1   **True UV color vision in a butterfly with two UV opsins**

2

3   Susan D. Finkbeiner[1,2*] and Adriana D. Briscoe[1*]

4

5   [1] Department of Ecology and Evolutionary Biology, University of California, Irvine,

6   CA 92697, USA

7

8   [2] Department of Biological Sciences, California State University, Long Beach, CA, 90840,

9   USA

10

11   *Authors for correspondence: abriscoe@uci.edu and susan.finkbeiner@csulb.edu

12

13

14   Running Title: UV color vision in butterflies

15

16   Keywords: Visual system, wavelength discrimination, ultraviolet, insect vision, behavior

17

18   Data Archival: DRYAD (doi: XXXXX)

19

20

21

22

23

24

25

26   ABSTRACT

27   1. True color vision in animals is achieved when wavelength discrimination occurs

28   based on chromatic content of the stimuli, regardless of intensity. In order to

29   successfully discriminate between multiple wavelengths, animals must use at least

30   two photoreceptor types with different spectral sensitivity peaks.

31   2. *Heliconius* butterflies have duplicate UV opsin genes, which encode two kinds of

32   photoreceptors with peak sensitivities in the ultraviolet and violet, respectively. In

33   *H. erato*, the ultraviolet photoreceptor is only expressed in females.

34   3. Evidence from intracellular recordings suggests female *H. erato* may be able to

35   discriminate between UV wavelengths, however, this has yet to be tested

36   experimentally.

37   4. Using an arena with a controlled light setting, we tested the ability of *H. erato,*

38   and two species lacking the violet receptor, *H. melpomene* and outgroup *Eueides*

39   *isabella,* to discriminate between two ultraviolet wavelengths, 380 and 390 nm, as

40   well as two blue wavelengths, 400 and 436 nm, after being trained to associate

41   each stimulus with a food reward. Wavelength stimuli were presented in varying

42   intensities to rule out brightness as a cue.

43   5. We found that *H. erato* females were the only butterflies capable of color vision

44   in the UV range; the other butterflies had an intensity-dependent preference for

45   UV stimuli. Across species, both sexes showed color vision in the blue-range.

46   6. Models of *H. erato* color vision suggest that females have an advantage over

47   males in discriminating the inner UV-yellow corolla of *Psiguria* pollen flowers

48   from the surrounding outer orange petals, while previous models (McCulloch et

49 al. 2017) suggested that *H. erato* males have an advantage over females in

50 discriminating *Heliconius* 3-hyroxykynurenine (3-OHK) yellow wing coloration

51 from non-3-OHK yellow wing coloration found in mimics.

52 7. These results provide some of the first behavioral evidence for UV color

53 discrimination in *Heliconius* females in the context of foraging, lending support to

54 the hypothesis (Briscoe et al. 2010) that the duplicated UV opsin genes function

55 together in UV color vision. Taken together, the sexually dimorphic visual system

56 of *H. erato* appears to have been shaped by both sexual selection and sex-specific

57 natural selection.

58

59

60 INTRODUCTION

61 Color vision in animals is characterized by wavelength discrimination based on

62 spectral composition of the stimuli, independent of intensity (Kelber and Pfaff 1999).

63 Animals that have true color vision must use at least two types of photoreceptor, with

64 different spectral sensitivities, to successfully discriminate between wavelengths where

65 their sensitivity curves overlap.

66 Insects use color vision for multiple tasks including foraging (Spaethe et al. 2001,

67 Muth et al. 2015), host plant detection (Scherer and Kolb 1987), and conspecific

68 recognition (Kemp and Rutowski 2011). Most insects have at least one ultraviolet, one

69 blue, and one green photoreceptor, many insects lack red receptors (Briscoe and Chittka

70 2001), and some have lost their blue receptors (Sharkey et al. 2017). Numerous

71   butterflies, however, have visual systems with more than three photoreceptor classes (van

72   der Kooi et al. 2021).

73   While butterflies typically have only one kind of UV opsin (Briscoe et al. 2003,

74   Koshitaka et al. 2008), and variable numbers of blue and green opsins, *Heliconius* have

75   duplicated UV opsins (Briscoe et al. 2010). The two UV opsin-encoded photoreceptors

76   have peak sensitivities or λ_{max} values at 355 and 390 nm as measured by intracellular

77   recordings (McCulloch et al. 2016 *a*). Although the gene encoding UVRh2, which

78   together with the chromophore produces a violet receptor, is present throughout the

79   genus, the UVRh2 protein, is only expressed at detectable levels in the eye in certain

80   *Heliconius* clades (specifically the *sara* and *erato* clades); it is also sex-specific. In *H.*

81   *erato,* adult females express both UVRh1 and UVRh2 opsins but males only express the

82   violet receptor with sensitivity at 390 nm (McCulloch et al. 2017).

83   *Heliconius* butterflies also have a genus-specific wing pigment, 3-hydroxy-DL-

84   kynurenine (3-OHK), found in the yellow scales of the wings (Brown 1967). Together,

85   the pigment and the wing ultrastructure reflect UV light in the 300-400 nm range and

86   have a step-like reflectance starting about 440 nm. This wing pigment has evolved in

87   *Heliconius* along with their duplicated UV opsins (Briscoe et al. 2010), and close

88   relatives to this genus lack both the opsin duplication and the 3-OHK wing pigment

89   (Yuan et al. 2010). It has been proposed that the second UV opsin might allow for better

90   discrimination of yellow-winged *Heliconius* conspecifics from yellow-winged non-

91   *Heliconius* mimics (Bybee et al. 2012), perhaps because these butterflies' yellows differ

92   with respect to UV wavelengths; recent experiments lend some support to this hypothesis

93   (Finkbeiner et al. 2017, Dell'Aglio et al. 2018) but more behavioral experiments

94   examining the functional significance of the duplicate UV opsins are needed.

95   In *Heliconius* or passion-vine butterflies, adults have large heads relative to body

96   mass (compared to other butterflies) with notable investment in the visual neuropile

97   (Jiggins 2017), implying selective pressures for increased visual function. *Heliconius*

98   vision has been investigated using a variety of broad and narrow-band stimuli (Crane

99   1955, colored paper flowers; Swihart 1967, narrow band interference filters; Swihart

100   1972, narrow-spectrum color fibers; Zaccardi et al. 2006, narrow band interference

101   filters). Available evidence demonstrates that *Heliconius* have true color vision in the

102   long wavelength range (Zaccardi et al. 2006) but so far investigations in the short

103   wavelength range have been limited.

104   Here we test whether *Heliconius erato* are capable of discriminating between

105   narrow band wavelengths within in the UV range in the context of foraging. We use male

106   and female *H. erato* butterflies, and as controls, male and female *H. melpomene* and

107   *Eueides isabella* butterflies. Both *H. melpomene* and *E. isabella* lack a second UV opsin

108   protein expressed in the eye but for different reasons: protein expression of UVRh2 was

109   lost in *H. melpomene* (McCulloch et al. 2017), and *E. isabella* —a closely-related

110   outgroup —never evolved a second UV opsin (Yuan et al. 2010). By confirming UV

111   color discrimination in *H. erato* butterflies, and ruling it out in *H. melpomene* and *E.*

112   *isabella,* we demonstrate the functional significance of their UV opsin duplication.

113

114   METHODS

115   *Animals*

116   Butterflies were purchased as pupae from the Costa Rica Entomological Supply

117   (La Guácima, Costa Rica). The pupae were kept in a humidified chamber until they

118   eclosed, then they were sexed and marked with a unique number. The butterflies were fed

119   using a 10% honey solution with one bee pollen granule dissolved per 2 ml of solution.

120   Butterflies were only allowed to feed on the positive stimulus during the training and

121   testing. A total of 362 butterflies were used in the study, of which 200 were successfully

122   trained and used in complete trials: 80 *H. erato* (40 females, 40 males); 80 *H. melpomene*

123   (40 females, 40 males); and 40 *E. isabella* (20 females, 20 males).

124

125   *Behavioral experiments and apparatus*

126   The experiments and training took place indoors in a mesh enclosure constructed

127   from PVC pipes, measuring 1 m × 75 cm × 75 cm, and the room temperature was 24° C.

128   The top of the enclosure was lined with 8 fluorescent tubes (Philips TLD 965 18 W;

129   Eindhoven, The Netherlands). Our apparatus for training and experiments was based on a

130   design described in Zaccardi et al. (2006) and previously used to test color vision in the

131   monarch butterfly (Blackiston et al. 2011; see also Swihart and Swihart 1970, Weiss and

132   Papaj 2003, Takeuchi et al. 2006, Rodrigues et al. 2010, Kinoshita and Arikawa 2014,

133   and Drewniak et al. 2020 for other apparatus' used in butterfly visual learning). It

134   consists of two 3.0 cm diameter stimuli presented side-by-side, separated by 6 cm on two

135   black platforms set on a larger black plate, measuring a total of 20 cm × 10 cm (see

136   Figure 2 and Supporting Video 1). The apparatus was positioned vertically at the far end

137   of the enclosure. Two wavelength stimuli were presented to the butterflies at a time.

138   Light was emitted from two KL2500 Schott cold light sources (Mainx, Germany) into

139   light guides held stable with a light guide holder. The light from each guide passed

6

140   through a diffusor, a 10 nm narrow band-pass filter (Edmund Optics; Barrington, NJ,

141   USA), and then through a transparent Plexiglass feeder disk (see Figure 3 in Zaccardi et

142   al. 2006 for a diagram). For our experiments we used four narrow band-pass filters in

143   paired choice tests: 380 nm versus 390 nm, and 400 nm versus 436 nm. We use 380 and

144   390 nm as the UV stimuli because the sensitivity curves of the two UV photoreceptors

145   overlap in this range (McCulloch et al. 2016*a*) (Figure 1). If the butterflies have UV color

146   discrimination using the UV and the violet photoreceptors together then we would expect

147   that they would be able to discriminate between these two wavelengths. We also chose

148   400 nm and 436 nm as a control for color vision in all three species using the UV and

149   blue photoreceptors. The light intensities for each wavelength were adjusted so that

150   between these four wavelengths of light, the intensities for the experiments ranged from

151   9.56×10^{15} to 1.71×10^{17}; quanta s^{-1} steradian^{-1} cm^{-2}.

152

153   *Butterfly training*

154   Butterflies were trained and fed for the first time within 15 hours of eclosion.

155   Before training, they were allowed to acclimate to the experimental cage up to one

156   minute, and only one butterfly was trained at a time. A droplet of food was placed in a

157   small trough attached to the front of the feeder disk for the rewarded stimulus (+). The

158   unrewarded stimulus (-) feeder trough remained empty. Each butterfly was trained by

159   having its wings held together with forceps, and then slowly moved from the rear of the

160   enclosure toward the apparatus to simulate a flying motion. The butterfly was then slowly

161   waved in front of both the rewarded and unrewarded stimuli, and finally held in front of

162   the rewarded stimulus where its proboscis was uncoiled with an insect pin until it came

163   into contact with the food solution. At this point the butterfly would begin to drink. After

164   the proboscis was manually uncoiled 2-3 times, the butterfly was able to uncoil the

165   proboscis on its own in response to the stimulus. The procedure of carrying the butterfly

166   with forceps from the rear of the cage to the light sources to feed was repeated 5 times

167   per training session, with two training sessions per day separated by approximately 6

168   hours. Each time the butterfly fed from the rewarded stimulus, it was allowed to drink for

169   10 seconds, except for the very last training segment of the day where it was allowed to

170   drink for several minutes. During training and between training sessions, the placement

171   of the rewarded and unrewarded stimuli was randomly switched so that the butterfly did

172   not learn to associate the left or right light with a food source. The apparatus was also

173   cleaned thoroughly after each training session to minimize the association of chemical

174   cues to the stimulus. After about 4-5 days of training, butterflies were capable of

175   independently flying toward the apparatus when released from the rear of the cage and

176   making a choice to fly to one of the two light stimuli (Supporting Video 1). At this point,

177   the trained butterflies were starved for 20-24 hours then their choice trials began.

178

179   *Experimental trials*

180   A separate cohort of butterflies was trained with each wavelength pair because the

181   butterflies did not survive long enough to be trained multiple times. Both sexes of each

182   species were first trained to 390 nm (+), and then tested for UV discrimination ability

183   between 390 nm (+) and 380 nm (-) (ten per sex for *H. erato* and *H. melpomene* and five

184   per sex for *E. isabella*). The same number of individuals was trained to 380 nm (+) and

185   given the choice between the two UV stimuli. Two new cohorts of butterflies were used

8

186   for reciprocal training to 400 nm and to 436 nm. Three different approximate ratios of the

187   physical intensities, or absolute brightnesses, of the +/- (rewarded/unrewarded) stimuli

188   were used: 0.067, 1.0, and 15.0 (or 1:15, 1:1, and 15:1). The calculated ratios are 0.062,

189   1.0, 16.213 for 380 vs. 390 nm; and 0.0635, 1.0, 15.741 for 400 vs. 436 nm. These

190   intensity ratios are described throughout the rest of this study as 1:15, 1:1, and 15:1, i.e.

191   the rewarded stimulus (+) at 15 times less bright than the unrewarded stimulus (-), equal

192   intensities for both stimuli, and the rewarded stimulus (+) at 15 times brighter than

193   unrewarded stimulus (-). Butterflies first completed trials at an intensity combination of

194   1:1 (15 choices each). Following this test they were given random choices between

195   intensities of 1:15 or 15:1 (rewarded:unrewarded) until they had completed 15 choices

196   with each intensity combination.

197   The number of correct versus incorrect choices each butterfly made at different

198   intensity combinations was modeled as dependent upon wavelength using general linear

199   models in R statistical software (R Development Core Team 2010). We compared the

200   ability of each category of butterfly to discriminate between the wavelength combinations

201   at the different intensities. We also examined how discrimination abilities differed

202   between all three butterfly species used in the study.

203

204   *Reflectance spectrometry*

205   Live tissue was collected by accessing the butterfly and plant collection of Dr. Lawrence

206   Gilbert at the Brackenridge Field Laboratory at the University of Texas, Austin on July

207   20, 2010. Reflectance spectra of *Heliconius erato petiverana* eggs, *Passiflora biflora* egg

208   mimics, *Psychotria tomentosa* yellow infloresences, red bracts, and green leaves and

9

209   *Psiguria warcewiczii* yellow and orange inflorescences and green leaves were measured

210   by placing a probe holder (Ocean Optics RPH-1) over the specimen such that the axis of

211   the illuminating and detecting fiber (Ocean Optics R400-7-UV/VIS) was at an elevation

212   of 45 degrees to the plane of the tissue surface. Illumination was by a DH-2000

213   deuterium-halogen lamp, and reflectance spectra were measured with an Ocean Optics

214   USB2000 spectrometer. Data were processed in MATLAB. Four to nine biological

215   replicates per taxon were measured for each tissue type.

216

217   *Discriminability Modeling*

218   To examine whether male or female *H. erato* eyes perform differently when viewing

219   ecologically relevant objects, we constructed visual models. Models of color vision take

220   into account how receptor signals contribute to chromatic (e.g., color opponent)

221   mechanisms (Kelber et al. 2003). For *H. erato* males, we calculated discriminabilities for

222   a trichromatic system consisting of UV2, blue and green receptors. For *H.* erato females,

223   we calculated discriminabilities for a tetrachromatic system consisting of UV1, UV2,

224   blue and green receptors. We excluded the red receptor from our calculations for both

225   sexes because we do not have count data for this receptor class. Equations from Kelber et

226   al. (2003) and Vorobyev & Osorio (1998) were used to model discriminabilities. This

227   model incorporates a von Kries's transformation, that is, normalization by the

228   illumination spectrum, which models the way in which low-level mechanisms such as

229   photoreceptor adaptation give color constancy (Kelber et al. 2003). Endler's daylight

230   illumination spectrum (Endler 1993) was used in the model. *H. erato* photoreceptor

231   spectral sensitivity curves with λ_{max} values=355 nm (UV1)(female only), 390 nm (UV2),

232   470 nm (B), and 555 nm (L) from (McCulloch et al. 2016*a*) were used. Parameters for

233   the butterfly visual models were as follows: Weber fraction=0.05 (Koshitaka et al. 2008)

234   and relative abundances of photoreceptors, V=0.13, B=0.2, L=1 (male) or UV=0.09,

235   V=0.07, B=0.17, L=1 (females) (McCulloch et al. 2016*a*).

236

237   RESULTS

238   *UV discrimination*

239   At the intensity of 1:1 for 390 and 380 nm light, female *H. erato* chose the

240   rewarded light stimulus, 390 nm (+), significantly more than the unrewarded stimulus,

241   380 nm (-) (z-value = 6.791, p < 0.0001, Figure 3A). This indicates the ability of female

242   *H. erato* to distinguish between the two UV wavelengths. The females continued to

243   choose the correct, rewarded color stimulus under varying light intensity combinations.

244   At an intensity ratio of 1:15 (rewarded : unrewarded), females significantly chose 390 nm

245   (+) over 380 nm (-) (z-value = 5.19, p < 0.0001); and at an intensity of 15:1 (rewarded :

246   unrewarded), females also chose 390 nm (+) over 380 nm (-) (z-value = 7.35, p <

247   0.0001). There was no difference between female preference for the correct stimulus with

248   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

249   (z-value = 0.319, p = 0.749), showing that females chose the correct light stimulus (390

250   nm) equally across all tested light intensity combinations.

251   With respect to male behavior, at the intensity of 1:1 for 390 (+) and 380 nm (-),

252   male *H. erato* chose both the rewarded and unrewarded light stimuli equally (z-value = -

253   0.49, p = 0.624, Figure 3B). This suggests they cannot distinguish between the two UV

254   wavelengths. However, the males significantly preferred the correct, rewarded stimulus

11

255   (390 nm)(+) when it was presented 15x brighter than the unrewarded stimulus (ratio 15:1

256   for rewarded : unrewarded; z-value = 6.421, p < 0.0001); and they significantly preferred

257   the incorrect, unrewarded stimulus, 380 nm (-), at the intensity of 1:15 (rewarded :

258   unrewarded; z-value = -6.671, p < 0.0001). These results imply that males prefer the

259   brighter stimulus regardless of light wavelength, and further support their inability to

260   discriminate between 390 and 380 nm. Comparing male and female performance,

261   females significantly prefer the correct stimulus (390 nm)(+) more than males when 390

262   vs. 380 nm are at intensities of 1:1 (z-value = -3.427, p = 0.0006), and at intensities of

263   1:15 (z-value = -6.126, p < 0.0001), respectively. However, males and females equally

264   chose the correct stimulus, 390 nm (+), when the rewarded:unrewarded intensity ratio

265   was at 15:1 (z-value = -0.514, p = 0.607, Figure 3 A,B).

266   With *H. melpomene* and *E. isabella*, at the intensity of 1:1 for 390 and 380 nm,

267   both sexes had similar wavelength discrimination behavior to male *H. erato* in that they

268   chose both the rewarded (390 nm)(+) and unrewarded (380 nm)(-) light stimuli equally

269   (z-value = 0.923, p = 0.356 for *H. melpomene*, Figure 3 C,D; z-value = 0.327, p = 0.744

270   for *E. isabella*, Figure 3 E,F). They were able to significantly choose the correct stimulus

271   (390 nm)(+) only when it was 15x brighter than the unrewarded stimulus (z-value = -

272   10.79, p < 0.0001 for *H. melpomene*; z-value = 6.791, p < 0.0001 for *E. isabella*), and

273   they chose the unrewarded stimulus (380 nm)(-) significantly more when it was 15x

274   brighter than the rewarded, correct stimulus (z-value = 10.460, p < 0.0001 for *H.*

275   *melpomene*; z-value = -6.293, p < 0.0001 for *E. isabella*). No behavioral differences

276   between sexes of either species were detected with statistical analyses (all p > 0.05),

277   indicating that discrimination ability was consistent between both males and females of

278   *H. melpomene* and *E. isabella*.

279   For the reciprocally rewarded tests, female *H. erato* butterflies were again

280   consistent in discriminating between the rewarded (380 nm)(+) and unrewarded (390

281   nm)(-) stimuli when intensities were the same (z-value = -6.671, $p < 0.0001$, Supporting

282   Figure 1A), when the rewarded stimulus was 15x brighter (z-value = -7.793, $p < 0.0001$),

283   and when the rewarded stimulus was 15x less bright (z-value = -5.194, $p < 0.0001$).

284   Male *H. erato* butterflies were incapable of discriminating between the different

285   wavelengths when presented at equal intensities (z-value = -0.327, $p = 0.744$, Supporting

286   Figure 1B), and chose the incorrect stimulus when it was 15x brighter than the correct,

287   rewarded stimulus (z-value = 6.162, $p < 0.0001$). Males did, however, choose the correct

288   stimulus when presented at an intensity ratio of 15x brighter than the unrewarded

289   stimulus (z-value = -5.194, $p < 0.0001$). Females correctly chose the rewarded stimulus

290   (380 nm)(+) significantly more than males at intensity ratios of 1:1 (z-value = -2.976, $p =$

291   0.00292) and 1:15 (z-value = -5.793, $p < 0.0001$), but at a ratio of 15:1 male and female

292   *H. erato* chose the correct wavelength at similar rates (z-value = -1.424, $p = 0.154$,

293   Supporting Figure 1 A,B).

294   Like male *H. erato*, *H. melpomene* and *E. isabella* could not distinguish between

295   the two UV wavelengths presented at a 1:1 intensity ratio (z-value = 0.462, $p = 0.644$ for

296   *H. melpomene*, Supporting Figure 1 C,D; z-value = 0.327, $p = 0.744$ for *E. isabella*,

297   Supporting Figure 1 E,F). They significantly preferred the rewarded stimulus only when

298   15x brighter (z-value = -11.12, $p < 0.0001$ for *H. melpomene*; z-value = -7.024, $p <$

299   0.0001 for *E. isabella*), and preferred the unrewarded stimulus also only when 15x

300 brighter (z-value = 7.793, p < 0.0001 for *H. melpomene*; *z*-value = 7.346, p < 0.0001 for

301 *E. isabella*). Male and female discrimination behavior did not differ between *H.*

302 *melpomene* or *E. isabella* (p > 0.05). In summary, female *H. erato* always discriminated

303 between 380 and 390 nm light, consistently preferring the correct, rewarded stimulus,

304 whereas male *H. erato*, male and female *H. melpomene*, and male and female *E. isabella*

305 struggled with UV discrimination and only chose the correct stimulus when it was at a

306 brighter intensity than the incorrect, unrewarded stimulus.

307

308 *Short wavelength discrimination*

309   To investigate color vision in the blue range, we repeated the series of

310 discrimination tests using 400 nm and 436 nm which would allow short wavelength

311 discrimination using a UV or violet photoreceptor and a blue photoreceptor. As expected,

312 when trained to 400 nm (+), female *H. erato* chose the correct stimulus when offered

313 both light wavelengths at equal intensities (z-value = -7.93, p < 0.0001, Figure 4A), at an

314 intensity of 1:15 for rewarded:unrewarded (z-value = -7.54, p < 0.0001), and at an

315 intensity of 15:1 of rewarded:unrewarded light (z-value = -8.099, p < 0.0001). Male *H.*

316 *erato*, male and female *H. melpomene*, and *E. isabella* behavior paralleled female

317 discrimination behavior between the two blue wavelengths, with male *H. erato* choosing

318 the correct wavelength at intensity combinations of 1:1 (z-value = -7.93, p < 0.0001,

319 Figure 4B), 1:15 (z-value = -7.54, p < 0.0001), and 15:1 (z-value = -7.987, p < 0.0001);

320 and *H. melpomene* and *E. isabella* males and females also choosing the correct, rewarded

321 wavelengths at intensity ratios of 1:1 (z-value = -11.46, p < 0.0001 for *H. melpomene*,

322 Figure 4 C,D; z-value = -7.63, p < 0.0001 for *E. isabella*, Figure 4 E,F), 1:15 (z-value = -

323   11.07, p < 0.0001 for *H. melpomene*; z-value = -6.671, p < 0.0001 for *E. isabella*), and

324   15:1 (z-value = -11.47, p < 0.0001 for *H. melpomene*; z-value = -7.445, p < 0.0001 for *E.*

325   *isabella*).

326   When trained to 436 nm (+), all butterflies continued to show a significant

327   preference for the correct wavelength stimulus regardless of intensity. Female and male

328   *H. erato* preferred the rewarded stimulus at equal intensities (z-value = 7.930, p < 0.0001

329   for females, Supporting Figure 2A; z-value = 7.714, p < 0.0001 for males, Supporting

330   Figure 2B), at an intensity combination of 1:15 (z-value = 7.242, p < 0.0001 for females;

331   z-value = 6.909, p < 0.0001 for males), and at 15:1 (z-value = 7.987, p < 0.0001 for

332   females; z-value = 7.865, p < 0.0001 for males). *H. melpomene* and *E. isabella* followed

333   the same trend and significantly preferred the correct wavelength (436 nm) (+) at an

334   intensity combination of 1:1 (z-value = -10.85, p < 0.0001 for *H. melpomene* Supporting

335   Figure 2 C,D; z-value = 7.793, p < 0.0001 for *E. isabella*, Supporting Figure 2 E,F), 1:15

336   (z-value = -9.853, p < 0.0001 for *H. melpomene*; z-value = 6.293, p < 0.0001 for *E.*

337   *isabella*), and 15:1 (z-value = -11.07, p < 0.0001 for *H. melpomene*; z = 7.930, p < 0.0001

338   for *E. isabella*). There was no difference between *H. erato* male and female behavior,

339   between *H. melpomene* male and female behavior, or between *H. erato, H. melpomene,*

340   and *E. isabella* behavior (all p > 0.05) for selecting the correct light wavelength when

341   trained to either 400 nm or 436 nm. All butterflies expressed the same ability to

342   discriminate between 400 nm and 436 nm across all three intensity combinations.

343

344   DISCUSSION

345   We conclude that *Heliconius erato* butterflies have true color vision in the UV

346   range, between 380 nm and 390 nm, and that this is a female-limited behavior. Our

347   results provide behavioral evidence that these butterflies can discriminate between more

348   than one UV color using an ultraviolet and a violet photoreceptor, which suggests that the

349   *UVRh1* (ultraviolet) and *UVRh2* (violet) opsin genes in *H. erato* function in the context of

350   UV color discrimination. We also show that *H. erato, H. melpomene,* and *E. isabella*

351   have color vision in the blue range between 400 nm and 436 nm, using both an UV and

352   blue photoreceptor.

353   True UV color discrimination in *H. erato* is possible because of the evolution of a

354   violet-sensitive photoreceptor, UVRh2*,* which has been present since the genus originated

355   (Briscoe et al. 2010). As noted above, some clades (e.g., *H. melpomene*) do not express

356   the UVRh2 protein at detectable levels in the adult compound eye despite expressing the

357   *UVRh2* mRNA, due to ongoing pseudogenization (McCulloch et al. 2017). Opsin

358   duplication events are not uncommon in butterflies (Sison-Mangus et al. 2006, Lienard et

359   al. 2020). For example, the lycaenid butterfly *Polyommatus icarus* uses its duplicated

360   blue opsin to see green, perhaps for discrimination of oviposition sites (Sison-Mangus et

361   al. 2008). The pierid butterfly *Pieris rapae* has both a duplicated blue opsin and

362   spectrally tuned filtering pigments: photoreceptor modifications that may be crucial for

363   mate recognition by males (Arikawa et al. 2005; Wakakuwa et al. 2010). Yet another

364   study has found that while both sexes of the wood tiger moth, *Arcia plantaginis* can

365   distinguish between white and yellow male morphs (and females prefer to mate with

366   white males), variation in female orange and red coloration is indiscriminable by both

367   sexes, suggesting the moths' visual system has evolved to facilitate female choice (Henze

368   et al. 2018).

369   In *Heliconius erato* females, duplicate UV opsin genes encoding a UV and a

370   violet receptor allow for UV color discrimination. The diversity of duplicated UV opsin

371   presence or absence and spatial expression across the genus *Heliconius* is nonetheless

372   thought-provoking. Male *H. erato* butterflies evidently use their duplicated UVRh2

373   (violet), blue, and long wavelength opsins in the context of mate choice discrimination of

374   3-OHK versus non-3-OHK yellow wing colors (Finkbeiner et al. 2017), an advantage

375   predicted by modeling the discrimination abilities of *H. erato* males in comparison with a

376   hypothetical male *H. erato* visual system in which UVRh1 takes the place of UVRh2

377   (Table 1) (McCulloch et al. 2017). Moreover, the loss of UVRh2 protein expression in *H.*

378   *melpomene* (which use their ancestral UVRh1 opsin and not UVRh2) may contribute to

379   increased attempts to mate with other species due to a reduction in visual ability to

380   recognize conspecifics (Bybee et al. 2012, Dell'Aglio et al. 2019). *Heliconius* are part of

381   a large mimicry complex that includes both unpalatable within-genus Müllerian mimics

382   (which display 3-OHK yellow wing pigments) and palatable Batesian mimics such as

383   *Eueides isabella* (which display unknown yellow wing pigments) (Srygley and Chai

384   1990; Bybee et al. 2012). Consequently, *Heliconius erato* (but not *H. melpomene*)

385   butterflies benefit from having the violet receptor, UVRh2, which facilitates

386   discrimination of yellow pigments of mimics from those of conspecifics.

387   Early visual modeling of the *Heliconius* visual system suggested an additional

388   benefit to *Heliconius'* displaying 3-OHK yellow pigments on the wing: with a second UV

389   opsin in their eyes, more colors can be discriminated among *Heliconius* yellows than can

17

390   be discriminated among the yellows of outgroup taxa (Briscoe et al. 2010). More recent

391   work suggests *Heliconius* species may indeed be more conspicuous to conspecifics in

392   their preferred habitats and light environment (Dell'Aglio et al. 2018, Dell'Aglio et al.

393   2019).

394   Both *H. erato* and *H. melpomene* may interact together by forming communal

395   roosts in the same home range, which would provide added anti-predatory benefits

396   through a similar visual signal (Finkbeiner et al. 2012). *Heliconius* co-mimics have been

397   observed foraging together (pers. obs.) and roosting together (although uncommon;

398   Mallet 1986, Finkbeiner 2014), and this could represent one instance where identifying a

399   *Heliconius* individual (whether or not a co-species) would be beneficial. Aside from

400   visual signals, *Heliconius* frequently use pheromone cues for conspecific recognition,

401   especially for short-range signaling, for example during courtship behavior (Estrada and

402   Jiggins 2008, Darragh et al. 2017, van Schooten et al. 2020).

403   It is possible that the adaptive function of UV color discrimination in female *H.*

404   *erato* butterflies extends beyond intra- and interspecific communication to include host

405   plant or pollen plant recognition. Within *Heliconius*, different species are specialists on

406   *Passiflora* host plants for oviposition, and some of these *Passiflora* species contain

407   extrafloral nectaries that resemble yellow *Heliconius* eggs (Williams and Gilbert 1981).

408   *Heliconius* are known to avoid ovipositing on host plants that already have eggs because

409   larvae have cannibalistic tendencies (Brown 1982, De Nardin and Araújo 2011), and

410   fresh, new shoots that are the most edible for larvae can be of limited quantity (Gilbert

411   1982). While it is possible that the egg mimic structures differ spectrally from actual eggs

412   in their UV reflectance, thus potentially allowing the additional UV opsin to provide

413   discrimination between natural and mimic eggs, our preliminary investigation of the

414   reflectance spectra of *H. erato petiverana* eggs and *Passiflora biflora* egg mimics,

415   indicates that there is little to no UV reflectance for either the eggs or the egg mimics

416   (Figure 5, top). Moreover, visual models indicate that both male and female *H. erato*

417   visual systems are both able to discriminate *H. erato* eggs from *Passiflora biflora* egg

418   mimics (Table 1), and *P. biflora* egg mimics from *P. biflora* leaves but not *H. erato* eggs

419   from *P. biflora* leaves (Figure 5, bottom).

420   There is also the possibility that the leaves of caterpillar host plants, or even the

421   petals of adult pollen flowers (such as *Psychotria* and *Psiguria*) have unique spectral

422   properties in the UV range that would make a second UV/violet opsin beneficial.

423   Intriguingly, we found evidence of a UV component to the reflectance spectra of the

424   yellow inflorescences of *Psychotria tomentosa,* a plant from which *Heliconius*

425   prolifically collect pollen (Figure 6, top). Both male and female *H. erato* visual systems

426   appear adept, however, at discriminating between the yellow inflorescence from the red

427   bracts of *Psychotria tomentosa* and at discriminating the red bracts from the green

428   *Psychotria* leaf (Table 1, Figure 6, bottom). We also found that the yellow inner part of

429   the *Psiguria warcewiczii* inflorescence has an even brighter UV component (Figure 7,

430   top). Notably, the female *H. erato* visual system seems to have a bit of an advantage over

431   the male *H. erato* visual system in discriminating the inner yellow from the outer orange

432   petals of *Psiguria warcewiczii* flowers (Table 1, Figure 7 bottom). This difference is

433   intriguing in light of evidence that female *Heliconius charitonia* (which have similar

434   visual systems to *H. erato*)(McCulloch et al. 2017) collect significantly more pollen than

435   do male *H. charitonia* because of their higher protein requirements for egg production

19

436 (Boggs 1981; Boggs et al. 1981; Cardoso 2001; Estrada and Jiggins 2002; Mendoza-

437 Cuenca and Macías-Ordóñez 2005); *H. charitonia* also display a sexual dimorphism in

438 the flowers they collect pollen from with females preferring *Hamelia patens* pollen and

439 males preferring *Lantana camara* flowers in one study locality (Mendoza-Cuenca and

440 Macías-Ordóñez 2005).

441   An additional area ripe for exploration although not considered in the present

442 study is in the investigation of ultraviolet polarized light cues in the context of host plant

443 recognition. At least two studies of butterfly oviposition behavior have found that *Papilio*

444 and *Pieris* butterflies respond to visible wavelength polarized light cues (Kelber et al.

445 2001; Blake et al. 2020), and previous work on *Heliconius cydno* finds they are able to

446 use polarized light as a mating cue (Sweeney et al. 2003). Extending future investigations

447 of *Heliconius erato* behavior to include UV polarized cues in the context of oviposition

448 and mate choice seems likely to yield further insights into selective forces driving the

449 evolution of this visual system's sexual dimorphism.

450   Other animals that have photoreceptor spectral sensitivity in the UV range likely

451 have true UV color discrimination, although to rule out brightness discrimination further

452 experimentation is needed. Hummingbird hawkmoths (*Macroglossum stellatarum*) can

453 discriminate between 365 nm and 380 nm, but it is unclear whether they are able to do so

454 by means of true color vision or an achromatic cue (Kelber and Henique 1999). A

455 different study showed that these moths are indeed able to discriminate between long

456 wavelength stimuli under a range of intensities (Telles et al. 2016). In the case of the

457 mantis shrimp and similar stomatopods, whose compound eyes possess the largest

458 number of photoreceptor types known in any animal (including four UV-sensitive

20

459   photoreceptors, Marshall and Oberwinkler 1999), there is little indication that their

460   photoreceptors function with respect to true color vision at all (Thoen et al. 2014).

461   However, our study provides clear evidence that despite differences in light intensity, *H.*

462   *erato* female butterflies have the ability to discriminate between two UV wavelengths,

463   lending support to the hypothesis that the new UV opsin gene in *Heliconius* functions in

464   the context of UV color discrimination, and is one of the first to show that an animal can

465   see multiple UV wavelengths using true color vision. In conclusion, our current and prior

466   findings strongly suggest that both sexual selection and sex-specific natural selection

467   have shaped the sexually-dimorphic visual system of *Heliconius erato.*

468

469

470   ACKNOWLEDGEMENTS

480

481

482   LITERATURE CITED

483   Arikawa, K., Wakakuwa, M., Qiu, X., Kurasawa, M., & Stavenga, D. G. (2005). Sexual

484   dimorphism of short-wavelength photoreceptors in the Small White butterfly,

485   *Pieris rapae crucivora*. *Journal of Neuroscience,* 25, 5935–5942.

486   https://doi.org/10.1523/JNEUROSCI.1364-05.2005

487

488   Blackiston, D., Briscoe, A. D., & Weiss, M. R. (2011). Color vision and learning in the

489   monarch butterfly, *Danaus plexippus* (Nymphalidae). *Journal of Experimental Biology,*

490   214, 509-520. doi: 10.1242/jeb.048728

491

492   Blake, A. J., Hahn, G. S., Grey, H., Kwok, S. A., McIntosh, D., & Gries, G. (2020).

493   Polarized light sensitivity in *Pieris rapae* is dependent on both color and intensity.

494   *Journal of Experimental Biology,* 223, jeb220350. doi: 10.1242/jeb.220350

495

496   Boggs, C. L. (1981). Nutritional and life-history determinants of resource

497   allocation in holometabolous insects. *The American Naturalist,* 117, 692–709.

498

499   Boggs, C. L., Smiley, J. T. & Gilbert, L. E. (1981). Patterns of pollen exploitation by

500   *Heliconius* butterflies. *Oecologia,* 48, 284–289.

501

502

503   Boggs, C. L., Smiley, J. T., & Gilbert, L. E. (1981). Patterns of pollen exploitation by

504   *Heliconius* butterflies. *Oecologia,* 48, 284-289. https://doi.org/10.1007/BF00347978

505

506   Briscoe, A. D., Bernard, G. D., Szeto, A. S., Nagy, L. M., & White, R. H. (2003). Not all

507   butterfly eyes are created equal: Rhodopsin absorption spectra, molecular identification

508   and localization of ultraviolet-, blue-, and green-sensitive rhodopsin-encoding mRNAs in

509   the retina of *Vanessa cardui. Journal of Comparative Neurology,* 458, 334-349.

510   https://doi.org/10.1002/cne.10582

511

512   Briscoe, A. D., Bybee, S. M., Bernard, G. D., Yuan, F., Sison-Mangus, M. P., Reed, R.

513   D., Warren, A. D., Llorente-Bousquets, J., & Chiao, C.-C. (2010). Positive selection of a

514   duplicated UV-sensitive visual pigment coincides with wing pigment evolution in

515   *Heliconius* butterflies. *Proceedings of the National Academy of Sciences, U.S.A.,* 107,

516   3628-3633. https://doi.org/10.1073/pnas.0910085107

517

518   Briscoe, A. D., & Chittka, L. (2001). The evolution of color vision in insects. *Annual*

519   *Review of Entomology,* 46, 471-510. doi:10.1146/annurev.ento.46.1.471

520

521   Brown, K. S. Jr. (1981). The biology of *Heliconius* and related genera. *Annual Review of*

522   *Entomology,* 26, 427-456. https://doi.org/10.1146/annurev.en.26.010181.002235

523

524   Brown, K. S. (1967). Chemotaxonomy and chemomimicry: The case of 3-

525   hydroxykynurenine. *Systematic Zoology,* 16, 213–216. https://doi.org/10.2307/2412068

526

527   Bybee, S. M., Yuan, F., Ramstetter, M. D., Llorente-Bousquets, J., Reed, R. D., Osorio,

528   D., & Briscoe, A. D. (2012). UV photoreceptors and UV-yellow wing pigments in

529   *Heliconius* butterflies allow a color signal to serve both mimicry and intraspecific

530   communication. *The American Naturalist,* 179, 38-51. doi: 10.1086/663192

531

532   Cardoso, M. Z. (2001). Patterns of pollen collection and flower visitation by *Heliconius*

533   butterflies in southeastern Mexico. *Journal of Tropical Ecology,* 17, 763–768.

534

535   Crane, J. (1955). Imaginal behaviour of a Trinidad butterfly, *Heliconius erato hydara*

536   Hewitson, with special reference to the social use of color. *Zoologica,* 40, 167–196.

537

538   Darragh, K., Vanjari, S., Mann, F., Gonzalez-Rojas, M. F., Morrison, C. R., Salazar, C.,

539   et al. (2017). Male sex pheromone components in *Heliconius* butterflies released by the

540   androconia affect female choice. *Peer J*, 5, e3953.

541

542   Dell'Aglio, D. D., Troscianko, J., McMillan, W. O., Stevens, M., & Jiggins, C. D. (2018).

543   The appearance of mimetic *Heliconius* butterflies to predators and conspecifics.

544   *Evolution*, 72, 2156-2166.

545

546 Dell'Aglio, D. D., Troscianko, J., Stevens, M., McMillan, W. O., & Jiggins, C. D. (2019).

547 The conspicuousness of the toxic *Heliconius* butterflies across time and habitat. *BioRxiv*.

548 doi: https://doi.org/10.1101/662155

549

550 De Nardin, J., & Araújo, A. (2011). Kin recognition in immatures of *Heliconius erato*

551 *phyllis* (Lepidoptera; Nymphalidae). *Journal of Ethology, 23*, 499-503.

552

553 Drewniak, M. E., Briscoe, A. D., Cocucci, A. A., Beccacece, H. M., Zapata, A. I., &

554 Moré, M. (2020). From the butterfly's point of view: learned colour association

555 determines differential pollination of two co-occurring mock verbains by *Agraulis vanilla*

556 (Nymphalidae). *Biological Journal of the Linnaen Society, 130*, 715-725. doi:

557 10.1093/biolinnean/blaa066

558

559 Endler, J.A. (1993). The color of light in forests and its implications. *Ecological*

560 *Monographs*, 63, 1–27.

561

562 Estrada, C., & Jiggins, C. D. (2002). Patterns of pollen feeding and habitat preference

563 among *Heliconius* species. *Ecologial Entomology, 27*, 448–456.

564

565 Estrada, C., & Jiggins, C. D. (2008). Interspecific sexual attraction because of

566 convergence in warning colouration: is there a conflict between natural and sexual

567 selection in mimetic species? *Journal of Evolutionary Biology, 21*, 749-760.

568

569   Finkbeiner, S. D. (2014). Communal roosting in *Heliconius* butterflies (Nymphalidae):

570   Roost recruitment, establishment, fidelity, and resource use trends based on age and sex.

571   *Journal of the Lepidopterists' Society,* 68, 10-16.

572

573   Finkbeiner, S. D., Briscoe, A. D., & Reed, R. D. (2012). The benefit of being a social

574   butterfly: communal roosting deters predation. *Proceedings of the Royal Society of*

575   *London B.,* 279, 2769-2776. doi:10.1098/rspb.2012.0203

576

577   Finkbeiner, S. D., Fishman, D. A., Osorio, D., & Briscoe, A. D. (2017). Ultraviolet and

578   yellow reflectance but not fluorescence is important for visual discrimination of

579   conspecifics by *Heliconius erato*. *Journal of Experimental Biology,* 220, 1267-1276.

580   doi: 10.1242/jeb.153593

581

582   Gilbert, L. E. (1972). Pollen feeding and reproductive biology *of Heliconius* butterflies.

583   *Proceedings of the National Academy of Sciences U.S.A*., 69, 1403–1407.

584

585   -------. (1982). The coevolution of a butterfly and a vine. *Scientific American,* 247, 110-

586   121.

587

588   Henze, M. J., Lind, O., Mappes, J. M., Rojas, B., & Kelber, A. (2018). An aposematic

589   colour-polymorphic moth seen through the eyes of conspecifics and predators –

590   sensitivity and colour discrimination in a tiger moth. *Functional Ecology*, 32, 1797-1809.

591   doi:10.1111/1365-2435.13100

592

593   Jiggins, C. D. (2017). *The Ecology and Evolution of Heliconius Butterflies*. Oxford

594   University Press, Oxford, U.K.

595

596   Kelber, A., & Hénique, U. (1999). Trichromatic colour vision in the hummingbird

597   hawkmoth, *Macroglossum stellatarum* L. *Journal of Comparative Physiology A.* 184,

598   535-541.

599

600   Kelber, A., & Pfaff, M. (1999). True colour vision in the orchard butterfly, *Papilio*

601   *aegeus*. *Naturwissenschaften,* 86, 221-224.

602

603   Kelber, A., Thunell, C., & Arikawa, K. (2001). Polarisation-dependent colour vision in

604   *Papilio* butterflies. *Journal of Experimental Biology,* 204, 2469-2480.

605

606   Kelber A, Vorobyev M, Osorio D. (2003). Animal colour vision: behavioural tests and

607   physiological concepts. *Biological Reviews,* 78, 81–118.

608

609   Kemp, D. J., & Rutowski, R. L. (2011). The role of coloration in mate choice and sexual

610   interactions in butterflies. *Advances in the Study of Behavior,* 43, 55-92.

611

612   Kinoshita, M., & Arikawa, K. (2014). Color and polarization vision in foraging *Papilio*.

613   *Journal of Comparative Physiology A.,* 200, 513-526. doi:10.1007/s00359-014-0903-5

614

615 Koshitaka, H., Kinoshita, M., Vorobyev, M., & Arikawa, K. (2008). Tetrachromacy in a

616 butterfly that has eight varieties of spectral receptors. *Proceedings of the Royal Society of*

617 *London B., 275*, 947-954.

618

619 Lienard, M. A., Bernard, G. D., Allen, A. A, Lassance, J-M., Song, S., Rabideau

620 Childers, R. A., Yu, N., Ye, D., Stephenson, A., Valencia-Montoya, W. A., Salzman, S.,

621 Whitaker, M. R. L., Calonje, M., & Pierce, N. E. (2020). The evolution of red colour

622 vision is linked to coordinated rhodopsin tuning in lycaenid butterflies. *bioRxiv*

623 doi:https://doi.org/10.1101/2020.04.06.027102

624

625 Mallet, J. (1986). Gregarious roosting and home range in *Heliconius* butterflies. *National*

626 *Geographic Research, 2*, 198-215.

627

628 Marshall, J., & Oberwinkler, J. (1999). Ultraviolet vision: The colourful world of the

629 mantis shrimp. *Nature, 401*, 873-874.

630

631 McCulloch, K. J., Osorio, D. C., & Briscoe, A. D. (2016*a*). Sexual dimorphism in the

632 compound eye of *Heliconius erato*: a nymphalid butterfly with at least five spectral

633 classes of photoreceptor. *Journal of Experimental Biology, 219*, 2366-2387.

634 doi: 10.1242/jeb.136523

635

636   McCulloch, K., Osorio, D., & Briscoe, A. D. (2016*b*). Determination of photoreceptor

637   cell spectral sensitivity in an insect model from in vivo intracellular recordings. *Journal*

638   *of Visualized Experiments,* 108, e53829. doi:10.3791/53829

639

640   McCulloch, K. J., Yuan, F., Zhen, Y., Aardema, M. L., Smith, G., Llorente-Bousquets, J.,

641   Andolfatto, P., & Briscoe, A. D. (2017). Sexual dimorphism and retinal mosaic

642   diversification following the evolution of a violet receptor in butterflies. *Molecular*

643   *Biology and Evolution,* 34, 2271-2284. doi:10.1093/molbev/msx163

644

645   Mendoza-Cuenca, L., & Macías-Ordóñez, R. (2005). Foraging polymorphism in

646   *Heliconius charitonia* (Lepidoptera: Nymphalidae): morphological constraints and

647   behavioural compensation. *Journal of Tropical Ecology*, 21, 407-415.

648   doi:10.1017/S0266467405002385

649

650   Muth, F., Papaj, D. R. , & Leonard, A. S. (2015). Colour learning when foraging for

651   nectar and pollen: bees learn two colours at once. *Biology Letters,* 11, 20150628.

652   https://doi.org/10.1098/rsbl.2015.0628

653

654   Rodrigues, D., Goodner, B. W., & Weiss, M. R. (2010). Reversal learning and risk-averse

655   foraging behavior in the monarch butterfly, *Danaus plexippus* (Lepidoptera:

656   Nymphalidae). *Ethology,* 116, 270-280.

657

658   R Development Core Team. (2010). R: A language and environment for statistical

659   computing. R Foundation for Statistical Computing, Vienna, Austria.

660

661   Scherer, C., & Kolb, G. (1987). Behavioral experiments on the visual processing of color

662   stimuli in *Pieris brassicae* L. (Lepidoptera). *Journal of Comparative Physiology A,* 160,

663   645-656.

664

665   Sharkey, C. R., Fujumoto, M. S., Lord, N. P., Shin, S., McKenna, D. D., Suvorov, A.,

666   Martin, G. J., & Bybee, S. M. (2017). Overcoming the loss of blue sensitivity through

667   opsin duplication in the largest animal group, beetles. *Scientific Reports,* 7, 8.

668

669   Sison-Mangus, M. P., Bernard, G. D., Lampel, J., & Briscoe, A. D. (2006). Beauty in the

670   eye of the beholder: The two blue opsins of lycaenid butterflies and the opsin gene-driven

671   evolution of sexually dimorphic eyes. *Journal of Experimental Biology,* 209, 3079-3090.

672   doi:10.1242/jeb.02

673

674   Sison-Mangus, M. P., Briscoe, A. D., Zaccardi, G., Knüttel, H., & Kelber, A. (2008). The

675   lycaenid butterfly *Polyommatus icarus* uses a duplicated blue opsin to see green. *Journal*

676   *of Experimental Biology*, 211, 361-369. doi: 10.1242/jeb.012617

677

678   Spaethe, J., Tautz, J., & Chittka, L. (2001). Visual constraints in foraging bumblebees:

679   flower size and color affect search time and flight behavior. *Proceedings of the National*

680   *Academy of Sciences U.S.A.,* 98, 3898-3903. https://doi.org/10.1073/pnas.071053098

681

682   Srygley, R. B., & Chai, P. (1990). Flight morphology of Neotropical butterflies:

683   palatability and distribution of mass to the thorax and abdomen. *Oecologia,* 84, 491-499.

684

685   Sweeney, A., Jiggins, C. D., & Johnsen, S. (2003). Polarized light as a butterfly mating

686   signal. *Nature,* 423, 31-32. https://doi.org/10.1038/423031a

687

688   Swihart, S. L. (1967). Neural adaptations in the visual pathway of certain heliconiine

689   butterflies and related forms to variations in wing coloration. *Zoologica, New York,* 52, 1-

690   14.

691

692   -------. (1972). The neural basis of color-vision in the butterfly, *Heliconius erato*. *Journal*

693   *of Insect Physiology,* 18, 1015-1025.

694

695   Swihart, C. A., & Swihart, S. L. (1970). Colour selection and learned feeding preferences

696   in the butterfly, *Heliconius charitonius* Linn. *Animal Behavior,* 18, 60-64.

697

698   Takeuchi, Y., Arikawa, K., & Kinoshita, M. (2006). Color discrimination at the spatial

699   resolution limit in a swallowtail butterfly, *Papilio xuthus*. *Journal of Experimental*

700   *Biology,* 209, 2873-2879. doi:10.1242/jeb.02311

701

702   Telles, F. J., Kelber, A., & Rodriguez-Gironés, M. A. (2016). Wavelength discrimination

703   in the hummingbird hawkmoth *Macroglossum stellatarum*. *Journal of Experimental*

704   *Biology,* 219, 553-560.

705

706   Thoen, H. H., How, M. J., Chiou, T.-H., & Marshall, J. (2014). A different form of color

707   vision in mantis shrimp. *Science*, 343, 411-413.

708

709   van der Kooi, C. J., Stavenga, D. G., Arikawa, K., Belusic, G., & Kelber, A. *In press*.

710   Evolution of insect colour vision – from spectral sensitivity to visual ecology. *Annual*

711   *Review of Entomology* doi: 10.1146/annurev-ento-061720-071644.

712

713   van Schooten, B., Meléndez-Rosa, J., Van Belleghem, S. M., Jiggins, C. D., Tan, J. D.,

714   McMillan, W. O., & Papa, R. (2020). Divergence of chemosensing during the early

715   stages of speciation. *Proceedings of the National Academy of Sciences U.S.A.,* 117,

716   16438-16447 doi.org/10.1073/pnas.1921318117

717

718   Vorobyev, M., &Osorio, D. (1998). Receptor noise as a determinant of colour thresholds.

719   *Proceedings of the Royal Society of London B*, 265, 351–358.

720

721   Wakakuwa, M., Terakita, A., Koyanagi, M., Stavenga, D. G., Shichida, Y., & Arikawa,

722   K. (2010). Evolution and mechanism of spectral tuning of blue-absorbing visual pigments

723   in butterflies. *PLoS One,* 5, e15015.

724

725   Weiss, M. D., & Papaj, D. R. (2003). Colour learning in two behavioural contexts: how

726   much can a butterfly keep in mind? *Animal Behavior,* 65, 425-434.

727

728   Williams, K. S., & Gilbert, L. E. (1981). Insects as selective agents on plant vegetative

729   morphology - egg mimicry reduces egg-laying by butterflies. *Science,* 212, 467-469.

730

731   Yuan, F., Bernard, G. D., Le, J., & Briscoe, A. D. (2010). Contrasting modes of evolution

732   of the visual pigments in *Heliconius* butterflies. *Molecular Biology and Evolution,* 27,

733   2392-2405. doi: 10.1093/molbev/msq12.

734

735   Zaccardi, G., Kelber, A., Sison-Mangus, M. P., & Briscoe, A. D. (2006). Color

736   discrimination in the red range with only one long-wavelength sensitive opsin. *Journal of*

737   *Experimental Biology,* 209, 1944-1955. doi: 10.1242/jeb.02207

738

739

740   TABLE 1. Percentage of *Heliconius* egg, egg mimic, pollen flower and wing colors with

741   chromatic just noticeable difference (JND) values >1 for male and female *H. erato* eyes.

	N	Male (%)	Female (%)
H. erato eggs. vs. *Passiflora biflora* egg mimics	16	100%	100%
Psychotria tomentosa yellow flowers vs. red bracts	16	100%	100%
Psychotria tomentosa red bracts vs. green leaves	16	100%	100%
Psiguria warcewiczii inner corolla yellow vs. outer orange petals	25	80%	96%
Psiguria warcewiczii outer orange petals vs. green leaves	25	100%	100%
Heliconius vs. *Eueides* wing dorsal yellow†	144	78.5%	45.1%
Heliconius vs. *Eueides* wing ventral yellow†	117	87.2%	84.6%

742

743   NOTE.—Two systems are modeled: male and female *H. erato* eyes under high light,

744   sunny illumination. The male eye includes UV1, B and L opsins, the female eye includes

745   UV1, UV2, B and L opsins. The red receptor found in both sexes is not included in the

746   visual modeling because their relative abundance is unknown.

747   †From Table 1 of McCulloch et al. (2017).

748

749   FIGURE LEGENDS

750   Figure 1: Normalized spectral sensitivities of the photoreceptors in adult (A) female and

751   (B) male *H. erato* based on recorded intracellular spectral sensitivity maxima (McCulloch

752   et al. 2016 *a, b*). The UV photoreceptor (dark purple), encoded by UVRh1, has a peak

753   sensitivity at 355 nm, the violet photoreceptor (light purple), encoded by UVRh2, has a

754   peak sensitivity at 390 nm, the blue photoreceptor (blue), encoded by the blue opsin has a

755   peak sensitivity of 470 nm and the green photoreceptor (green), encoded by the LWRh

756   opsin, has a peak sensitivity at 555 nm. A fifth known receptor class, with a peak at 600

757   nm due to filtering of the green rhodopsin by a red filtering pigment is not shown. Dotted

758   lines represent the wavelength of peak transmission of the narrow bandpass fibers, 380

759   nm, 390 nm, 400 nm and 436 nm, used in discrimination tests. Male *H. erato* (B)*,* lacking

760   the UV photoreceptor (dark purple) are unable to discriminate between 380 and 390 nm

761   light. *H. melpomene* and *Eueides isabella,* for different reasons, do not express the

762   UVRh2 (light purple) opsin protein in their eye.

763

764   Figure 2: Experimental design of behavioral trials and experimental apparatus. (A)

765   Female and male butterflies of three species, *Heliconius erato, H. melpomene* and *E.*

766   *isabella* were reciprocally trained to associate honey water with a rewarded light (+) and

767   tested using an apparatus (B) consisting of a rewarded light and an unrewarded light (-).

768   Butterflies were trained and tested on their ability to discriminate 380 nm (right) from

769   390 nm (left) and 400 nm from 436nm lights (not shown). Shown is a male *H. erato*

770   butterfly that has just landed on the light source apparatus during a trial.

771

772   Figure 3: Percent correct choices to the rewarded (+) wavelength of 390 nm by *H. erato*

773   females (A) and males (B), *H. melpomene* females (C) and males (D), and *E. isabella*

774   females (E) and males (F) when given a choice between 390 nm (+) and 380 nm (-) light

775   under varying intensities.

776

777   Figure 4: Percent correct choices to the rewarded (+) wavelength of 400 nm by *H. erato*

778   females (A) and males (B), *H. melpomene* females (C) and males (D), and *E. isabella*

35

779   females (E) and males (F) when given a choice between 400 nm (+) and 436 nm (-) light

780   under varying intensities.

781

782   Figure 5: Reflectance spectra (top) and color contrasts (bottom) of *H. erato* eggs (dark

783   grey line) and egg mimics (orange line) found on the leaves (green line) of *H. erato* host

784   plant, *Passiflora biflora.* Shaded areas correspond to 95% confidence intervals, N=4.

785   Black lines indicate UV1, UV2, blue, and green photoreceptors' normalized spectral

786   sensitivities. Not shown is the red receptor that is the result of filtering the green receptor

787   with a red filtering pigment. Data from McCulloch et al. (2016 *a*, *b*). Bottom: Color

788   contrasts between *H. erato* eggs and *P. biflora* leaves (N=16)(left) and between *P. biflora*

789   egg mimics and *P. biflora* leaves (N=16)(right) in just noticeable differences (JNDs).

790   Whiskers correspond to upper and lower limits. The absolute threshold is 1 JND;

791   however in butterflies, the receptor noise levels are not well known so this is an

792   approximation. Inset: *P. biflora* photograph with yellow arrow indicating egg mimic by C

793   T Johansson. Source: Wikimedia: CC BY (https://creativecommons.org/licenses/by/3.0).

794

795   Figure 6: Reflectance spectra (top) and color contrasts (bottom) of *Psychotria tomentosa*

796   yellow inflorescence (yellow line), red bracts (red line) and green leaves (green line)*,* a

797   plant from which *Heliconius* butterflies collect pollen*.* Shaded areas correspond to 95%

798   confidence intervals, N=4-5. Black lines indicate UV1, UV2, blue, and green

799   photoreceptors' normalized spectral sensitivities. Data from McCulloch et al. (2016 *a*, *b*).

800   Bottom: Color contrasts between yellow inflorescence and red bracts (N=16)(left) and

801   red bracts and green leaves (N=16)(right) in just noticeable differences (JNDs). Whiskers

802   correspond to upper and lower limits. The absolute threshold is 1 JND; however in

803   butterflies, the receptor noise levels are not well known so this is an approximation. Inset:

804   *P. tomentosa* photograph with yellow arrow indicating the yellow inflorescence.

805   Surrounding the inflorescence are red bracts.

806

807   Figure 7: Reflectance spectra (top) and color contrasts (bottom) of *Psiguria*

808   *warcewiczii*—yellow flower center (yellow line), outer orange petals (red line), light

809   green corolla (dotted green line) and green leaves (solid green line)—a plant from which

810   adult *Heliconius* butterflies collect pollen. Shaded areas correspond to 95% confidence

811   intervals, N=5-9. Black lines indicate UV1, UV2, blue, and green photoreceptors'

812   normalized spectral sensitivities. Data from McCulloch et al. (2016 *a*, *b*). Bottom: Color

813   contrasts between yellow flower center and outer orange petals (N=25) (left) and between

814   outer orange petals and green leaves (N=25) (right) in just noticeable differences (JNDs).

815   Whiskers correspond to upper and lower limits. The absolute threshold is 1 JND;

816   however in butterflies, the receptor noise levels are not well known so this is an

817   approximation. Inset: *P. warcewiczii* photograph. Photo credit: Steven Paton,

818   Smithsonian Tropical Research Institute. Reprinted with permission.

819

820   SUPPORTING INFORMATION

821   Supporting Figure 1: Percent correct choices to the rewarded (+) wavelength of 380 nm

822   by *H. erato* females (A) and males (B), *H. melpomene* females (C) and males (D), and *E.*

823   *isabella* females (E) and males (F) when given a choice between 380 nm (+) and 390 nm

824   (-) light under varying intensities.

825

826   Supporting Figure 2: Percent correct choices to the rewarded (+) wavelength of 436 nm

827   by *H. erato* females (A) and males (B), *H. melpomene* females (C) and males (D), and *E.*

828   *isabella* females (E) and males (F) when given a choice between 436 nm (+) and 400 nm

829   (-) light under varying intensities.

830

831   Supporting Video 1: A male *H. erato* butterfly is shown flying towards, and landing, on

832   the light source apparatus during a trial. The light wavelengths presented are 390 nm

833   (left) and 380 nm (right). The male chose 380 nm, the unrewarded stimulus, while it was

834   presented at 15x brighter than the rewarded light stimulus of 390 nm.

Figure 1

A Experimental design

B

390 nm (+)   380 nm (-)

honey water (reward)   H₂O (control)
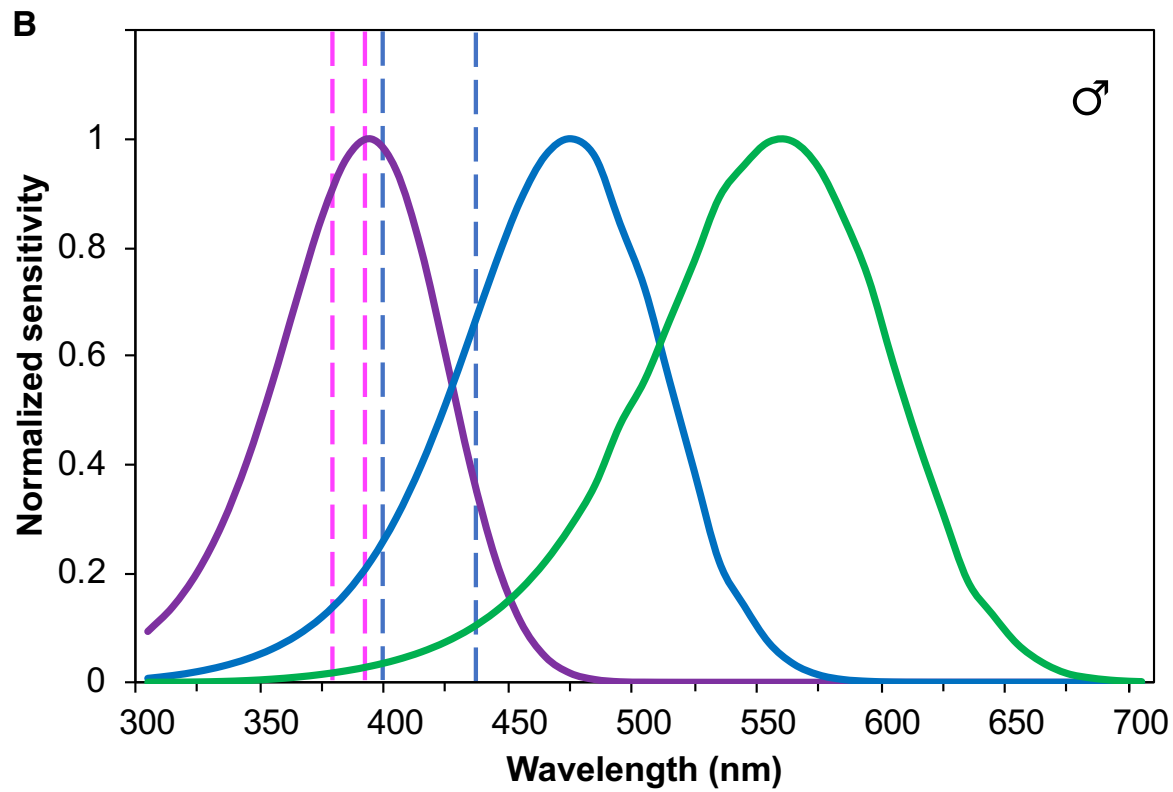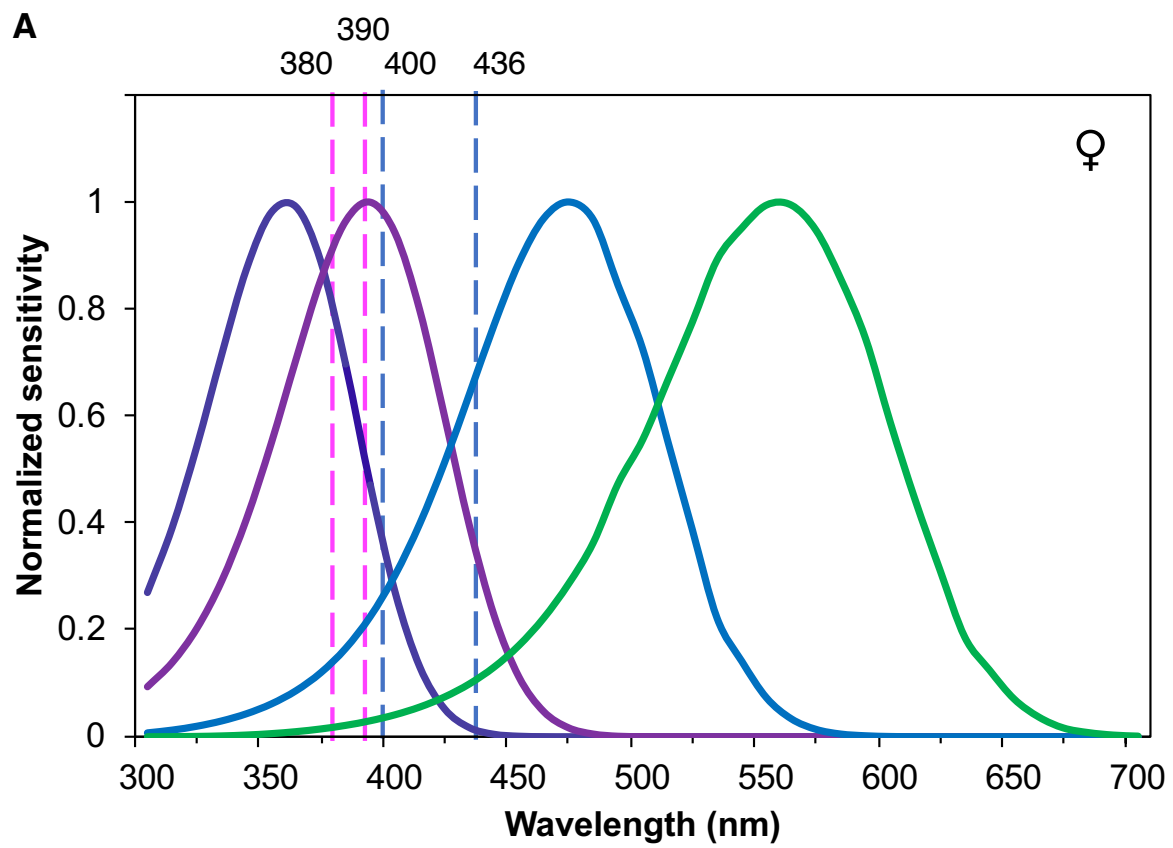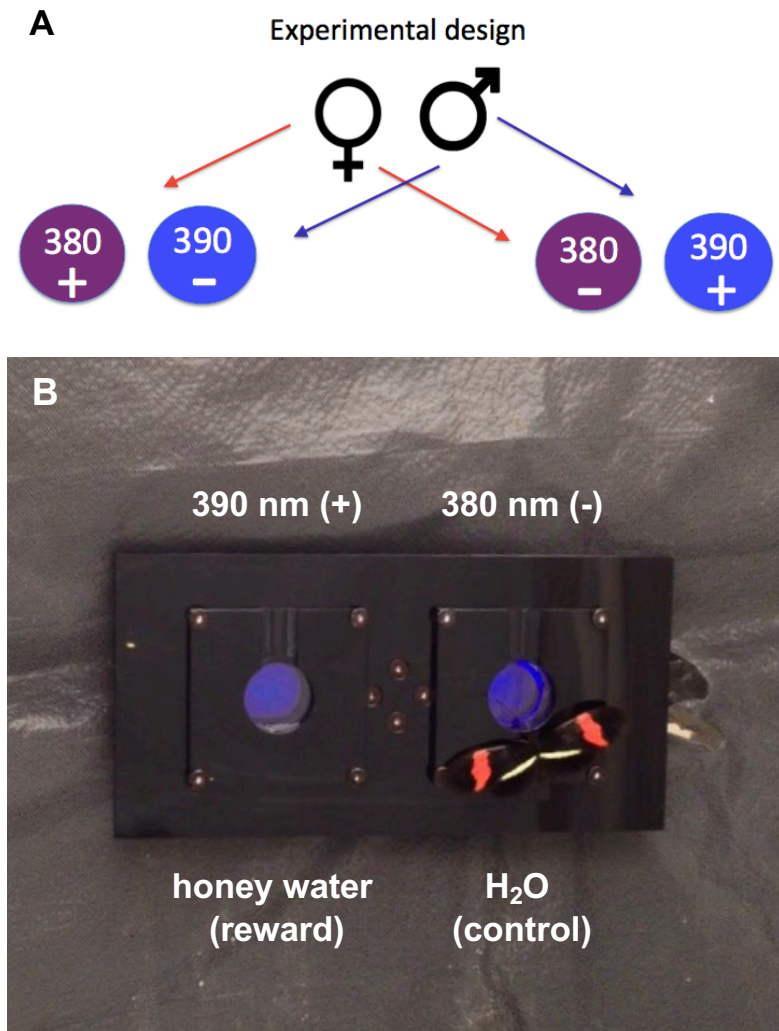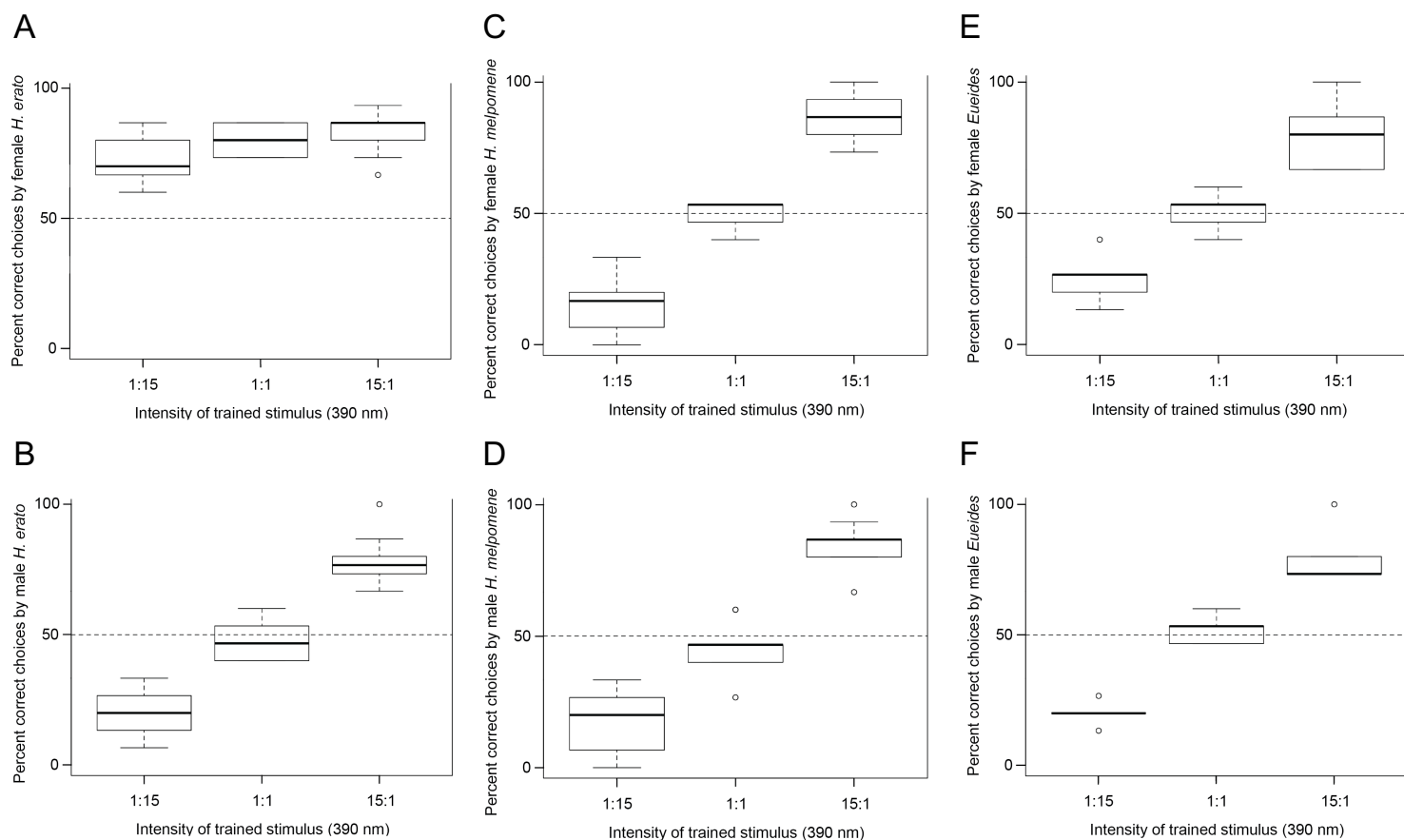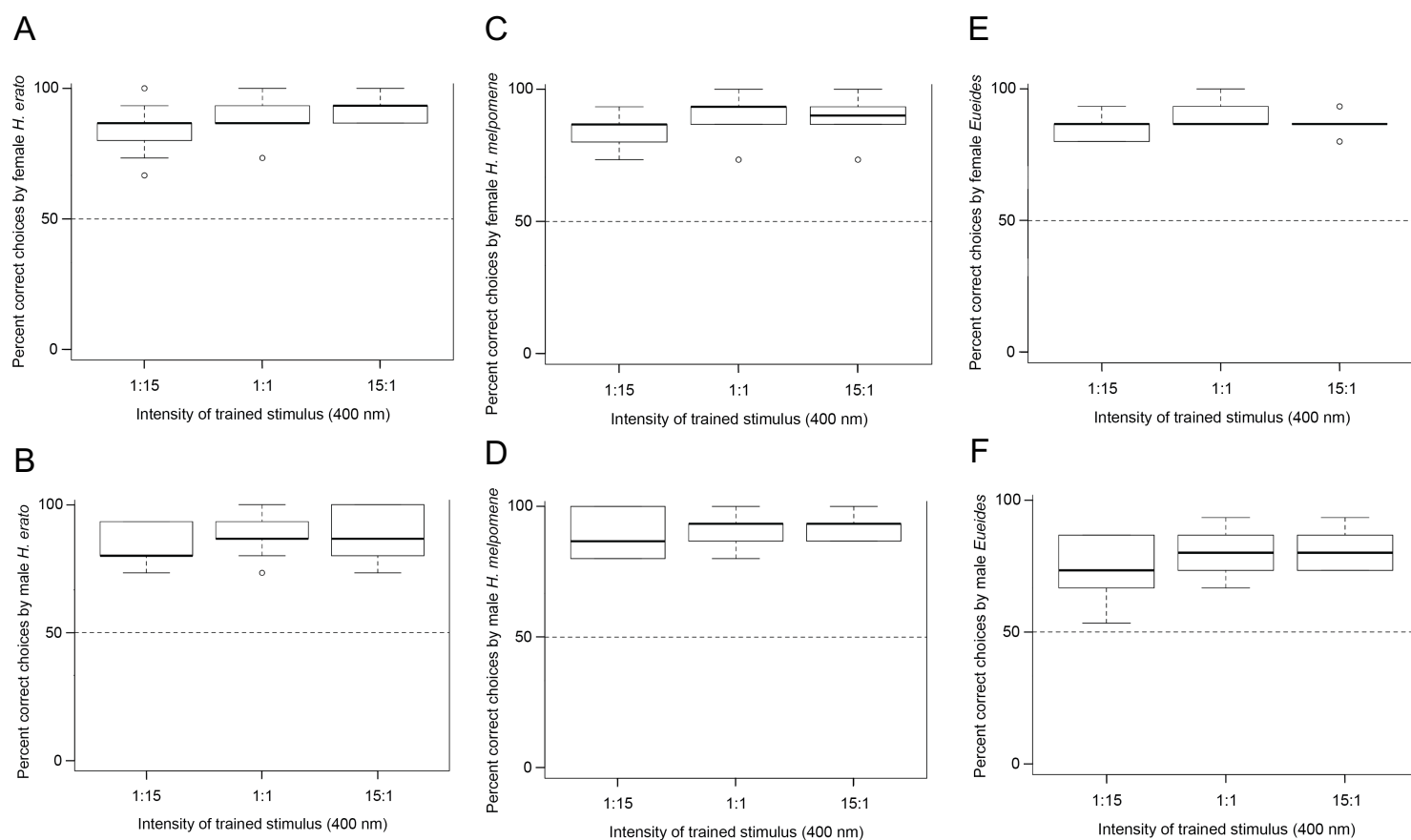
Figure 2

Figure 3

Figure 4
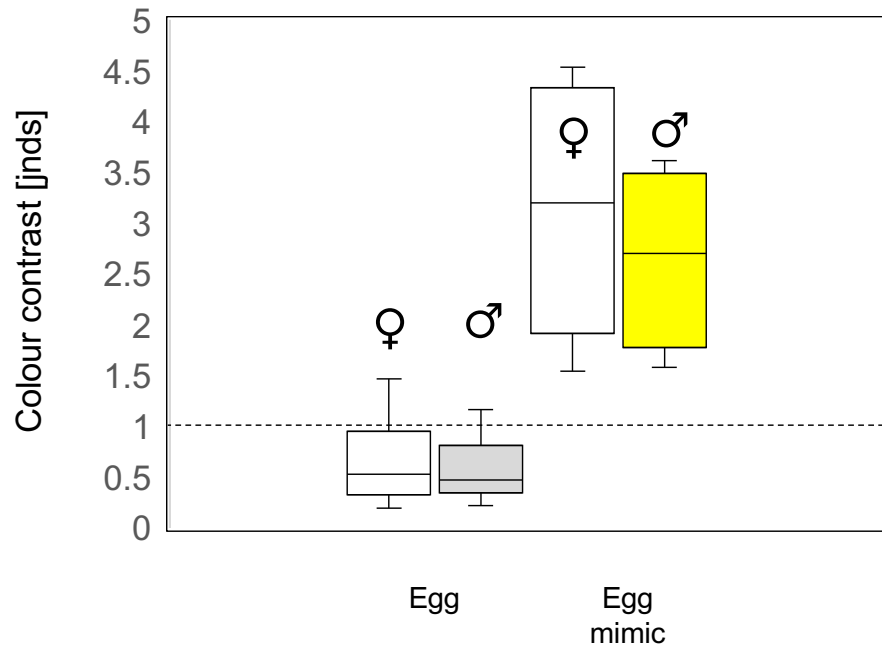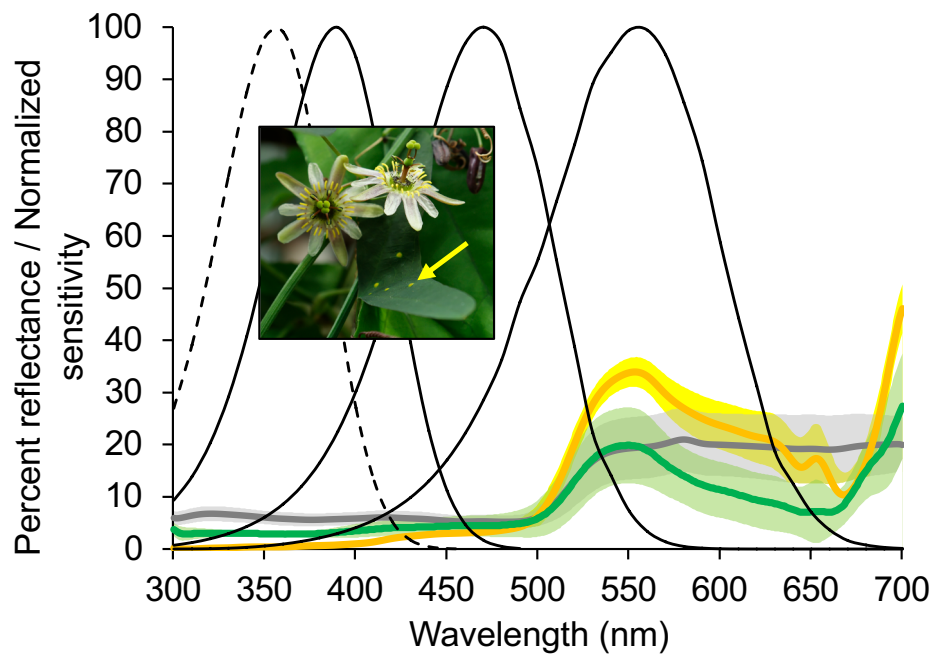
Figure 5

Figure 6

Figure 7