig. 3E,F). They were able to significantly choose the correct stimulus (390 nm) (+) only when it was 15 times brighter than the unrewarded stimulus (z-value=−10.79, $P<0.0001$ for *H. melpomene*; z-value=6.791, $P<0.0001$ for *E. isabella*), and they chose the unrewarded stimulus (380 nm) (−) significantly more when it was 15 times brighter than the rewarded, correct stimulus (z-value=10.460, $P<0.0001$ for *H. melpomene*; z-value=−6.293, $P<0.0001$ for *E. isabella*). No
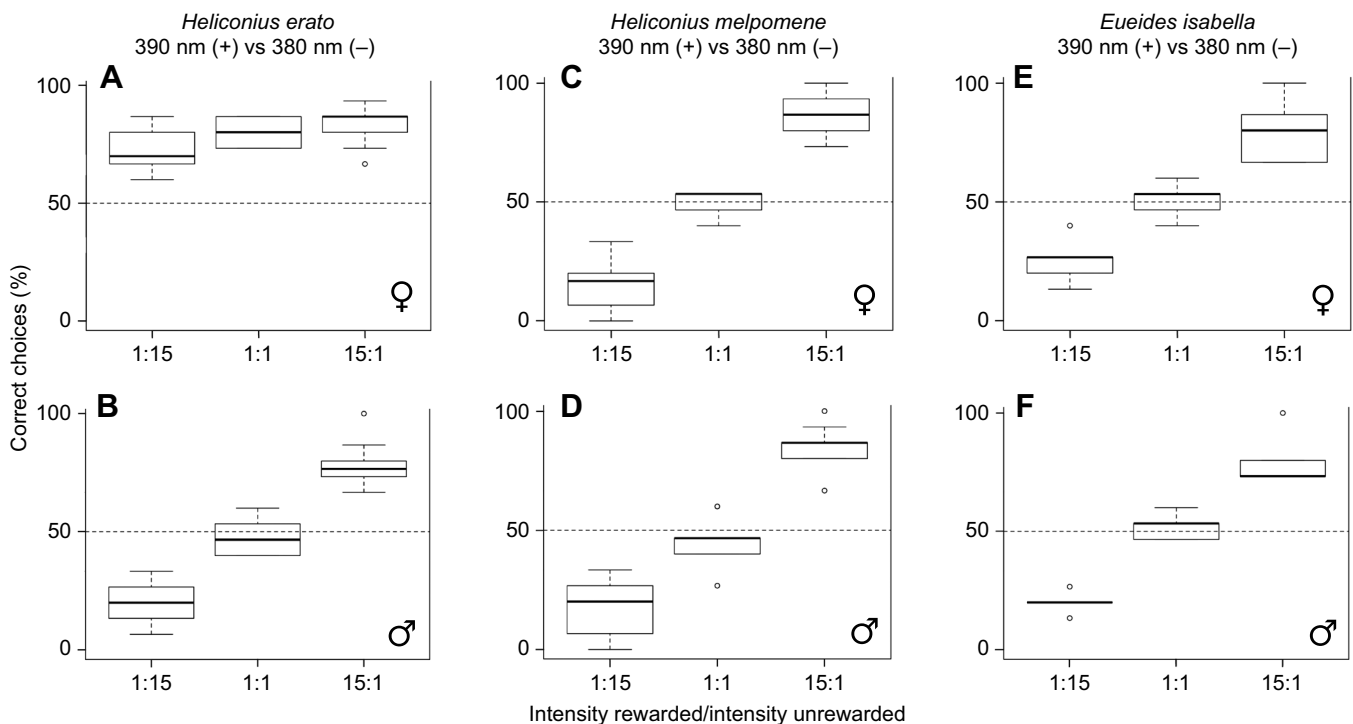

Fig. 3. Percentage of correct choices for the rewarded (+) wavelength of 390 nm by species and sex. *Heliconius erato* females (A) and males (B), *H. melpomene* females (C) and males (D), and *Eueides isabella* females (E) and males (F) when given a choice between 390 nm (+) and 380 nm (−) light under varying intensities. For *H. erato* and *H. melpomene* N=10 and for *E. isabella* N=5 biological replicates per species and sex under which 15 choice trials were completed at each light intensity combination. Non-overlapping box plots indicate where $P<0.01$ from a general linear model calculated in R. Spectra of the filtered lights under each of the intensity ratios are given in Fig. S1. Boxes represent upper and lower quartiles with median; whiskers indicate 25th and 75th percentiles.

behavioral differences between sexes of either species were detected with statistical analyses (all *P*>0.05), indicating that discrimination ability was consistent between both males and females of *H. melpomene* and *E. isabella*.

For the reciprocally rewarded tests, female *H. erato* butterflies were again consistent in discriminating between the rewarded (380 nm) (+) and unrewarded (390 nm) (−) stimuli when intensities were the same (*z*-value=−6.671, *P*<0.0001, Fig. S2A), when the rewarded stimulus was 15 times brighter (*z*-value=−7.793, *P*<0.0001), and when the rewarded stimulus was 15 times less bright (*z*-value=−5.194, *P*<0.0001). Male *H. erato* butterflies were incapable of discriminating between the different wavelengths when presented at equal intensities (*z*-value=−0.327, *P*=0.744, Fig. S2B), and chose the incorrect stimulus when it was 15 times brighter than the correct, rewarded stimulus (*z*-value=6.162, *P*<0.0001). Males did, however, choose the correct stimulus when presented at an intensity ratio of 15 times brighter than the unrewarded stimulus (*z*-value=−5.194, *P*<0.0001). Females correctly chose the rewarded stimulus (380 nm) (+) significantly more than males at intensity ratios of 1:1 (*z*-value=−2.976, *P*=0.00292) and 1:15 (*z*-value=−5.793, *P*<0.0001), but at a ratio of 15:1 male and female *H. erato* chose the correct wavelength at similar rates (*z*-value=−1.424, *P*=0.154, Fig. S2A,B).

Like male *H. erato*, *H. melpomene* and *E. isabella* could not distinguish between the two UV wavelengths presented at a 1:1 intensity ratio (*z*-value=0.462, *P*=0.644 for *H. melpomene*, Fig. S2C,D; *z*-value=0.327, *P*=0.744 for *E. isabella*, Fig. S2E,F). They significantly preferred the rewarded stimulus only when 15 times brighter (*z*-value=−11.12, *P*<0.0001 for *H. melpomene*; *z*-value=−7.024, *P*<0.0001 for *E. isabella*), and preferred the

unrewarded stimulus also only when 15 times brighter (*z*-value=7.793, *P*<0.0001 for *H. melpomene*; *z*-value=7.346, *P*<0.0001 for *E. isabella*). Male and female discrimination behavior did not differ within *H. melpomene* or *E. isabella* (*P*>0.05). In summary, female *H. erato* always discriminated between 380 and 390 nm light, consistently preferring the correct, rewarded stimulus, whereas male *H. erato*, male and female *H. melpomene*, and male and female *E. isabella* struggled with UV discrimination and only chose the correct stimulus when it was at a brighter intensity than the incorrect, unrewarded stimulus.

Blue discrimination
To investigate color vision in the blue range, we repeated the series of discrimination tests using 400 nm and 436 nm which would allow short wavelength discrimination using a UV or violet photoreceptor and a blue photoreceptor. As expected, when trained to 400 nm (+), female *H. erato* chose the correct stimulus when offered both light wavelengths at equal intensities (*z*-value=−7.93, *P*<0.0001, Fig. 4A), at an intensity of 1:15 for rewarded:unrewarded (*z*-value=−7.54, *P*<0.0001), and at an intensity of 15:1 of rewarded:unrewarded light (*z*-value=−8.099, *P*<0.0001). Male *H. erato*, male and female *H. melpomene*, and *E. isabella* behavior paralleled female discrimination behavior between the two blue wavelengths, with male *H. erato* choosing the correct wavelength at intensity combinations of 1:1 (*z*-value=−7.93, *P*<0.0001, Fig. 4B), 1:15 (*z*-value=−7.54, *P*<0.0001) and 15:1 (*z*-value=−7.987, *P*<0.0001); and *H. melpomene* and *E. isabella* males and females also choosing the correct, rewarded wavelengths at intensity ratios of 1:1 (*z*-value=−11.46, *P*<0.0001 for *H. melpomene*, Fig. 4C,D; *z*-value=−7.63, *P*<0.0001 for
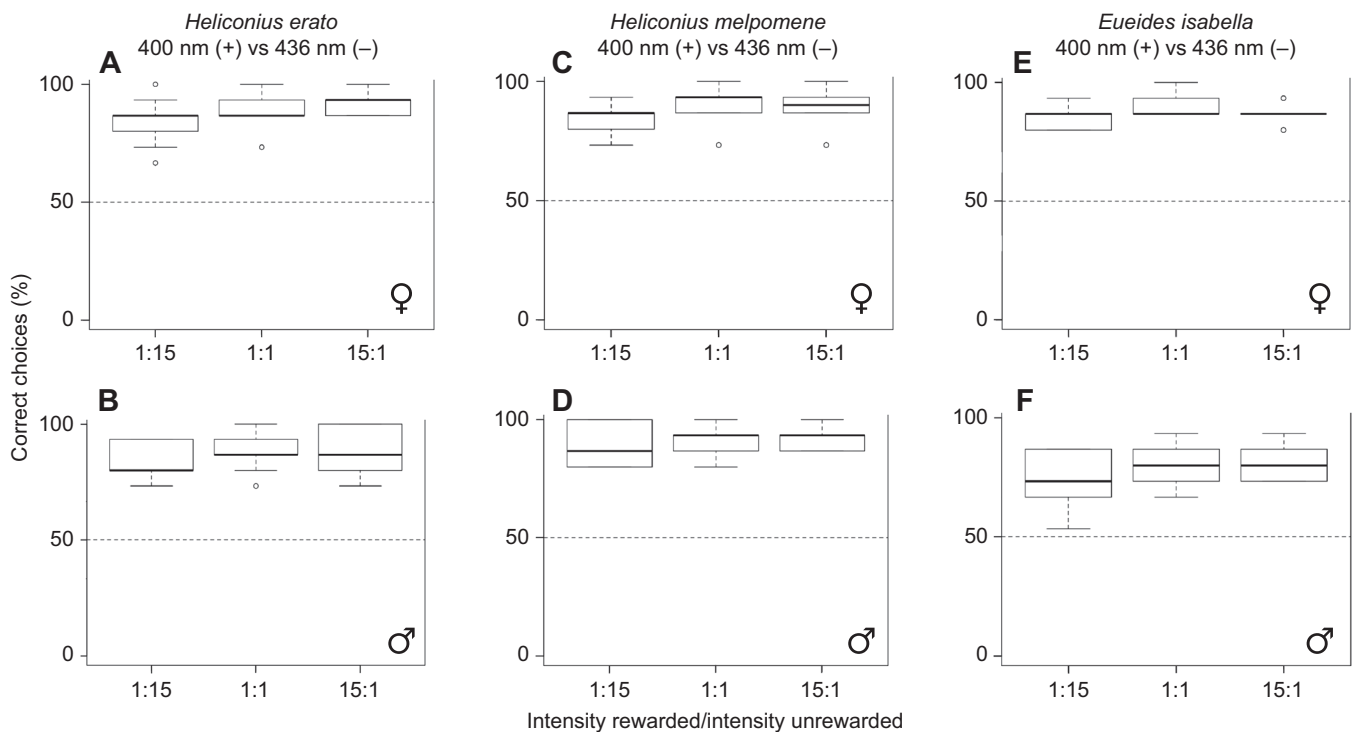

Fig. 4. Percentage of correct choices for the rewarded (+) wavelength of 400 nm by species and sex. *Heliconius erato* females (A) and males (B), *H. melpomene* females (C) and males (D), and *E. isabella* females (E) and males (F) when given a choice between 400 nm (+) and 436 nm (−) light under varying intensities. For *H. erato* and *H. melpomene* N=10 and for *E. isabella* N=5 biological replicates per species and sex under which 15 choice trials were completed at each light intensity combination. Non-overlapping box plots indicate where *P*<0.01 from a general linear model calculated in R. Spectra of the filtered lights under each of the intensity ratios are given in Fig. S1. Boxes represent upper and lower quartiles with median; whiskers indicate 25th and 75th percentiles.

E. isabella, Fig. 4E,F), 1:15 (z-value=−11.07, *P*<0.0001 for *H. melpomene*; z-value=−6.671, *P*<0.0001 for *E. isabella*), and 15:1 (z-value=−11.47, *P*<0.0001 for *H. melpomene*; z-value=−7.445, *P*<0.0001 for *E. isabella*).

When trained to 436 nm (+), all butterflies continued to show a significant preference for the correct wavelength stimulus regardless of intensity. Female and male *H. erato* preferred the rewarded stimulus at equal intensities (z-value=7.930, *P*<0.0001 for females, Fig. S3A; z-value=7.714, *P*<0.0001 for males, Fig. S3B), at an intensity combination of 1:15 (z-value=7.242, *P*<0.0001 for females; z-value=6.909, *P*<0.0001 for males) and at 15:1 (z-value=7.987, *P*<0.0001 for females; z-value=7.865, *P*<0.0001 for males). *H. melpomene* and *E. isabella* followed the same trend and significantly preferred the correct wavelength (436 nm) (+) at an intensity combination of 1:1 (z-value=−10.85, *P*<0.0001 for *H. melpomene* Fig. S3C,D; z-value=7.793, *P*<0.0001 for *E. isabella*, Fig. S3E,F), 1:15 (z-value=−9.853, *P*<0.0001 for *H. melpomene*; z-value=6.293, *P*<0.0001 for *E. isabella*) and 15:1 (z-value=−11.07, *P*<0.0001 for *H. melpomene*; *z*=7.930, *P*<0.0001 for *E. isabella*). There was no difference between *H. erato* male and female behavior, between *H. melpomene* male and female behavior, or between *H. erato*, *H. melpomene* and *E. isabella* behavior (all *P*>0.05) for selecting the correct light wavelength when trained to either 400 nm or 436 nm. All butterflies expressed the same ability to discriminate between 400 nm and 436 nm across all three intensity combinations.

DISCUSSION

In this study we used a range of relative intensities to test for UV and blue color discrimination in three species of butterfly, although we note the subjective brightnesses of the stimuli presented here are not necessarily proportional to the photon fluxes of the filtered lights. Among other variables that influence subjective brightness, we do not know the sensitivity of the eyes of each species and sex to the illuminant conditions. To control for this variable, we performed reciprocal sets of training and testing for each pair of wavelengths (Fig. 3 and Fig. S2, Fig. 4 and Fig. S3). For instance, in one set of trials the butterflies were first trained to associate a sugar reward with 390 nm (+) light and then given the choice between 390 nm (+) and 380 nm (−) light (Fig. 3). In another trial, the butterflies were trained to associate 380 nm light (+) with a sugar reward and then given the choice between 380 (+) and 390 nm (−) light (Fig. S2). We conclude that *Heliconius erato* butterflies have true color vision in the UV range, between 380 nm and 390 nm, and that this is a female-limited behavior. Our results provide behavioral evidence that these butterflies can discriminate between more than one UV color using an ultraviolet and a violet photoreceptor, which suggests that the *UVRh1* (ultraviolet) and *UVRh2* (violet) opsin genes in *H. erato* function in the context of UV color discrimination. We also show that *H. erato*, *H. melpomene* and *E. isabella* have color vision in the blue range between 400 nm and 436 nm, using a UV or violet receptor in combination with a blue receptor.

True UV color discrimination in *H. erato* females is possible because of the evolution of a violet-sensitive photoreceptor, UVRh2, which has been present since the genus originated (Briscoe et al., 2010). As noted above, some clades (e.g. *melpomene* and silvaniform) do not express the UVRh2 protein at detectable levels in the adult compound eye despite expressing the *UVRh2* mRNA, due to ongoing pseudogenization (McCulloch et al., 2017). Opsin duplication events are not uncommon in butterflies (Sison-Mangus et al., 2006; Frentiu et al., 2007; Liénard et al., 2021; Sondhi et al., 2021). For example, the lycaenid butterfly *Polyommatus icarus* uses its duplicated blue opsin to see green,

perhaps for discrimination of oviposition sites (Sison-Mangus et al., 2008). The pierid butterfly *Pieris rapae* has both a duplicated blue opsin and spectrally tuned filtering pigments: photoreceptor modifications that may be crucial for mate recognition by males (Arikawa et al., 2005; Wakakuwa et al., 2010). Yet another study has found that while both sexes of the wood tiger moth, *Arcia plantaginis* can distinguish between white and yellow male morphs (and females prefer to mate with white males), variation in female orange and red coloration is indiscriminable by both sexes, suggesting the moths' visual system has evolved to facilitate female choice (Henze et al., 2018).

In *Heliconius erato* females, duplicate UV opsin genes encoding a UV and a violet receptor, respectively, allow for UV color discrimination. The diversity of duplicated UV opsin presence or absence and spatial expression across the genus *Heliconius* is nonetheless thought-provoking (McCulloch et al., 2017). Male *H. erato* butterflies evidently use their duplicated UVRh2 (violet), blue and long wavelength opsins in the context of mate choice discrimination of 3-OHK versus non-3-OHK yellow wing colors (Finkbeiner et al., 2017), an advantage predicted by modeling the discrimination abilities of *H. erato* males in comparison with a hypothetical male *H. erato* visual system in which UVRh1 takes the place of UVRh2 (Table 1) (McCulloch et al., 2017). Moreover, the loss of UVRh2 protein expression in *H. melpomene* (whose eyes express the UVRh1 opsin) may contribute to increased attempts to mate with other species because of a reduction in visual ability to recognize conspecifics (Bybee et al., 2012; Dell'Aglio et al., 2019 preprint). *Heliconius* are part of a large mimicry complex that includes both unpalatable within-genus Müllerian mimics (which display 3-OHK yellow wing pigments) and somewhat palatable Batesian mimics such as *Eueides isabella* (which display unknown and distinct yellow wing pigments) (Srygley and Chai, 1990; Bybee et al., 2012). Consequently, male *H. erato* (but not *H. melpomene*) butterflies benefit from having the violet receptor, UVRh2, which facilitates discrimination of yellow pigments of mimics from those of conspecifics.

Early visual modeling of the *Heliconius* visual system suggested an additional benefit of the display of 3-OHK yellow pigments on the *Heliconius* wing: with a second UV opsin in their eyes, more colors can be discriminated among *Heliconius* yellows than can be discriminated among the yellows of outgroup taxa (Briscoe et al., 2010). More recent work suggests *Heliconius* species may indeed

Table 1. Percentage of pairs of *Heliconius* egg, egg mimic, pollen flower and wing colors that differ with chromatic just noticeable difference (JND) values >1 as modeled for male and female *H. erato* eyes

	N	Male (%)	Female (%)
H. erato egg vs *P. biflora* egg mimics	16	100	100
P. tomentosa yellow flowers vs red bracts	20	100	100
P. tomentosa red bracts vs green leaves	20	100	100
P. warscewiczii inner corolla yellow vs outer orange petals	81	76.5	93.8
P. warscewiczii outer orange petals vs green leaves	45	100	100
Heliconius vs *Eueides* wing dorsal yellow*	144	78.5	45.1
Heliconius vs *Eueides* wing ventral yellow*	117	87.2	84.6

Note that two systems are modeled: male and female *H. erato* eyes under high levels of illumination. The male eye includes UV2, B and L opsins, the female eye includes UV1, UV2, B and L opsins. The red receptor found in both sexes is not included in the visual modeling because its relative abundance is unknown. *From table 1 in McCulloch et al. (2017).

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be more conspicuous to conspecifics in their preferred habitats and light environment (Dell'Aglio et al., 2018, 2019 preprint).

Both *H. erato* and *H. melpomene* may interact together by forming communal roosts in the same home range, which would provide added anti-predatory benefits through a similar visual signal (Finkbeiner et al., 2012). *Heliconius* co-mimics have been observed foraging together (our personal observations) and roosting together (although uncommon; Mallet, 1986; Finkbeiner, 2014), and this could represent one instance where identifying a *Heliconius* individual (whether or not a co-species) would be beneficial. Aside from visual signals, *Heliconius* frequently use pheromone cues for conspecific recognition, especially for short-range signaling, for example during courtship behavior (Estrada and Jiggins, 2008; Darragh et al., 2017; van Schooten et al., 2020).

It is possible that the adaptive function of UV color discrimination in female *H. erato* butterflies is shaped more by host plant or pollen plant recognition than by intraspecific and interspecific communication. Within *Heliconius*, different species are specialists on *Passiflora* host plants for oviposition, and some of these *Passiflora* species contain extrafloral nectaries that resemble yellow *Heliconius* eggs (Williams and Gilbert, 1981). *Heliconius* are known to avoid ovipositing on host plants that already have eggs because larvae have cannibalistic tendencies (Brown, 1981; De Nardin and de Araújo, 2011), and fresh, new shoots that are the most edible for larvae can be of limited quantity (Gilbert, 1982). While it is possible that the egg mimic structures differ spectrally from actual eggs in their UV reflectance, thus potentially allowing the additional UV opsin to provide discrimination between natural and mimic eggs, our preliminary investigation of the reflectance spectra of *H. erato petiverana* eggs and *Passiflora biflora* egg mimics, indicates that there is little to no UV reflectance for either the eggs or the egg mimics (Fig. 5, top) (Gilbert, 1972). Moreover, visual models employing the UV (in the case of females), violet, blue and green receptors (but excluding the red receptor; see the Materials and Methods) indicate that both male and female *H. erato* visual systems are both able to discriminate *H. erato* eggs from *P. biflora* egg mimics (Table 1), and *P. biflora* egg mimics from *P. biflora* leaves but not *H. erato* eggs from *P. biflora* leaves (at least using the receptors modeled here) (Fig. 5, bottom).

There is also the possibility that the leaves of caterpillar host plants, or even the petals of adult pollen flowers (such as *Psychotria* and *Psiguria*) have unique spectral properties in the UV range that would make a second UV/violet opsin beneficial. Intriguingly, we found evidence of a UV component to the reflectance spectra of the yellow inflorescences of *Psychotria tomentosa*, a plant from which *Heliconius* prolifically collect pollen (Fig. 6, top) (Gilbert, 1972). Both male and female *H. erato* visual systems appear adept, however, at discriminating between the yellow inflorescence from the red bracts of *P. tomentosa* and at discriminating the red bracts from the green leaf (Table 1, Fig. 6, bottom). We also found that the yellow inner part of the *Psiguria warscewiczii* inflorescence has an even brighter UV component (Fig. 7, top). Notably, the female *H. erato* visual system is predicted to have an advantage over the male *H. erato* visual system in discriminating the inner yellow from the outer orange petals of *P. warscewiczii* flowers (Table 1, Fig. 7 bottom). This difference is intriguing in light of evidence that female *Heliconius charithonia* (which have similarly sexually dimorphic eyes as *H. erato*) (McCulloch et al., 2017) collect significantly more pollen than do male *H. charithonia* because of their higher protein requirements for egg production (Boggs, 1981; Boggs et al., 1981; Cardoso, 2001; Estrada and Jiggins, 2002; Mendoza-Cuenca and Macías-Ordóñez, 2005); *H. charithonia* also
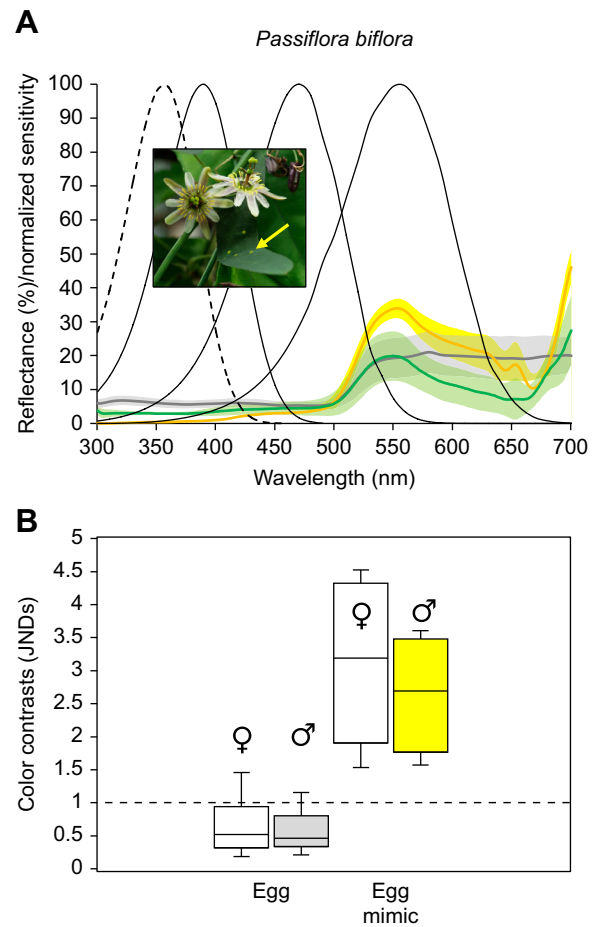

Fig. 5. Reflectance spectra and color contrasts for *Heliconius erato* eggs, egg mimics and leaves. (A) Reflectance spectra of *H. erato* eggs (dark grey line) and egg mimics (orange line) found on the leaves (green line) of *H. erato* host plant, *Passiflora biflora*. Shaded areas correspond to 95% confidence intervals, N=4 biological replicates per species/tissue. Black curves indicate UV1, UV2, blue and green photoreceptor normalized spectral sensitivities (left to right). Not shown is the red receptor that is the result of filtering the green receptor with a red filtering pigment. Photoreceptor data from McCulloch et al. (2016a,b). (B) Color contrasts between *H. erato* eggs and *P. biflora* leaves (N=16 pairs) (left) and between *P. biflora* egg mimics and *P. biflora* leaves (right) (N=16 pairs) in just noticeable differences (JNDs). Boxes represent upper and lower quartiles with median; whiskers correspond to upper and lower limits. The absolute threshold is 1 JND; however, in butterflies, the receptor noise levels are not well known so this is an approximation. Inset: *P. biflora* with yellow arrow indicating egg mimic by C. T. Johansson. Source: Wikimedia: CC BY (https://creativecommons.org/licenses/by/3.0).

display a sexual dimorphism in the flowers from which they collect pollen with females preferring *Hamelia patens* pollen and males preferring *Lantana camara* pollen in one study locality (Mendoza-Cuenca and Macías-Ordóñez, 2005).

An additional area ripe for exploration although not considered in the present study is in the investigation of ultraviolet polarized light cues in the context of host plant recognition. At least two studies of butterfly oviposition behavior have found that *Papilio* and *Pieris* butterflies respond to visible wavelength polarized light cues (Kelber et al., 2001; Blake et al., 2020), and previous work on *Heliconius cydno* finds they are able to use polarized light as a mating cue (Sweeney et al., 2003). Extending future investigations of *H. erato* behavior to include UV polarized cues in the context of oviposition and mate choice seems likely to yield further insights
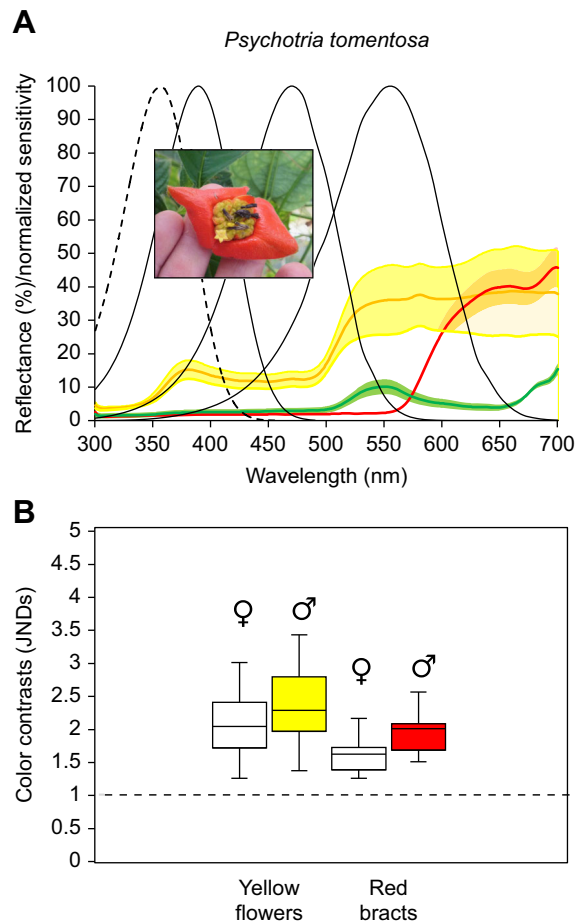
Fig. 6. Reflectance spectra and color contrasts of *Psychotria tomentosa* inflorescences, bracts and leaves. (A) Reflectance spectra of *P. tomentosa* yellow inflorescence (yellow line), red bracts (red line) and green leaves (green line). *P. tomentosa* is a plant from which *Heliconius* butterflies collect pollen. Shaded areas correspond to 95% confidence intervals, N=4–5 biological replicates per tissue. Black curves indicate UV1, UV2, blue, and green photoreceptor normalized spectral sensitivities (left to right). Not shown is the red receptor that is the result of filtering the green receptor with a red filtering pigment. Data from McCulloch et al. (2016a,b). (B) Color contrasts between yellow inflorescence and red bracts (N=20 pairs) (left) and red bracts and green leaves (right) (N=20 pairs) in just noticeable differences (JNDs). Boxes represent upper and lower quartiles with median; whiskers correspond to upper and lower limits. Inset: *P. tomentosa* with yellow arrow indicating the yellow inflorescence; surrounding the inflorescence are red bracts.

Fig. 7. Reflectance spectra and color contrasts of *Psiguria warscewiczii* flower petals, corolla and leaves. (A) Reflectance spectra of *P. warscewiczii* yellow flower center (yellow line), outer orange petals (red line), light green corolla (dotted green line) and green leaves (solid green line). *P. warscewiczii* is a plant from which adult *Heliconius* butterflies collect pollen. Shaded areas correspond to 95% confidence intervals, N=5–9 biological replicates per tissue. Black lines indicate UV1, UV2, blue, and green photoreceptor normalized spectral sensitivities (left to right). Not shown is the red receptor that is the result of filtering the green receptor with a red filtering pigment. Data from McCulloch et al. (2016a,b). (B) Color contrasts between yellow flower center and outer orange petals (N=81 pairs) (left) and between outer orange petals and green leaves (right) (N=45 pairs) in just noticeable differences (JNDs). Boxes represent upper and lower quartiles with median; whiskers correspond to upper and lower limits. Inset: *P. warscewiczii* photograph. Photo credit: Steven Paton, Smithsonian Tropical Research Institute. Reprinted with permission.

into selective forces driving the evolution of this visual system's sexual dimorphism.

Other animals that have photoreceptor spectral sensitivity in the UV range likely have true UV color discrimination, although further experimentation is needed to rule out brightness discrimination. Hummingbird hawkmoths (*Macroglossum stellatarum*) can discriminate between 365 nm and 380 nm, but it is unclear whether they are able to do so by means of true color vision or an achromatic cue (Kelber and Hénique, 1999). A different study showed that these moths are indeed able to discriminate between long wavelength stimuli under a range of intensities (Telles et al., 2016). In the case of the mantis shrimp and similar stomatopods whose compound eyes possess the largest number of photoreceptor types known in any animal (including four UV-sensitive photoreceptors, Marshall and Oberwinkler, 1999), color vision is
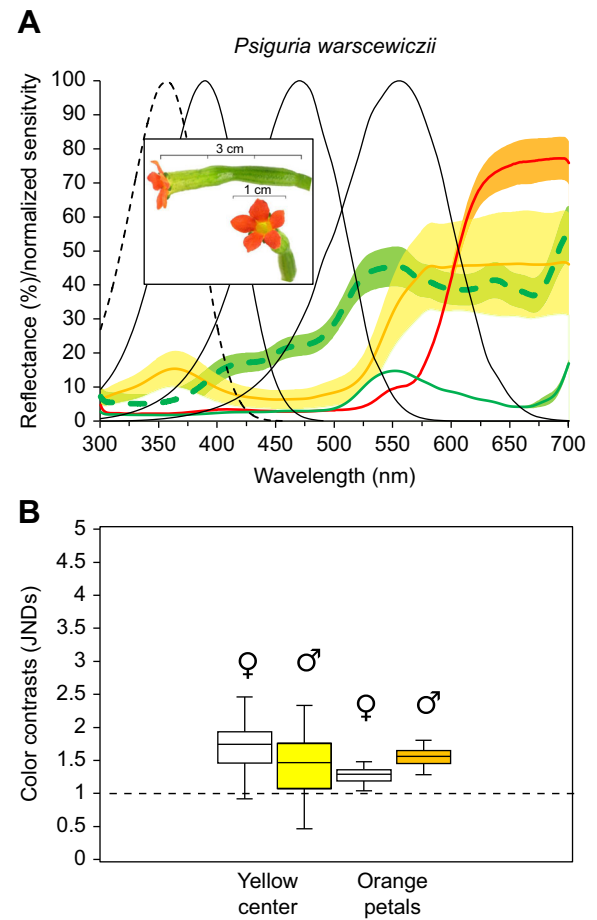
complicated (Thoen et al., 2014). However, these animals do appear to discriminate between different wavelengths of UV light independently of intensity, an ability described as 'polychromatic UV sensitivity' (as opposed to the UV color vision described here in *Heliconius* butterflies) (Bok et al., 2018). Taken together, our study provides clear evidence that despite differences in light intensity, *H. erato* female butterflies have the ability to discriminate between two UV wavelengths, lending support to the hypothesis that the new UV opsin gene in *Heliconius* functions in the context of UV color discrimination. Our study is one of the first to show that an animal can see multiple UV wavelengths using true color vision. In conclusion, our current and prior findings strongly suggest that both sexual selection and sex-specific natural selection have shaped the sexually dimorphic visual system of *H. erato*.

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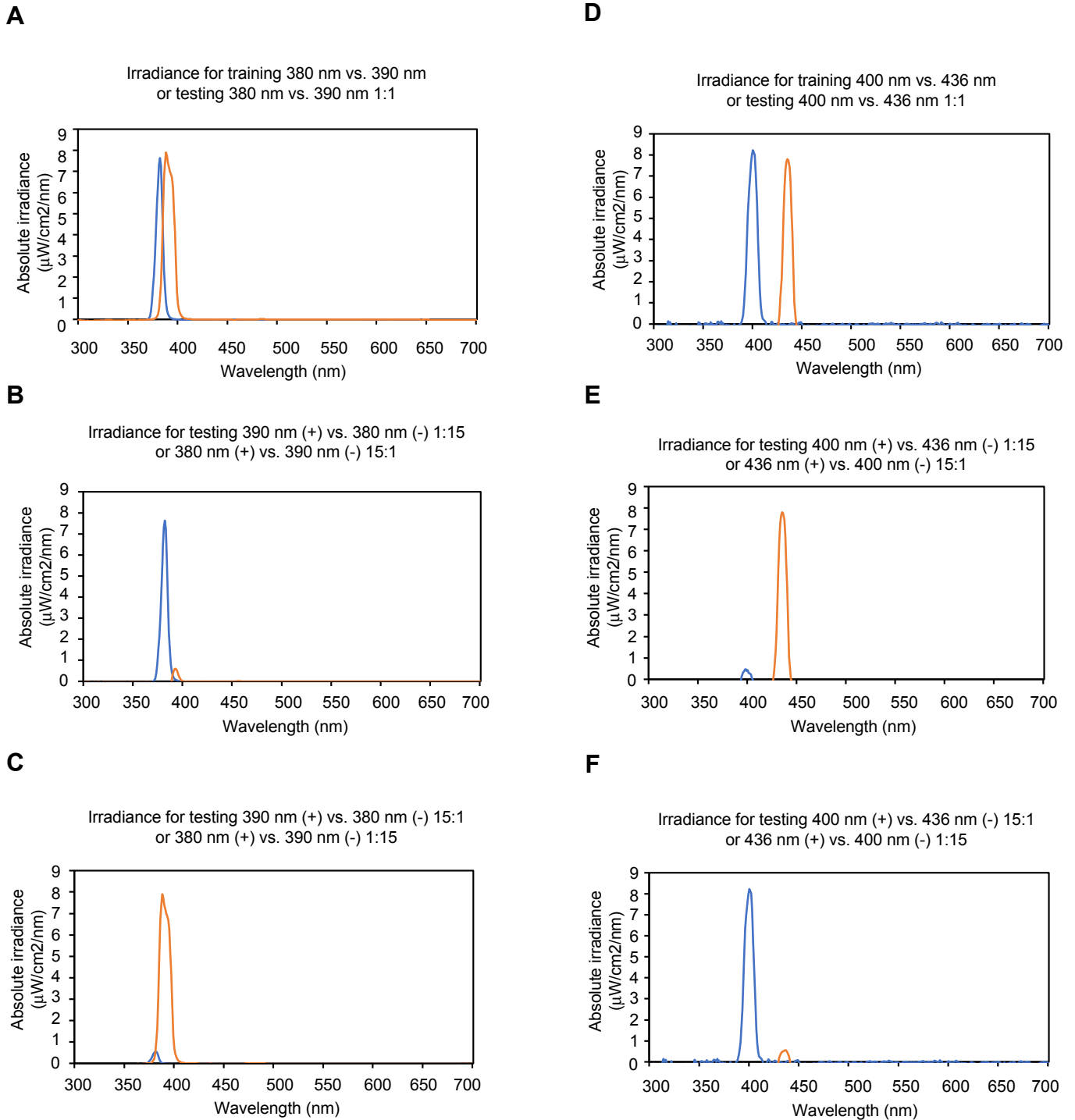
Fig. S1. Absolute irradiance spectra for 380 nm, 390 nm, 400 nm and 436 nm filtered lights used for butterfly behavior training and testing. Lights measured using an Ocean Optics USB2000 spectrometer and 100 µm-diameter fiber optic cable. (A) Irradiance for training 380 nm vs. 390 nm 1:1 or testing 380 nm vs. 390 nm 1:1. (B) Irradiance for testing 390 nm (+) vs. 380 nm (-) 1:15 or 380 nm (+) vs. 390 nm (-) 15:1. (C) Irradiance for testing 390 nm (-) vs. (380 nm) (-) 15:1 or 380 nm (+) vs. 390 nm (-) 1:15. (D) Irradiance for training 400 nm vs. 436 nm or testing 400 nm vs. 436 nm 1:1. (E) Irradiance for testing 400 nm (+) vs. 436 nm (1) 1:15 or 436 nm (+) vs. 400 nm (-) 15:1. (F) Irradiance for testing 400 nm (+) vs. 436 nm (-) 15:1 or 436 nm (+) vs. 400 nm (-) 1:15.
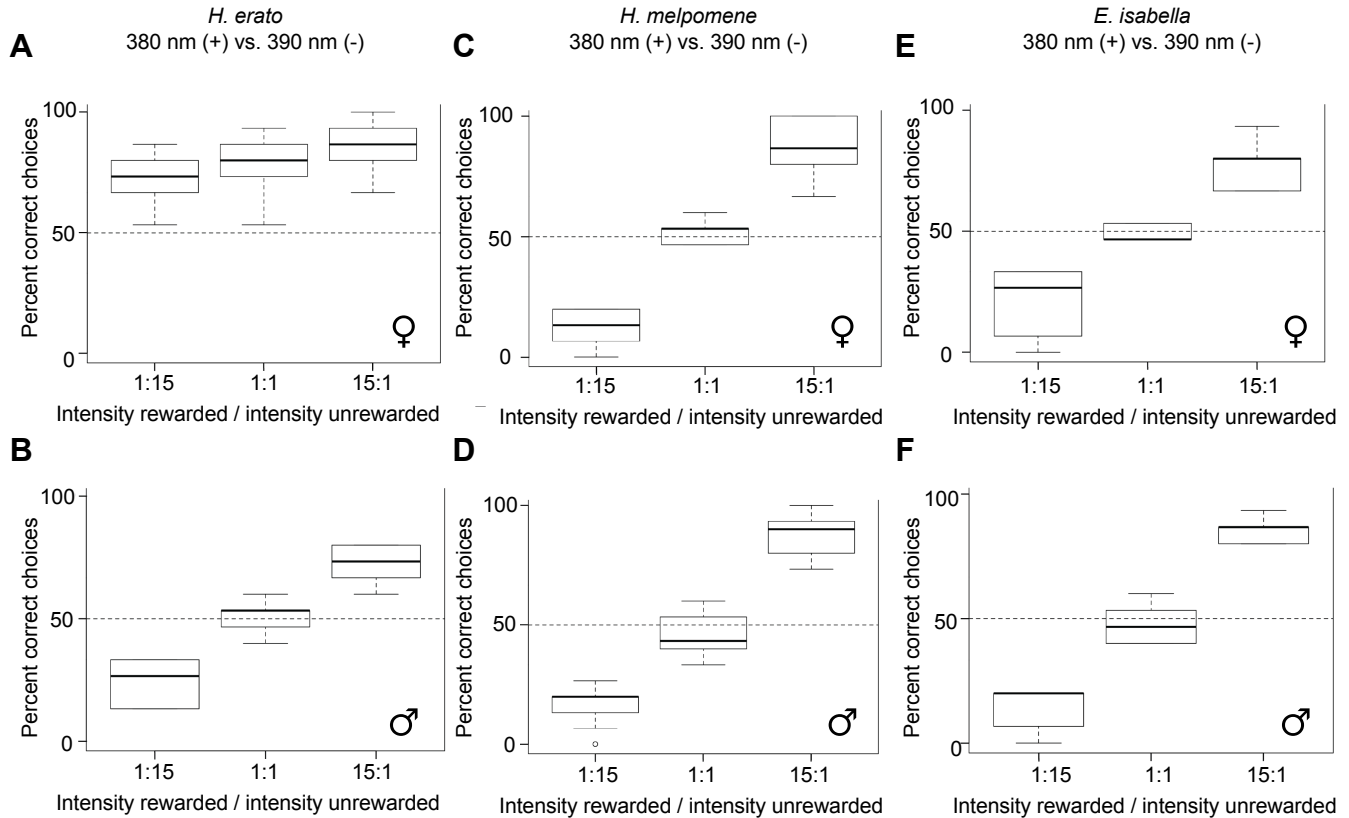
Fig. S2. **Percent correct choices for the rewarded (+) wavelength of 380 nm by species and sex.** *Heliconius erato* females (A) and males (B), *H. melpomene* females (C) and males (D), and *Eueides isabella* females (E) and males (F) when given a choice between 380 nm (+) and 390 nm (-) light under varying intensities. Spectra of the filtered lights under each of the intensity ratios are given in Fig. S1. For *H. erato* and *H. melpomene* N=10 and for E. isabella N=5 biological replicates per species and sex under which 15 choice trials were completed at each light intensity combination. Non-overlapping box plots indicate where P<0.01 from a general linear model calculated in R. Boxes represent upper and lower quartiles with median; whiskers indicate 25th and 75th percentiles
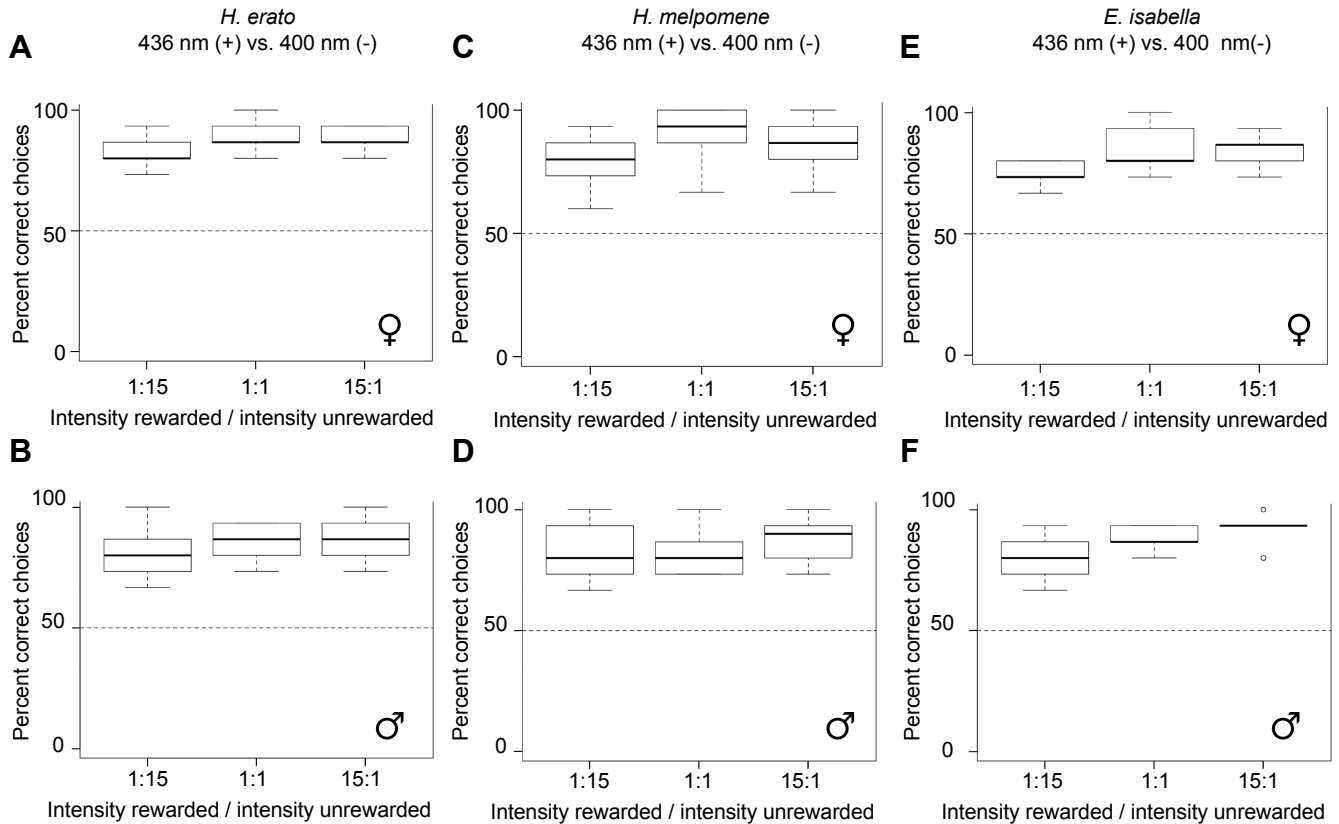
Fig. S3. Percent correct choices for the rewarded (+) wavelength of 436 nm by species and sex. *H. erato* females (A) and males (B), *H. melpomene* females (C) and males (D), and *Eueides isabella* females (E) and males (F) when given a choice between 436 nm (+) and 400 nm (-) light under varying intensities. Spectra of the filtered lights under each of the intensity ratios are given in Fig. S1. For *H. erato* and *H. melpomene* N=10 and for E. isabella N=5 biological replicates per species and sex under which 15 choice trials were completed at each light intensity combination. Non-overlapping box plots indicate where P<0.01 from a general linear model calculated in R. Boxes represent upper and lower quartiles with median; whiskers indicate 25th and 75th percentiles.
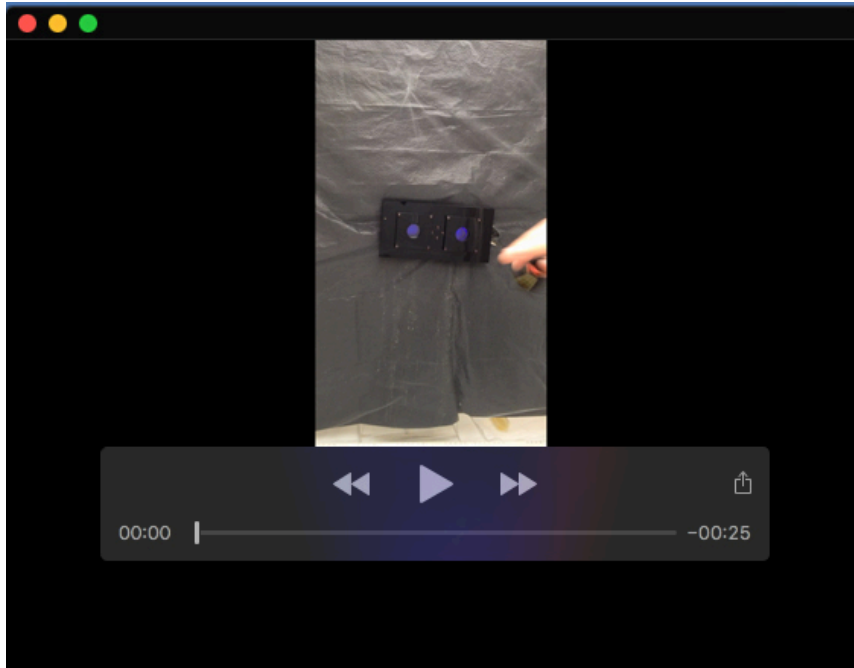
Movie 1. Apparatus for training and experiments based on a design described in Zaccardi et al. (2006). It consists of two 2.5 cm diameter stimuli presented side-by-side, separated by 6 cm on two black platforms set on a larger black plate, measuring 20x10 cm.