

### 3-OHK and UV signaling in *Heliconius*

## 1 **Ultraviolet and yellow reflectance but not fluorescence is important for visual** 2 **discrimination of conspecifics by *Heliconius erato***

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18 Key words: Visual signal, fluorescence, ultraviolet, predation, mate preference, light  
19 environment

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28 **Summary statement:** *Heliconius* butterflies use a co-opted yellow pigment for communication,  
29 while predators are fooled by non-*Heliconius* mimics using ancestral yellow pigments.

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## 3-OHK and UV signaling in *Heliconius*

### 31 **Abstract**

32 Toxic *Heliconius* butterflies have yellow hindwing bars that – unlike their closest relatives –  
33 reflect ultraviolet (UV) and long wavelength light, and also fluoresce. The pigment in the yellow  
34 scales is 3-hydroxy-DL-kynurenine (3-OHK), found also in the hair and scales of a variety of  
35 animals. In other butterflies including pierids, which similarly display wing colors that vary in  
36 both the UV and the human-visible range, behavioral experiments have indicated that only the  
37 UV component is most relevant to mate choice. Whether in *Heliconius* butterflies it is the UV,  
38 the human-visible yellow, and/or the fluorescent component of yellow wing coloration that is  
39 relevant to mate choice is unknown. In field studies with butterfly paper models we show that  
40 both UV and 3-OHK yellow act as signals for *H. erato* but attack rates by birds do not differ  
41 significantly between the models. Furthermore, measurement of the quantum yield and  
42 reflectance spectra of 3-OHK indicates that fluorescence does not contribute to the visual signal  
43 under broad-spectrum illumination. Our results suggest that the use of 3-OHK pigmentation  
44 instead of ancestral yellow was driven by sexual selection rather than predation.

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#### 52 **Introduction**

53           Color patches of animals are complex traits composed of multiple components (Grether  
54 et al., 2004). The pigment cells known as chromatophores in the skin of fishes, reptiles and  
55 amphibians for example are color-generating structures comprised of distinct pigmentary and  
56 structural layers that vary in their ability to reflect light. The feather barbs or integument of birds  
57 or the wing scales of butterflies similarly have diverse nano-structure architectures, thin films,  
58 and pigments, which produce a dazzling array of colors (Prum and Torres, 2003; Vukusic and  
59 Sambles, 2003; Shawkey and Hill, 2005; Stavenga et al., 2011, 2014). These pigmentary and  
60 structural components of color patches work in tandem to produce signals used in a variety of  
61 contexts (e.g., crypsis, mimicry, aposematism, and mate choice). Since the biochemical and  
62 developmental mechanisms underlying pigmentary and structural properties of color differ, each  
63 of these components may be subject to different selective pressures, and hence independent  
64 evolutionary trajectories (Grether et al., 2004). Here we look specifically at how two components  
65 of a butterfly visual display, UV reflectance and human-visible yellow reflectance due to  
66 selective filtering by a specific wing pigment, may function as a signal in mate choice and  
67 predation. We also look at what contribution fluorescence makes, if any, to the signal.

68           Many butterfly species have colorful wing patterns in both the human-visible (400-700  
69 nm) and in the UV (300-400 nm) ranges (Silberglied and Taylor, 1978; Eguchi and Meyer-  
70 Rochow, 1983; Meyer-Rochow, 1991; Rutowski et al., 2005; Briscoe et al., 2010). While the  
71 idea that UV coloration—invisible to humans—may serve as a 'private channel' of  
72 communication has been challenged (Cronin and Bok, 2016; but see Cummings et al., 2003),  
73 there is ample evidence that UV signals are important in animal communication (Rutowski,  
74 1977; Johnsen et al., 1998; Smith et al., 2002; Cummings et al., 2003; Robertson and Monteiro,  
75 2005; Kemp, 2008; Obara et al., 2008; Detto and Blackwell, 2009; Painting et al., 2016). On the  
76 other hand, although many butterflies have UV-visible color patches, in the absence of  
77 behavioral evidence, it is unclear whether the UV reflectance functions as a signal, or if it is  
78 simply an epi-phenomenon of the scale structure overlaying pigment granules. The same  
79 question can of course be applied to the colors produced by the pigments.

80           Studies of several butterfly groups suggest in fact that for color patches with both UV and  
81 visible reflectance, only variation in the UV component of the signal affects mate choice. Pierid  
82 butterfly males, *Colias eurytheme* and *C. philodice*, have forewing colors with both UV-

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83 iridescence due to the structural scattering of light by the scale lamellae (Ghiradella, 1974) and  
84 yellow-orange due to pterin pigments (Watt, 1964). In behavioral experiments, female *Colias*  
85 were shown to use the UV-reflection difference between the two species as a mate and species  
86 recognition cue, but not the human-visible color difference (Silberglied and Taylor, 1978).  
87 Female *Eurema hecuba* (Coliadinae: Pieridae) were similarly shown to prefer males with the  
88 brightest UV iridescence overlaying a diffuse pigment-based yellow (Kemp, 2007a). Given that  
89 many other butterflies have color patches with UV-visible reflectances, and that butterfly color  
90 vision systems are astonishingly diverse (Arikawa et al., 2005; Briscoe and Bernard, 2005;  
91 Stalleicken et al., 2006; Koshitaka et al., 2008; Sison-Mangus et al., 2008; Chen et al., 2013), it  
92 is worthwhile to investigate in other species whether it is the UV or the human-visible or both  
93 parts of the color patch reflectance spectrum that is being used for signaling. It is particularly  
94 interesting to investigate this question where there has been a phylogenetic transition from using  
95 one type of pigmentation to another, as for the yellow wing colors in the passion-vine butterflies  
96 of the genus *Heliconius* (Briscoe et al., 2010; Bybee et al., 2012)(see below).

97 *Heliconius erato* has yellow scales on its hindwings that contain the pigment 3-hydroxy-  
98 DL-kynurenine (3-OHK) (Tokyuama et al., 1967; Reed et al., 2008). The yellow bars reflect UV  
99 light and have a step-like reflectance at longer wavelengths—a rapid rise then a plateau in  
100 reflectance in the visible (400-700 nm) range (yellow lines, Fig. 1A,B)(see also Stavenga et al.,  
101 2004). Either the UV or the human-visible part of 3-OHK wing reflectance or both may serve as  
102 a signal for inter- and intra-specific communication. Intriguingly, 3-OHK's appearance in  
103 *Heliconius* co-occurred with the evolution of the butterflies' duplicated UV opsins, UV1 and  
104 UV2 (Briscoe et al., 2010; Yuan et al., 2010; Bybee et al., 2012). In some *Heliconius* species,  
105 UV1 and UV2 are found in both males and females (McCulloch and Briscoe, unpublished data).  
106 In *H. erato*, UV1 is a female-specific UV receptor with  $\lambda_{\max}$ =355 nm, while UV2 is a violet  
107 receptor with  $\lambda_{\max}$ =390 nm found in both sexes (Fig. 1, triangles and x marks, respectively)  
108 (McCulloch et al., 2016).

109 In addition to the components of the 3-OHK visual signal mentioned above, the yellow  
110 wing bars of *Heliconius* fluoresce under a hand-held blacklight (Movie S1). Fluorescence occurs  
111 when short-wavelength light is absorbed and then re-emitted as a longer wavelength, i.e. lower  
112 energy light. Fluorescent pigments are widespread in nature (Vukusic and Hooper, 2005; Lagorio  
113 et al., 2015) and are typically identified using spectrally narrow-band light; however, terrestrial

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114 illumination has a broad spectrum so it is unclear whether or not a pigment's fluorescence  
115 contributes much to a potential signal under natural conditions. The emission spectra of the 3-  
116 OHK pigment overlaps with the visible part of the reflectance spectrum of 3-OHK on *Heliconius*  
117 wings (see below) and so would be well-suited to being detected by the blue-sensitive receptor of  
118 *H. erato* with  $\lambda=470$  nm if it did (McCulloch et al., 2016).

119 Butterflies from the genus *Eueides*, which are a sister taxon to *Heliconius*, have mimetic  
120 wing patterns strikingly similar to some *Heliconius* species. These two genera co-occur in the  
121 same habitats, yet their yellow wing pigments lack the step-like reflectance spectrum of 3-OHK  
122 (grey line, Fig. 1A,B) (Bybee et al., 2012), and they do not fluoresce (data not shown). The  
123 yellow pigments in both butterflies appear similar to the human eye in natural light, but their  
124 spectra differ strongly (yellow and grey lines, Fig. 1A,B). Although modeling of wing colors  
125 suggests in principle that *Heliconius* can distinguish between *Heliconius* 3-OHK yellow and  
126 *Eueides* yellow (Bybee et al., 2012), it remains unknown whether *Heliconius* actually do so in  
127 nature. Previous work has shown that *H. erato* prefer chromatic over achromatic signals in the  
128 context of mate choice (Fig. S1) (Finkbeiner et al., 2014); but it is unclear whether the visible,  
129 the UV, or both parts of the reflectance spectrum of 3-OHK and fluorescence contribute to  
130 signaling. Prior work has also shown that avian predators will differentially attack achromatic  
131 compared to chromatic butterfly paper models (Fig. S1) (Finkbeiner et al., 2014; Dell'Aglio et  
132 al., 2016), but it is unknown whether avian predators will differentially attack butterfly paper  
133 models that vary in yellow coloration resembling the differences between *Heliconius* and  
134 *Eueides* yellow. While *Heliconius* wing color patterns warn avian predators of their toxicity  
135 (Benson, 1972; Chai, 1986), 3-OHK may further serve as a conspecific signal especially in  
136 courtship (Bybee et al., 2012; Llaurens et al., 2014). Demonstrating that *Heliconius* species can  
137 in fact discriminate 3-OHK yellow from other yellows in nature is an important step in  
138 elucidating the adaptive significance of 3-OHK pigmentation.

139 To further investigate the contribution of 3-OHK to *Heliconius erato* signaling, we  
140 carried out two sets of experiments: The first set tested responses of both male and female *H.*  
141 *erato* to four types of colored models, whose spectra were intended to approximate either those  
142 of *Heliconius* species or their mimics, such as *Eueides*. The first pair of spectra, which are  
143 designated Y+ or Y-, resemble 3-OHK (*Heliconius*) yellow or *Eueides* yellow. The second set of  
144 reflectance spectra have identical yellow and red coloration in the visible range, but UV

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145 reflectance is either present (UV+) or absent (UV-).

146 The second, complementary set of experiments tests the hypothesis that predatory birds  
147 will not differentially attack 3-OHK yellow from other yellows when presented with model  
148 butterflies due to the aposematic function of yellow in general. Together these experiments  
149 substantiate and elaborate our understanding of the function of 3-OHK yellow and UV  
150 coloration. We show also that fluorescence – although clearly visible in laboratory conditions,  
151 but with illumination restricted to the UV excitation wavelengths – is not likely to have any  
152 impact under the broadband and relatively low UV illumination found in nature.

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154

#### 155 **Material and methods**

##### 156 ***Butterfly Models, Wing Reflectance Spectra, Environmental Light and Discriminability***

157 Four paper model types of the *Heliconius erato petiverana* butterfly were made as  
158 described in Finkbeiner et al. (2012) with their colors modified as follows: with (Y+) and  
159 without (Y-) 3-OHK yellow, and with (UV+) and without (UV-) ultraviolet reflectance. The Y+  
160 treatment had 3-OHK on the yellow portion of the wing (0.010 mg/μl and 0.015 mg/μl 3-OHK in  
161 methanol applied to the ventral and dorsal sides, respectively). This provided the models with the  
162 same pigment as found in the butterfly yellow scales. The yellow portion of the non-3-OHK  
163 yellow models (Y-) was covered with yellow Manila paper (Creatology® Manila Drawing Paper,  
164 Item No. 410590). Manila paper has a reflectance spectra that resembles non-3-OHK yellow  
165 reflectance from the sister-genus to *Heliconius*, *Eueides*, which is a *Heliconius* mimic (Bybee et  
166 al., 2012) (Fig. 1A,B, grey and black lines). A thin film UV filter (Edmund Optics, Item No. 39-  
167 426) was placed over the Manila paper to create a closer match to *Eueides* yellow pigment. As a  
168 control, Mylar film was added to the yellow portions of models with 3-OHK for the Y+  
169 treatment. Mylar film resembles the UV filter but acts as a neutral-density filter. The red  
170 portions of the wings were identical in both Y+ and Y- treatments.

171 For the UV+ models, an odorless UV-reflective yellow paint (Fish Vision™) was added  
172 to the dorsal and ventral yellow band of the model wings to provide UV reflectance (Fig. 1A,B,  
173 blue line), and the red portions of the wings were printed as described in Finkbeiner et al. (2014).  
174 For UV- models, a thin film UV filter was placed over both the yellow and red/pink UV-  
175 reflective portions on the wings. The UV filter prevents any light reflectance up to 400 nm (Fig.

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176 1A-D, blue-green line). Mylar film was added to the yellow and red/pink portions of models  
177 used for the UV+ treatment to function as a control.

178 Reflectance spectra of the paper models and individual *Heliconius erato petiverana*  
179 (n=15), *Eueides isabella*, *E. surdus*, *E. thales* (n=3/species) and *E. heliconoides* (n=2) butterfly  
180 wings were measured by first aligning each measured wing in the same orientation as shown in  
181 appendix B of Bybee et al. (2012). If the viewer was looking directly from above at the oriented  
182 wings, the fixed probe holder (Ocean Optics RPH-1) was placed horizontally on top of the wing  
183 such that the axis of the illuminating and detecting bifurcating fiber (Ocean Optics R400-7-  
184 UV/VIS) was at an elevation of 45° to the plane of the wing and pointed left with respect to the  
185 body axis. Illumination was by a DH-2000 deuterium-halogen lamp, and reflectance spectra were  
186 measured with an Ocean Optics USB2000 spectrometer. A spectralon white standard (Ocean  
187 Optics WS-1) was used to calibrate the spectrometer. For the irradiance spectra measurements,  
188 the USB2000 spectrometer, a calibrated tungsten light source (Ocean Optics LS-1-CAL), a 100  
189 or 400 μm diameter fiber (Ocean Optics P100- or P400-2-UV-Vis) and cosine corrector (Ocean  
190 Optics CC-3-UV), which produces vector irradiance measures, were used (Cronin et al., 2014).  
191 Five irradiance spectra measurements of down-dwelling light were taken and averaged per site.

192 For the mate choice experiments, the von Kries' transformed quantum catches for stimuli  
193 (Kelber et al., 2003) were first calculated for *H. erato* males and *H. erato* females separately  
194 using high light intensity and sunny cage irradiance spectra. Pairwise discriminabilities between  
195 artificial models and natural wing reflectance spectra were determined using a trichromatic  
196 vision model for *H. erato* males and tetrachromatic vision models for *H. erato* females,  
197 respectively (Vorobyev and Osorio, 1998). Parameters for the butterfly visual models were as  
198 follows: Weber fraction=0.05 (Koshitaka et al., 2008), photoreceptor peak sensitivities,  
199  $\lambda_{\max}$ =355 nm (female only), 390 nm, 470 nm and 555 nm, and relative abundances of cones,  
200 VS=0.13, B=0.2, G=1 (male) or UV=0.09, VS=0.07, B=0.17, G=1 (female) (McCulloch et al.,  
201 2016). For the predation experiments, von Kries' transformed quantum catches for only ventral  
202 wing stimuli (since the butterflies were presented with their wings folded) were calculated using  
203 high light intensity and irradiance spectra from two of the four habitats where the models were  
204 placed: forest cover and forest edge. (The other two habitats, Pipeline Road and paved road,  
205 were found to have normalized spectra that were identical to forest cover). Discriminabilities  
206 between stimuli were determined using tetrachromatic models of bird vision representing two

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207 types of avian visual system, the UV- (blue tit, *Parus caeruleus*) and violet-type (chicken,  
208 *Gallus gallus*) systems. For chicken, we used ocular media of Toomey et al. (2016) and  
209 behaviorally-determined parameters of Olsson et al. (2015), namely, a Weber fraction=0.06 for  
210 the L cone, and relative abundances of cones (VS=0.25, S=0.5, M=1, L=1). For the blue tit, we  
211 followed the work of Hart et al. (2000) including the effects of blue tit ocular media and used a  
212 Weber fraction=0.05 for the L cone, and relative abundances of cones (UV=0.37, S=0.7,  
213 M=0.99, L=1).

214

#### 215 ***Mate Preference Experiments***

216 To test whether *Heliconius* 3-OHK yellow and UV serve as visual signals for conspecifics,  
217 mate preference experiments were carried out using insectary facilities in Gamboa, Panama  
218 from September 2013 through February 2014. Data were collected from 80 wild-caught *H.*  
219 *erato petiverana* butterflies: 40 males and 40 females. Each butterfly was introduced  
220 individually into experimental cages (2 m × 2 m × 2 m) and presented with one of two pairs of  
221 the artificial butterfly models: Y+ versus Y-, or UV+ versus UV-. The models were separated  
222 by 1 m and attached to an apparatus used to simulate flight (see Finkbeiner et al., 2014). Movies  
223 2 and 3 in Supplementary Information show an example of female butterfly trials with Y  
224 (Movie S2) and UV (Movie S3) models. Individual butterflies experienced six five-minute trials  
225 – three five-minute trials with each of the two pairs. During trials two variables were recorded:  
226 1) approaches, which consisted of flight unequivocally directed toward the model, and in which  
227 the butterfly came within 20 cm of the model, and 2) courtship events, which were classified as  
228 sustained hovering or circling behavior around the model (for examples see Videos 2 and 3 in  
229 Finkbeiner et al., 2014). Mate preference data were analyzed using a two-way ANOVA in R to  
230 examine the effects of model type and sex. Measurements of spectral irradiance (see above)  
231 were taken to provide quantitative information about the illumination conditions during the  
232 trials (Fig. S2).

233

#### 234 ***Predation Experiments***

235 Previously we have shown (Finkbeiner et al., 2014) that avian predators differentially  
236 attack achromatic local form butterfly models compared to chromatic models as well as models  
237 that display non-local or color-switched patterns (Fig. S1). Here we tested whether avian

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238 predators would differentially attack local wing color form paper models where UV or yellow is  
239 manipulated. Predation experiments were completed in Panama at the Smithsonian Tropical  
240 Research Institute Gamboa field station and at selected forest sites in Soberanía National Park  
241 (including Pipeline Road), from June through September in 2013. Models were fitted with  
242 plasticine abdomens and tied to branches with thread to represent natural resting postures in the  
243 following habitat types: forest cover (15 sites), forest edge (17 sites), Pipeline Road (unpaved  
244 road with partial forest cover, 55 sites), and paved road with partial forest cover (13 sites).  
245 Examples of foliage cover in each of these habitat types, along with corresponding spectral  
246 irradiance measurements, are presented in Fig. S3. For the 3-OHK yellow pigment study, five  
247 artificial models of each treatment (Y+ and Y-) were randomly placed in 100 forest sites  
248 (Finkbeiner et al., 2014). The sites were separated ~250 meters to account for avian predator  
249 home range (home ranges described in Finkbeiner et al., 2012). There were 500 Y+ models and  
250 500 Y- models for a total of 1000 models. The same methods were used for the UV study, using  
251 500 UV+ models and 500 UV- models in non-overlapping sites from the Y+/- models.

252 The models remained at their sites for four days, and each model was examined for  
253 evidence of predation. A butterfly was considered attacked if damage to the abdomen and wings  
254 appeared in the form of beak marks and/or large indentations in the abdomen (for examples of  
255 attacked models see Finkbeiner et al., 2012; Finkbeiner et al., 2014). The attack response was  
256 modeled as a binomial variable (yes or no) dependent upon butterfly model type using a zero-  
257 inflated Poisson regression model, including sites as a random effect, in R with the ‘pscl’  
258 package (Zeileis et al., 2008; R Development Core Team, 2010; Jackman, 2011). To examine  
259 whether forest light environment affected predator behavior, the same analysis was used to  
260 compare predation between model types in four main habitat types: forest cover, forest edge,  
261 Pipeline Road (unpaved road with partial forest cover), and paved road with partial forest cover.

262

#### 263 ***Fluorescence Experiments***

264 To determine the possible contribution of 3-OHK fluorescence to its yellow coloration  
265 we measured the absorption, excitation, and emission spectra of 1.5 mg 3-hydroxy-DL-  
266 kynurenine (3-OHK) (Sigma-Aldrich, Catalog No. H1771) in 3 ml methanol (Fisher Chemicals,  
267 Optima LC/MS grade, Catalog No. A456-1). The resultant solution was diluted to an optical  
268 density OD=0.3 to get it within the linear range for fluorescence measurement (Dhami et al.,

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269 1995). The absorption spectrum of the pigment was measured with a Cary-50 spectrometer  
270 (Varian), while the emission and excitation spectra was acquired with a Cary Eclipse fluorimeter  
271 (Varian). Quantum yield was determined using Coumarin 500 (Exciton, Catalog No. 05000) as a  
272 reference. The reflectance spectrum measurements of *H. erato* wings were made using an Ocean  
273 Optics USB2000 spectrometer, a UV-cut off filter (Edmund Optics #39-426), a 150 W Xenon  
274 Arc lamp (which resembles daylight illumination), and spectralon white standard.

275

276

## 277 **Results**

### 278 ***Discriminabilities of Model Spectra and Real Wings***

279 To test the hypothesis that our Y+ and UV+ paper models resembled real *H. erato* yellow  
280 wing colors, and that our Y- and UV- paper modeled resembled real *Eueides* yellow wing colors,  
281 we calculated pairwise discriminabilities between real wings and model spectra. We did so for  
282 the male and female *H. erato* visual system, and then for the UV- and violet-type avian visual  
283 systems. We found that for both male and female *H. erato* eyes, Y+ was an excellent match to *H.*  
284 *erato* dorsal and ventral yellows, and that Y- and UV- were excellent matches to *Eueides* dorsal  
285 and ventral yellows under high light illumination (Table 1, 66.7-100% of pairwise comparisons  
286 fell below 1 JND and 100% fell below 2 JNDs). This means that under lower light levels, model  
287 spectra would be an even better match to real wings. For the UV+ treatment, only ventral yellow  
288 was an excellent match to the *H. erato* ventral yellow for either *H. erato* sex. From this we  
289 conclude that the Y+ paper model bears a strong resemblance to real *H. erato* yellow wings and  
290 the Y- paper model bears a strong resemblance to real *Eueides* yellow wings for *H. erato*  
291 butterflies under the experimental illuminant conditions in which they were tested.

292 For the UV- and VS-type avian visual systems, the match between Y+ and UV+ and *H.*  
293 *erato* ventral yellow and between Y- and UV- *Eueides* ventral yellow was less good than if these  
294 same stimuli were viewed by the butterflies (Table 2). These results indicate that for birds at  
295 least, under forest shade or edge illumination, no pair of stimuli fully captured the spectral  
296 differences between *Heliconius* or *Eueides* yellow wing colors. All pairs of model spectra used  
297 in behavioral experiments, however, differed by >1JND for both birds and butterflies (except for  
298 Y+ vs. Y- for ventral yellow viewed through the male eye)(Table 3). This indicates that for both  
299 birds and butterflies, there was sufficient difference between the four model types to potentially

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300 elicit a behavioral response in the experiments described below.

301

#### 302 ***Experiment 1: Effect of model type on mate preference***

303 To determine how *Heliconius* yellow and UV affect conspecific recognition, we  
304 presented wild-caught *H. erato* butterflies with artificial butterfly models that had manipulated  
305 yellow and UV coloration. Preference toward models was measured in the form of approaches  
306 and courtship events. We found a strong model type effect on the number of butterfly approaches  
307 toward 3-OHK yellow and UV models. There were significantly more approaches toward Y+  
308 than Y- models (Two-way ANOVA,  $F=16.287$ ,  $p<0.0001$ ,  $n=80$ ), and toward UV+ than UV-  
309 models ( $F=10.469$ ,  $p=0.002$ ,  $n=80$ ; Fig. 2A, black lines). There was no apparent effect of sex on  
310 butterfly approach behavior ( $F=2.738$ ,  $p=0.099$ ,  $n=80$  for Y;  $F=0.049$ ,  $p=0.952$ ,  $n=80$  for UV),  
311 suggesting that males and females approach the models at equal rates. Specific male and female  
312 behaviors for all comparisons are illustrated in Fig. S4.

313 Regarding courtship behavior, we found a strong model type effect where Y+ models  
314 were courted much more than Y- models ( $F=11.731$ ,  $p=0.0008$ ,  $n=80$ ; Fig. 2A, red lines). The  
315 test for the main effect of sex shows that males court Y models at a significantly higher rate than  
316 females ( $F=9.211$ ,  $p=0.0002$ ,  $n=80$ ). However, we found no significant model type effect on the  
317 number of courtship events directed toward UV+ and UV- models ( $F=2.304$ ,  $p=0.131$ ,  $n=80$ ).  
318 There was also no effect of sex on butterfly courtship behavior toward the UV models ( $F=0.701$ ,  
319  $p=0.498$ ,  $n=80$ ).

320

#### 321 ***Experiment 2: Predator response to 3-OHK yellow and UV in different forest habitats***

322 Previously we showed that birds preferentially attack achromatic *H. erato* models over  
323 Y+ chromatic models (Fig. S1) (Finkbeiner et al., 2014), as expected if chromatic cues serve as  
324 aposematic signals to avian predators. To test whether birds differentially attack UV- or yellow-  
325 manipulated models, predation was measured as the frequency of avian attacks on models in the  
326 forest. A total of 110 avian attacks were recorded (over four days of predator exposure for 500  
327 models of each type): 27 and 24 attacks on Y+ and Y- models, and 27 and 32 attacks on UV+  
328 and UV- models, respectively. Using a zero-inflated Poisson regression model, we detected no  
329 difference in predation between Y+ and Y- models: (z-value=-0.014,  $p=0.989$ ,  $n=1000$ ; Fig. 2B,  
330 blue lines), and no difference in predation between UV+ and UV- models: (z-value=-0.536,

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331 p=0.592, n=1000; Fig. 2B). A test of whether forest type affected predator behavior found no  
332 difference in predation between the model types in forest cover, forest edge, Pipeline Road  
333 (unpaved road with partial forest cover), and paved road with partial forest cover (all p-values  
334 >0.10). Although our prior experiments indicate that avian predators differentially attack  
335 *Heliconius erato* paper models that differ in both red and yellow color and pattern (Finkbeiner et  
336 al., 2014), the results presented here indicate that avian predators do not differentially attack 3-  
337 OHK yellow and other yellow or UV+ and UV- models in field trials.

338

#### 339 ***Fluorescence does not contribute to the yellow signal***

340 The absorption spectrum of 3-OHK has a distinctive peak ( $\lambda_{\max}$ ) at 380 nm (Fig. 3B), so  
341 this wavelength was chosen as the excitation wavelength for fluorescence measurements (10 nm  
342 bandwidth). The excitation spectrum of the pigment (Fig. 3C, black line) is in full agreement  
343 with absorption measurements demonstrating that the 380 nm is the peak excitation wavelength.  
344 The fluorescence of the pigment has a broad spectrum with peak of the emission around 508 nm  
345 (Fig. 3C, green line). Notably, the emission spectra of 3-OHK overlaps well with the visible  
346 portion of *Heliconius* yellow, suggesting the fluorescence of 3-OHK might in principle  
347 contribute to the signal in the visible range.

348 In order to measure the efficiency of this emission, and hence understand if the  
349 fluorescence might contribute significantly to the signal, we determined the fluorescence  
350 quantum yield of 3-OHK. Quantum yield is characterized as the ratio of the number of photons  
351 emitted to the number of photons absorbed (Williams et al., 1983; Nad and Pal, 2003). Quantum  
352 yield was obtained by comparing 3-OHK to that of a standard and well-characterized fluorescent  
353 molecule, Coumarin 500 (Dhami et al., 1995), which has similar absorbance and fluorescence  
354 peaks as 3-OHK (Fig. S5). We were therefore surprised that the quantum yield of 3-OHK in  
355 methanol indicated that the emission is unlikely to be visible under normal illumination  
356 (quantum yield=5.1 x 10<sup>-4</sup>).

357 To be certain that these conclusions for 3-OHK in solution would also apply to 3-OHK  
358 on real wings in daylight illumination, additional experiments were carried out. Reflectance  
359 spectra of *H. erato* wings with and without a neutral-density filter (Mylar film) or a 400 nm cut-  
360 off filter (UV film), using a 150 W xenon arc lamp as a light source (which has a spectrum that  
361 resembles daylight illumination), were measured. If 3-OHK fluorescence does not contribute to

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362 the *Heliconius* yellow signal in broad-spectrum light, then measurements of *H. erato* wing  
363 reflectance spectra using a UV-cut off filter, which blocks excitation, should have no effect on  
364 the measured spectra in the visible range. That is indeed what we observed (Fig. S6). This series  
365 of experiments leads us to conclude that fluorescence does not contribute to the 3-OHK visual  
366 signal under broad-spectrum illumination.

367

368

## 369 **Discussion**

### 370 ***3-OHK coloration is preferred by *Heliconius erato****

371 Butterflies are astonishingly diverse in their coloration, but the phylogenetic origins of  
372 new pigmentary coloration and the evolutionary forces that may have governed the adoption of  
373 a new pigment have rarely been investigated. Previously we showed that 3-OHK pigmentation  
374 is a synapomorphy of the genus *Heliconius*, being an ancestral character for the genus, but  
375 absent for sister genera such as *Eueides* (Briscoe et al., 2010). Here we have attempted to  
376 investigate how 3-OHK pigmentation functions as a signal for *H. erato* mate choice and defense.  
377 *Heliconius* yellow coloration has a spectrum, which includes reflectance maxima in the  
378 ultraviolet and human-visible range as well as fluorescence (Figs. 1A,B; 3A). Evidence here  
379 indicates that both the UV and long wavelength components of the reflectance spectrum  
380 contribute to the visual signal *H. erato* butterflies use for conspecific recognition, but  
381 qualitatively that the UV part may be less important for *H. erato* courtship than it is for  
382 approach behavior. Specifically the butterflies demonstrated clear preferences under all  
383 circumstances for Y+ over Y- (Fig. 2A). It is notable that our discriminability modeling of male  
384 and female *H. erato* vision indicates that for the butterflies at least the Y+ yellows are a good  
385 match to real *H. erato* yellow wing colors and Y- yellows are a good match to real *Eueides*  
386 yellow wing colors (Table 1). These results provide the first empirical evidence that *H. erato*  
387 butterflies prefer 3-OHK yellows to yellows found on the wings of their sister-genera, *Eueides*,  
388 and the first empirical evidence that the evolution of 3-OHK pigmentation in *Heliconius* may  
389 have been driven by sexual selection.

390 The interpretation of the UV+ and UV- treatments is a little less clear. Both UV+ and UV-  
391 models had the same long wavelength reflectance, but differed in the UV. UV+ models were  
392 approached by both sexes more frequently than UV- models, but while there was a trend

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393 towards preferring UV+ models during mating attempts, this difference was non-significant.  
394 This observation is perhaps surprising in view of the idea that at least for birds UV may be a  
395 short-range signal (Stevens and Cuthill, 2007). On the other hand, our discriminability  
396 calculations indicate that the UV+ dorsal yellow model color was not a good match to real *H.*  
397 *erato* dorsal yellow (Table 1). Neither the long wavelength nor the UV reflectance for dorsal  
398 yellow UV treatments were as similar to natural *H. erato* dorsal yellow as was the Y+ treatment  
399 (Fig. 1A, Table 1). It may be that a closer match to the natural *H. erato* spectrum—including in  
400 the UV—is needed to elicit a stronger courtship response.

401 Many prior studies of butterfly mate choice have examined the preferences of one sex or  
402 the other but not both (Knüttel and Fiedler, 2001; Fordyce et al., 2002; Ellers and Boggs, 2003;  
403 Sweeney et al., 2003; Kemp, 2007b). We note that our mate preference results indicate equal  
404 responses to models by males and females with respect to approach behavior. This shows that  
405 females are ‘active’ during such preference studies (see Movies S2 and S3), and that females  
406 and males may share similar preferences for *Heliconius* yellow and UV in conspecifics. In  
407 nature, females may use approach behavior in non-mating related interactions (Crane, 1955;  
408 Crane, 1957), such as following between pollen resources or to new roosting locations (Waller  
409 and Gilbert, 1982; Finkbeiner, 2014).

410 Our field study results show that 3-OHK yellow and UV do not alter avian predation rates  
411 in themselves, despite studies showing that birds use UV for mate recognition and foraging  
412 (Bennett et al., 1996; Siitari et al., 1999; Lyytinen et al., 2004). Recent work has shown that  
413 birds have even lower-than-expected UV sensitivity when looking at stimuli against a UV-poor  
414 background (Chavez et al., 2014) and understory-dwelling birds may have lower UV opsin  
415 expression than canopy-dwelling birds (Bloch, 2015). Our results resemble those of Lyytinen et  
416 al. (2000), who also found no support for UV as an aposematic signal for bird predators.  
417 Moreover we provide experimental evidence that in natural conditions, the mimicry between  
418 *Heliconius* yellow/UV coloration and non-*Heliconius* yellow/non-UV coloration in butterflies is  
419 successful for deterring birds. Given that we found no indication that *Heliconius* yellow and UV  
420 enhance aposematic signaling toward avian predators, this reinforces the notion that the  
421 phylogenetic switch from using other yellow pigments to 3-OHK as a signal on *Heliconius*  
422 wings is significant exclusively in relation to intraspecific communication.

423

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#### 424 ***Fluorescence does not function as a signal***

425 Several studies have concluded that fluorescence is an important component of complex  
426 signals in aquatic animals because of the contrast between narrow-band down-welling blue light  
427 and long-wavelength fluorescence (Mazel et al., 2004; Gerlach et al., 2014), but the evidence  
428 that fluorescence contributes to signaling in terrestrial animals, where the illumination spectrum  
429 is broad-band, is much more limited and somewhat mixed. For instance, one laboratory study of  
430 fluorescence in parakeets (*Melopsittacus undulatus*) suggested that fluorescence contributed to  
431 sexual signaling (Arnold et al., 2002) while two other studies of the same species did not (Pearn  
432 et al., 2001; Pearn et al., 2003). In spiders, lab studies indicate that fluorescence plays a role in  
433 male mate choice while UV plays a role in female mate choice (Lim et al., 2007). A paper  
434 investigating UV and fluorescence in damselfly signaling (Guillermo-Ferreira et al., 2014)  
435 concluded that there might be a possible contribution of fluorescence to the signal, however,  
436 important controls necessary to confirm this were absent.

437 To our knowledge, we report here for the first time that the yellow wing coloration of  
438 *Heliconius* is fluorescent (Fig. 3A); although Rawson (1968) mentions anecdotally that *H. erato*  
439 and *H. charithonia* wings are fluorescent but without specification whether it is the yellow  
440 portion of the wings, and without identification of the fluorescent chemical. We find by  
441 measuring the absorption, excitation, and emission spectrum and quantum yield of 3-OHK,  
442 together with wing reflectance spectra using daylight-simulating illumination, however, no  
443 evidence that 3-OHK fluorescence enhances the reflectance spectrum of *Heliconius* yellow  
444 under broad-band illumination. Although the spectral sensitivity of the *H. erato* blue receptor  
445 (470 nm) is well-suited to detecting 3-OHK fluorescence (McCulloch et al., 2016) we found no  
446 evidence that under natural illumination, fluorescence contributes to the 3-OHK signal in the  
447 visible range. Our result highlights the importance of quantifying fluorescence using several  
448 methods, and specifically under broad-band daylight-simulating illumination, before concluding  
449 that it contributes to a signal under terrestrial environments (e.g. Andrews et al., 2007).

450

#### 451 ***Conclusion***

452 In summary, we demonstrate that *Heliconius* butterflies prefer 3-OHK yellow pigments in  
453 the context of conspecific signaling, these pigments have likely been selected for their  
454 reflectance properties in the visible range, and that fluorescence does not contribute to the visual

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455 signal. These results advance our understanding of the selective forces driving the transition  
456 from using other yellow pigments to using 3-OHK pigmentation in the genus *Heliconius*. We  
457 provide strong evidence that 3-OHK pigmentation is being maintained because it allows  
458 *Heliconius* species to recognize conspecifics for interspecific communication and sexual  
459 selection, whilst retaining the potential benefits of Müllerian mimicry with genera such as  
460 *Eueides*.

461

462

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472

473

#### 474 **Competing Interests**

475 The authors declare no competing or financial interests.

476

477

#### 478 **Author Contributions**

479 S.D.F. designed butterfly models, carried out, and analyzed field predation and mate preference  
480 experiments, and wrote the manuscript; D.A.F. contributed measurements and analysis of  
481 physical fluorescent properties; D.O. and A.D.B. conceived of the study and edited the  
482 manuscript; A.D.B. designed butterfly models, calculated discriminabilities, performed  
483 experiments, analyzed fluorescence data and wrote the manuscript. All authors gave final  
484 approval for publication.

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#### 493 **Data Availability**

494 DRYAD (doi: XXXXX)

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**Table 1.** Percentage of *H. erato* and *Eueides* wing colors compared to paper models with chromatic JND values <0.5, <1, <2 for male and female *H. erato* under high light, sunny cage illumination. n=15 *H. erato*; n=9 *Eueides* specimens measured.

		Percent below the threshold							
		Y+		Y-		UV+		UV-	
		Female	Male	Female	Male	Female	Male	Female	Male
Dorsal yellow	0.5JND	0.0	6.7	0.0	0.0	0.0	0.0	36.4	63.6
	1JND	86.7	100.0	81.8	9.1	0.0	0.0	100.0	100.0
	2JND	100.0	100.0	100.0	100.0	0.0	0.0	100.0	100.0
Ventral yellow	0.5JND	13.3	0.0	0.0	55.6	33.3	33.3	0.0	11.1
	1JND	86.7	86.7	100.0	77.8	100.0	100.0	88.9	66.7
	2JND	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Dorsal red	0.5JND	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	1JND	13.3	0.0	13.3	0.0	6.7	0.0	0.0	0.0
	2JND	86.7	46.7	86.7	46.7	86.7	46.7	93.3	80.0
Ventral red	0.5JND	46.7	46.7	46.7	46.7	46.7	46.7	0.0	0.0
	1JND	46.7	46.7	46.7	46.7	46.7	46.7	0.0	0.0
	2JND	100.0	66.7	100.0	66.7	100.0	73.3	60.0	100.0

**Table 2.** Percentage of *H. erato* and *Eueides* wing colors compared to paper models with chromatic JND values <0.5, <1, <2 for the UV-type, blue tit (*Parus caeruleus*) and violet-type chicken (*Gallus gallus*) under high light, partial forest shade illumination. n=15 *H. erato*; n=9 *Eueides* specimens measured. The percentages below the threshold were identical except for the number indicated in parentheses.

		Percent below the threshold							
		Y+		Y-		UV+		UV-	
		UV-type	Violet-type	UV-type	Violet-type	UV-type	Violet-type	UV-type	Violet-type
Ventral yellow	0.5JND	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	1JND	0.0	0.0	0.0	66.7	33.3	0.0	0.0	0.0
	2JND	86.7	6.7	88.9	77.8	100.0	100.0	0.0	0.0
Ventral red	0.5JND	0.0	20.0	0.0	20.0	6.7	13.3 (20.0)	0.0	0.0
	1JND	33.3	46.7	33.3	46.7	33.3	46.7	0.0	13.3
	2JND	46.7	46.7	46.7	46.7	46.7	46.7	0.0	46.7

**Table 3.** JNDs between model spectra through the eyes of male and female *H. erato* and representatives of the UV- and violet-type bird visual systems. For butterflies, sunny cage illumination and for birds, partial forest cover illumination was used. Numbers in parentheses represent spectra modeled with forest edge illumination.

	JNDs							
	Y+ vs. Y-				UV+ vs. UV+			
	Butterfly		Bird		Butterfly		Bird	
	Female	Male	UV-type	VS-type	Female	Male	UV-type	VS-type
Dorsal yellow	1.04	1.77	N/A	N/A	2.38	1.28	N/A	N/A
Dorsal Red	N/A	N/A	N/A	N/A	2.09	1.22	N/A	N/A
Ventral yellow	1.27	0.14	3.11 (3.37)	1.86 (1.91)	2.42	1.23	5.04 (5.38)	0.97 (1.03)
Ventral Red	N/A	N/A	N/A	N/A	2.28	1.23	4.73 (5.05)	1.01

### 3-OHK and UV signaling in *Heliconius*

#### 795 **Figure Legends**

#### 796 **Fig. 1. Reflectance spectra of *Heliconius erato* and *Eueides* wing colors and paper model**

797 **colors used in the mate choice and predation experiments.** (A) Dorsal yellow, (B) ventral

798 yellow, (C) dorsal red, (D) ventral red. Reflectance spectra correspond to: *H. erato* (yellow or

799 red), *Eueides* (grey), Y+ (orange), Y- (black), UV+ (dark blue), UV- (blue-green). Black

800 symbols correspond to the spectral sensitivities of *H. erato* photoreceptor cells with peak

801 sensitivities  $\lambda_{\max}$  at: 360 nm (triangles), 390 nm ( $\times$ ), 470 nm ( $\star$ ), and 555 nm (filled circles)

802 (McCulloch et al., 2016). The photoreceptor with a peak at 360 nm is found in female but not

803 male *H. erato*.

804

#### 805 **Fig. 2. UV- and 3-OHK-manipulated butterfly models experience different rates of** 806 **approach and courtship behavior by butterflies and similar rates of predation by birds.**

807 There are four model types that differ according to whether UV-yellow paint (UV+, UV-), 3-

808 OHK pigment (Y+) or Manila paper (Y-) was used to produce the yellow hindwing bar and

809 according to whether a neutral density filter (+ treatments) or a UV-blocking filter (- treatments)

810 was used. (A) Mean approach (left axis, black) and courtship (right axis, red) values with  $\pm$ s.e.m.

811 bars (each n = 80 butterflies: 40 males and 40 females). Asterisks represent the p-values from

812 pairwise comparisons where \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001. (B) Average proportion of

813 models attacked at each site (total n = 2000: 500 of each model type, 100 sites) with  $\pm$ s.e.m. bars.

814 The p-values from pairwise comparisons are >0.05.

815

#### 816 **Fig. 3. *Heliconius erato* fluorescence and 3-OHK absorption, excitation, and emission**

817 **spectra.** (A) Adult *H. erato* photographed under UV illumination to induce fluorescence (first

818 panel) and under white light (second panel). (B) Absorption spectrum of 3-OHK in methanol

819 ( $\lambda_{\max}$ =380 nm). (C) Excitation and emission spectrum of pigment 3-OHK. Emission has a broad

820 spectrum with a peak around 508 nm.

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825

### 3-OHK and UV signaling in *Heliconius*

#### 826 **Supplementary Material Legends**

827 **Fig. S1. Color- and pattern-manipulated butterfly models experience different predation**  
828 **rates (left axis) and different probabilities of inducing premating approach behavior in**  
829 **male butterflies (right axis).** There are four model types: a local *H. erato* type, a color-switched  
830 type, an achromatic type, and a nonlocal type.  $\pm$ s.e.m. bars for the predation data include 95%  
831 CIs and  $\pm$ s.e.m. bars for the mate preference data represent 95% credible intervals. Asterisks  
832 represent the p-values from pairwise comparisons between predation on the local model type and  
833 the three other model types where \* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.0001$ . All approach probability  
834 comparisons show that the preference means differ significantly between the model types  
835 (Reprinted with permission from Finkbeiner et al., 2014).

836  
837 **Fig. S2. Irradiance spectra of open sunlight and the experimental cage during open**  
838 **sunlight conditions.** Each graph represents the average from five measurements in each  
839 condition.

840  
841 **Fig. S3. Habitat types.** Irradiance spectra with photos of corresponding foliage cover, taken  
842 from the four major habitat types used in the predation study: forest cover (A-E); forest edge (F-  
843 J); Pipeline Road (unpaved road with partial forest cover), (K-T); and paved road with partial  
844 forest cover (U-Y). Five different sites were measured (repeated five times) for forest cover,  
845 forest edge, and paved road, whereas ten different sites were measured (repeated five times) for  
846 Pipeline Road because this was the dominant habitat type used in the study.

847  
848 **Fig. S4. Male and female *H. erato* approach and courtship behavior.** Male and female *H.*  
849 *erato* butterflies approach and court UV- and Y- manipulated artificial butterfly models at  
850 varying rates (A-D). All behaviors directed toward UV models are in the left column, and  
851 behaviors directed toward Y models are in the right column. Shown are the mean approach and  
852 courtship values  $\pm$  s.e.m. (n = 80 butterflies: 40 males and 40 females).

853  
854 **Fig. S5. Experimental data used to determine the quantum yield of 3-OHK in methanol.**  
855 (A) Absorption spectrum of 3-OHK pigment and Coumarin 500. Both dye and pigment have a  
856 very similar absorption spectrum making Coumarin 500 a good choice as a reference in quantum

### 3-OHK and UV signaling in *Heliconius*

857 yield measurements. (B) Quantum yield determination using Coumarin 500 dye (blue curve) and  
858 3-OHK pigment (orange curve). Coumarin 500 quantum yield is 0.46.

859

860 **Fig. S6. Reflectance spectrum of *H. erato* dorsal yellow hind wing with and without neutral**  
861 **density or UV-cutoff filters as measured using daylight-simulating illumination.** The neutral  
862 density filter (Mylar) has an identical spectrum to the UV-cutoff filter in the visible range (above  
863 400 nm) indicating that UV-induced fluorescence has no impact on the reflectance spectrum of  
864 3-OHK yellow.

865

866 **Movie S1:** Example of fluorescing 3-OHK pigment on a *H. erato* butterfly under a hand-held  
867 365 nm LED light.

868

869 **Movie S2:** A female *H. erato* butterfly directs approaches toward a Y+ model (right side).

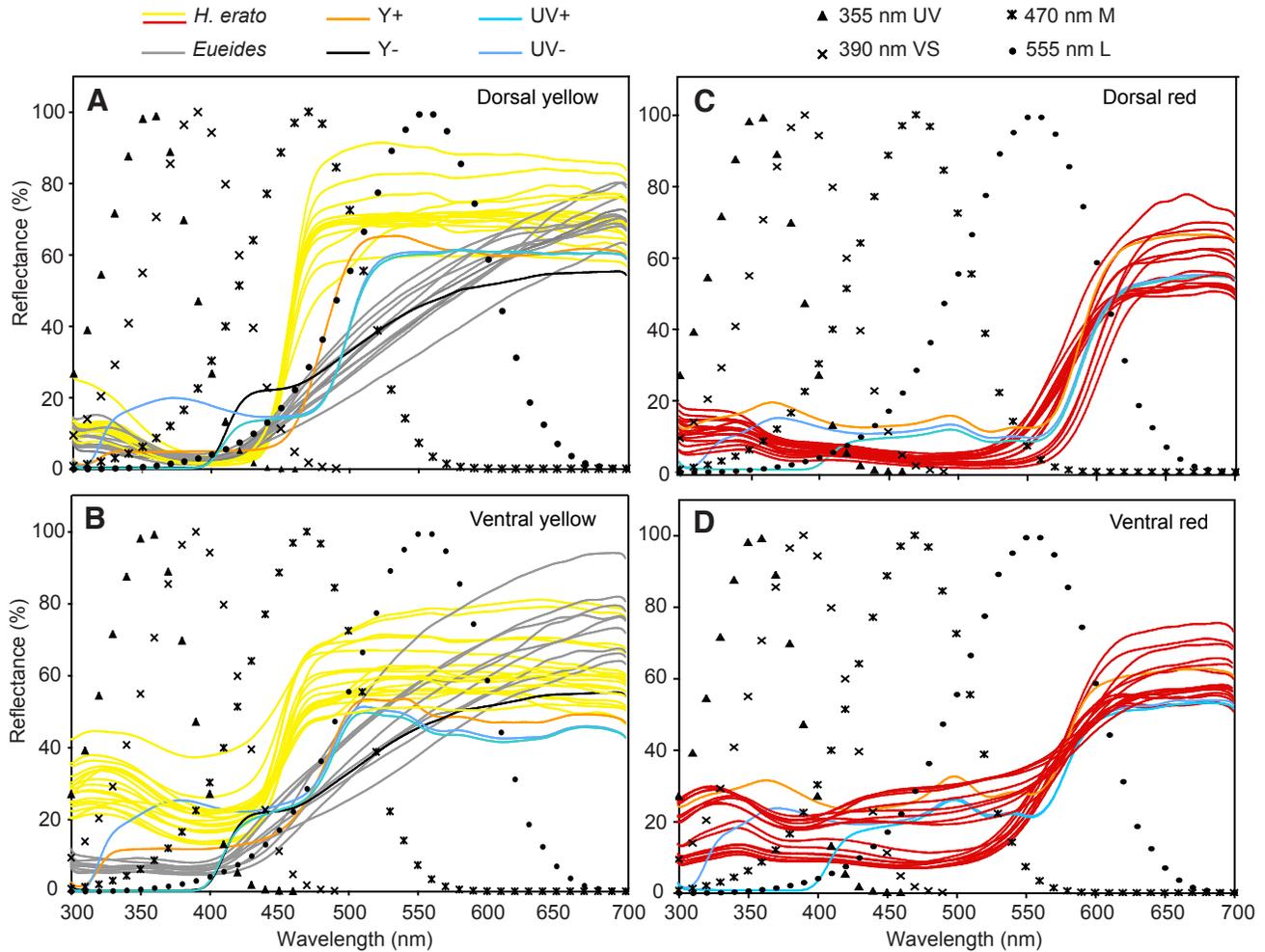
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871 **Movie S3:** A female *H. erato* butterfly directs approaches toward a UV+ model (left side).

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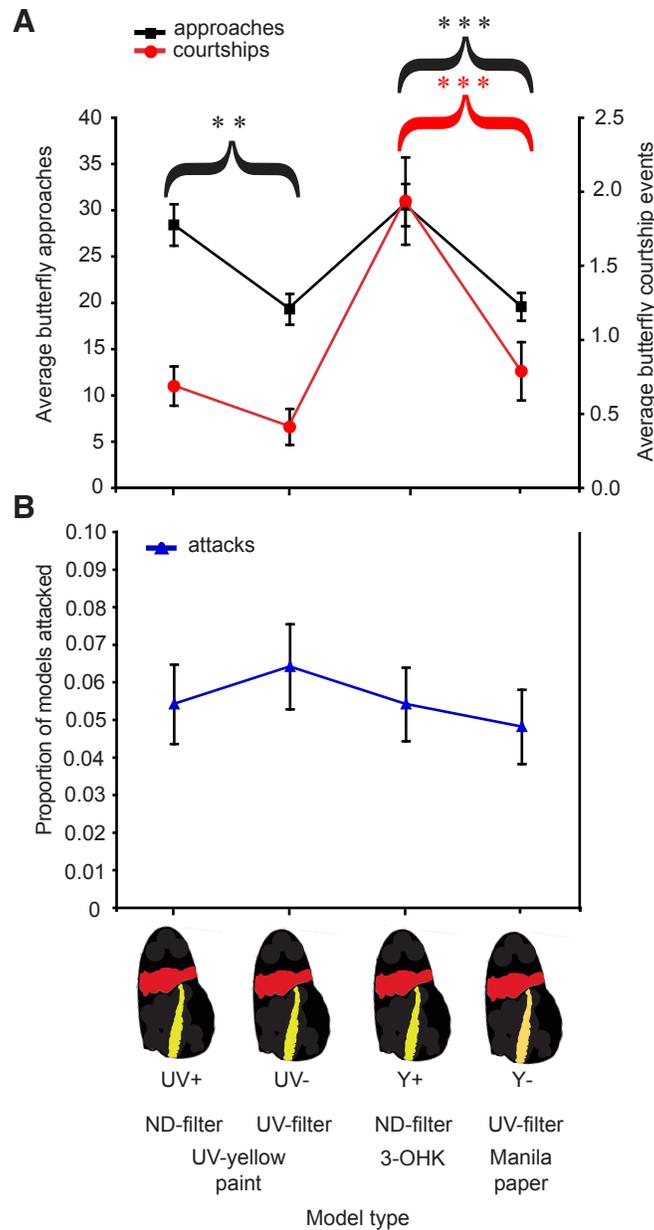
**Fig. 1**



**Fig. 1. Reflectance spectra of *Heliconius erato* and *Eueides* wing colors and paper model colors used in the mate choice and predation experiments.**

(A) Dorsal yellow, (B) ventral yellow, (C) dorsal red, (D) ventral red. Reflectance spectra correspond to: *H. erato* (yellow or red), *Eueides* (grey), Y+ (orange), Y- (black), UV+ (dark blue), UV- (blue-green). Black symbols correspond to the spectral sensitivities of *H. erato* photoreceptor cells with peak sensitivities  $\lambda_{\max}$  at: 360 nm (triangles), 390 nm (x), 470 nm (\*), and 555 nm (filled circles) (McCulloch et al., 2016). The photoreceptor with a peak at 360 nm is found in female but not male *H. erato*.

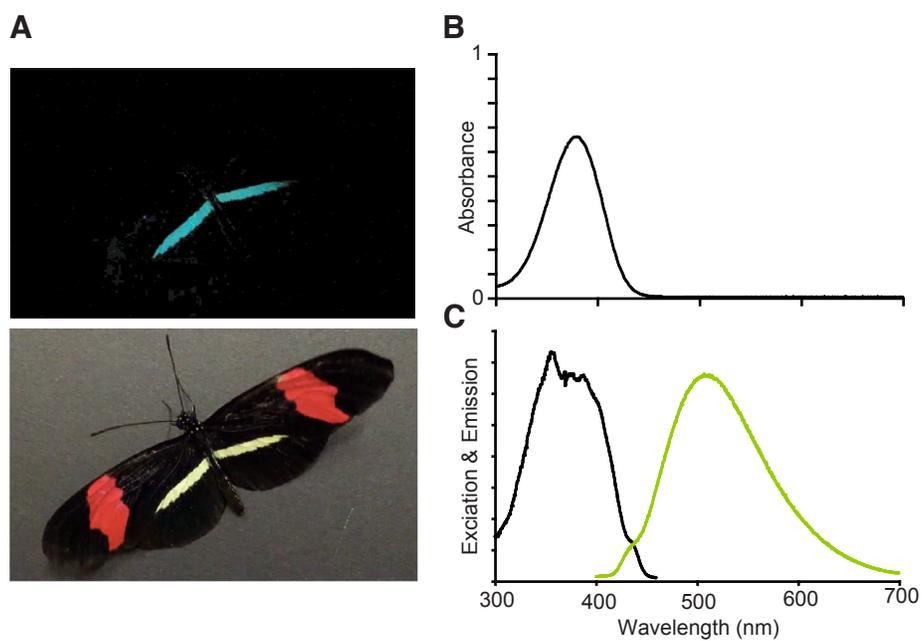
Fig. 2



**Fig. 2. UV- and 3-OHK-manipulated butterfly models experience different rates of approach and courtship behavior by butterflies and similar rates of predation by birds.**

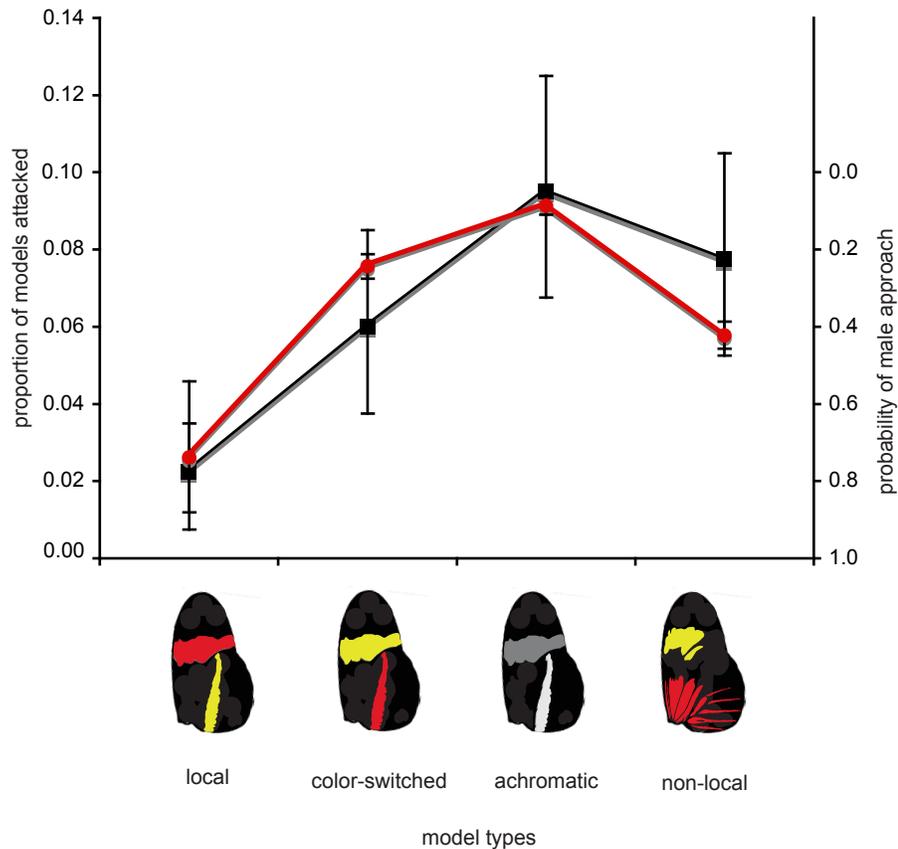
There are four model types that differ according to whether UV-yellow paint (UV+, UV-), 3-OHK pigment (Y+) or Manila paper (Y-) was used to produce the yellow hindwing bar and according to whether a neutral density filter (+ treatments) or a UV-blocking filter (- treatments) was used. (A) Mean approach (left axis black) and courtship (right axis, red) values with  $\pm$ s.e.m. bars (each  $n = 80$  butterflies: 40 males and 40 females). Asterisks represent the p-values from pairwise comparisons where \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . (B) Average proportion of models attacked at each site (total  $n = 2000$ : 500 of each model type, 100 sites) with  $\pm$ s.e.m. bars. The p-values from pairwise comparisons are  $> 0.05$ .

**Fig. 3**



**Fig. 3. *Heliconius erato* fluorescence and 3-OHK absorption, excitation, and emission spectra.** (A) Adult *H. erato* photographed under UV illumination to induce fluorescence (first panel) and under white light (second panel). (B) Absorption spectrum of 3-OHK in methanol ( $\lambda_{\max}$ =380 nm). (C) Excitation and emission spectrum of pigment 3-OHK. Emission has a broad spectrum with a peak around 508 nm.

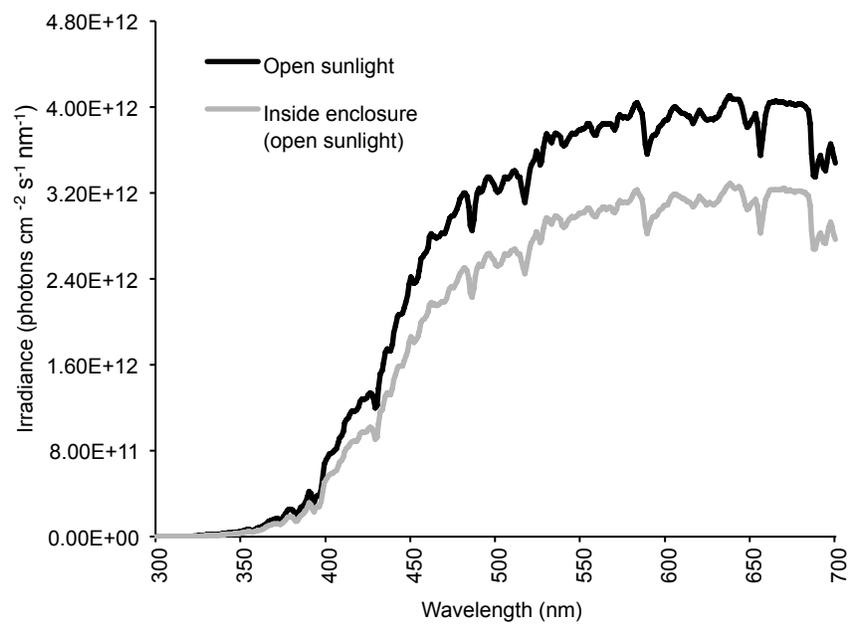
Fig. S1



**Fig. S1. Color- and pattern-manipulated butterfly models experience different predation rates (left axis) and different probabilities of inducing pre-mating approach behavior in male butterflies (right axis).**

There are four model types: a local *H. erato* type, a color-switched type, an achromatic type, and a nonlocal type.  $\pm$ s.e.m. bars for the predation data include 95% CIs and  $\pm$ s.e.m. bars for the mate preference data represent 95% credible intervals. Asterisks represent the p-values from pairwise comparisons between predation on the local model type and the three other model types where \* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.0001$ . All approach probability comparisons show that the preference means differ significantly between the model types (Reprinted with permission from Finkbeiner et al. 2014).

**Fig. S2**



**Fig. S2. Irradiance spectra of open sunlight and the experimental cage during open sunlight conditions.** Each graph represents the average from five measurements in each condition.

Fig. S3

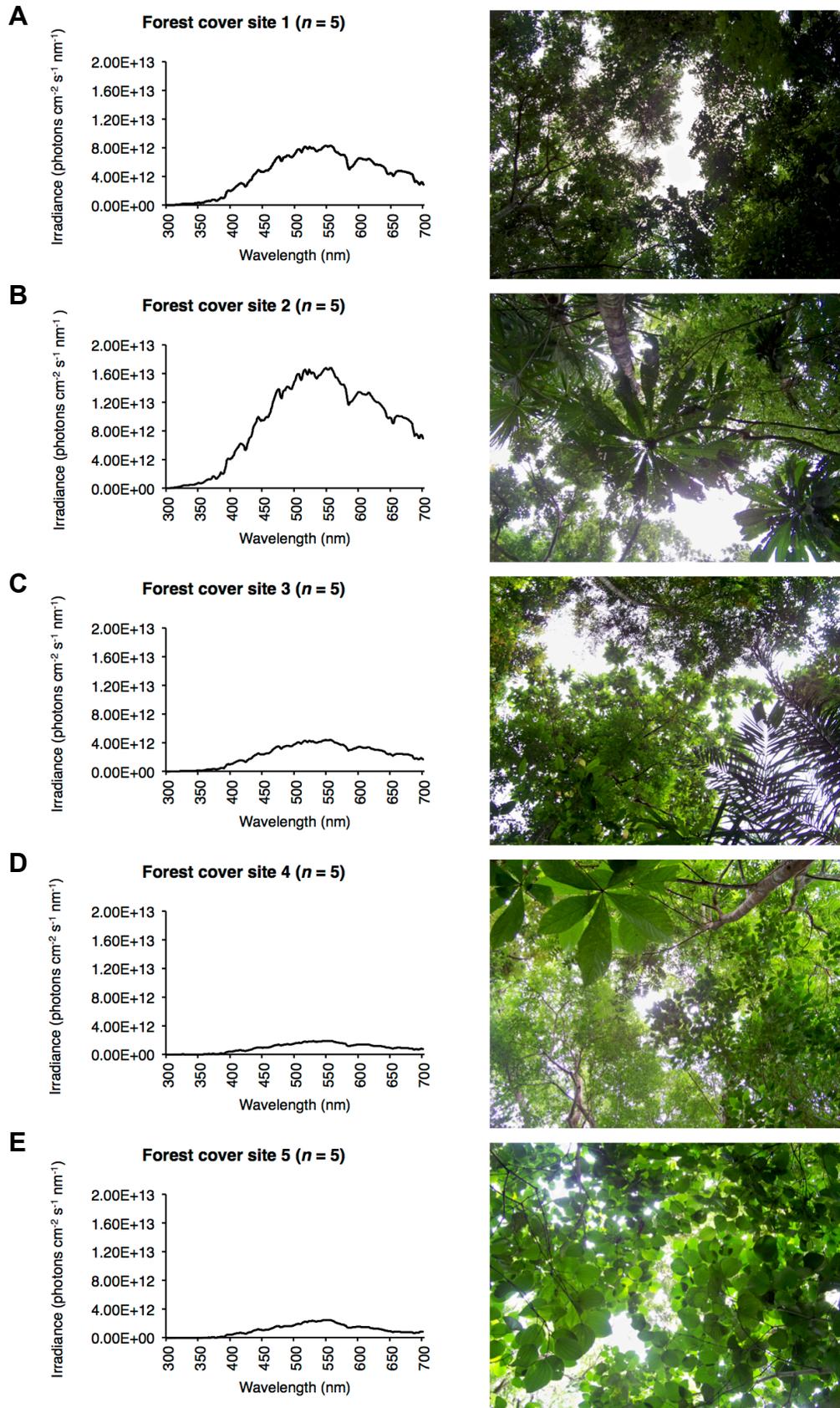


Fig. S3

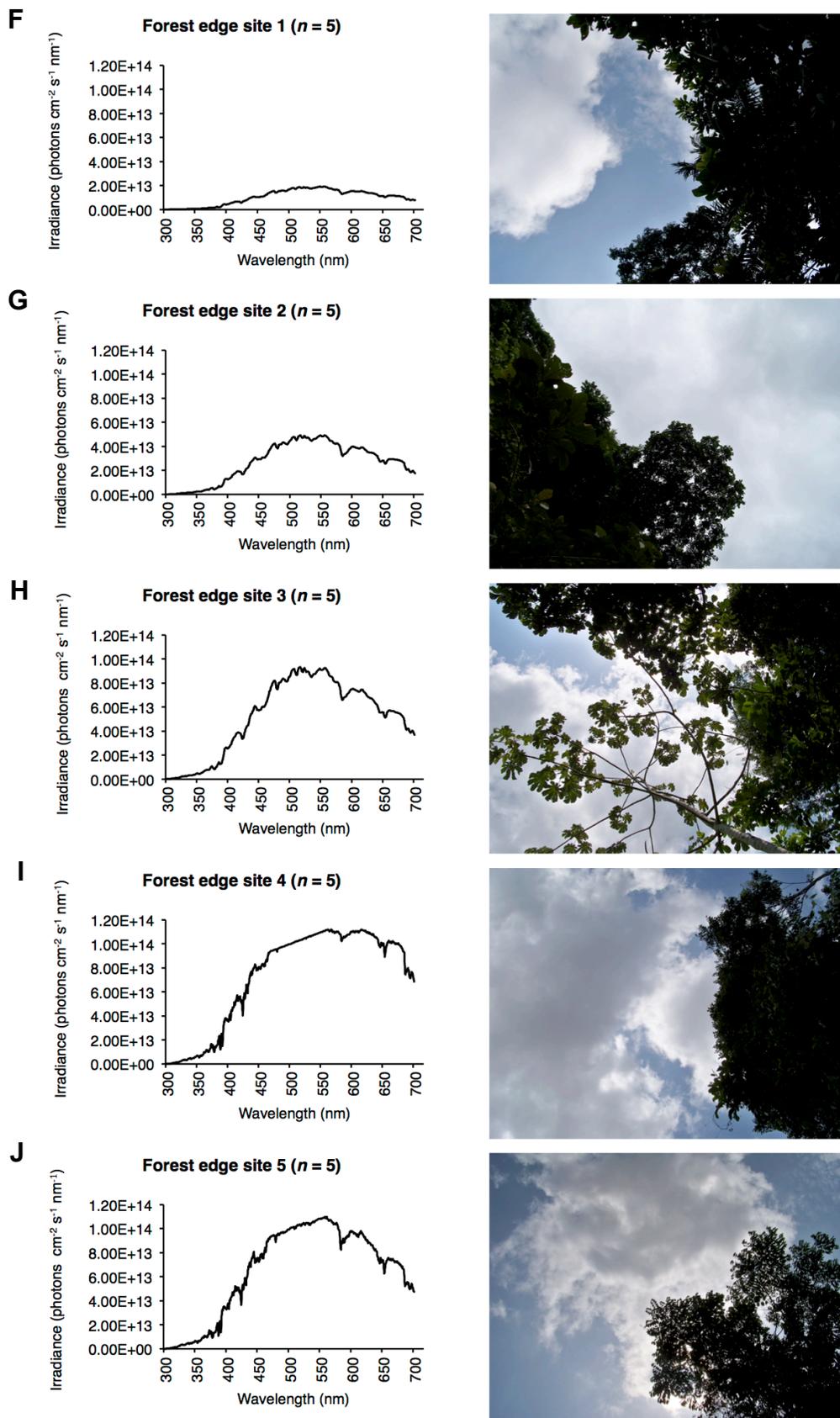


Fig. S3

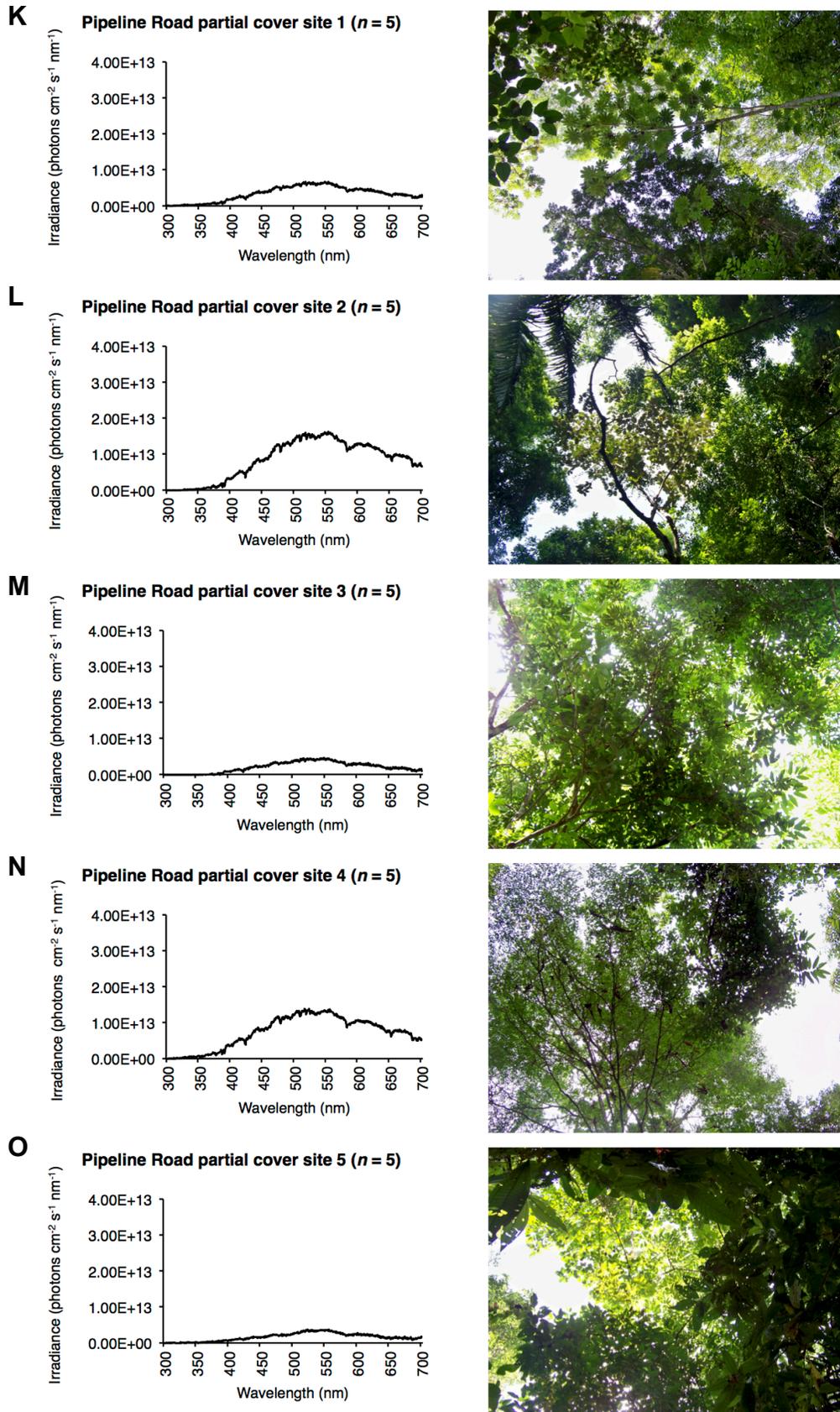
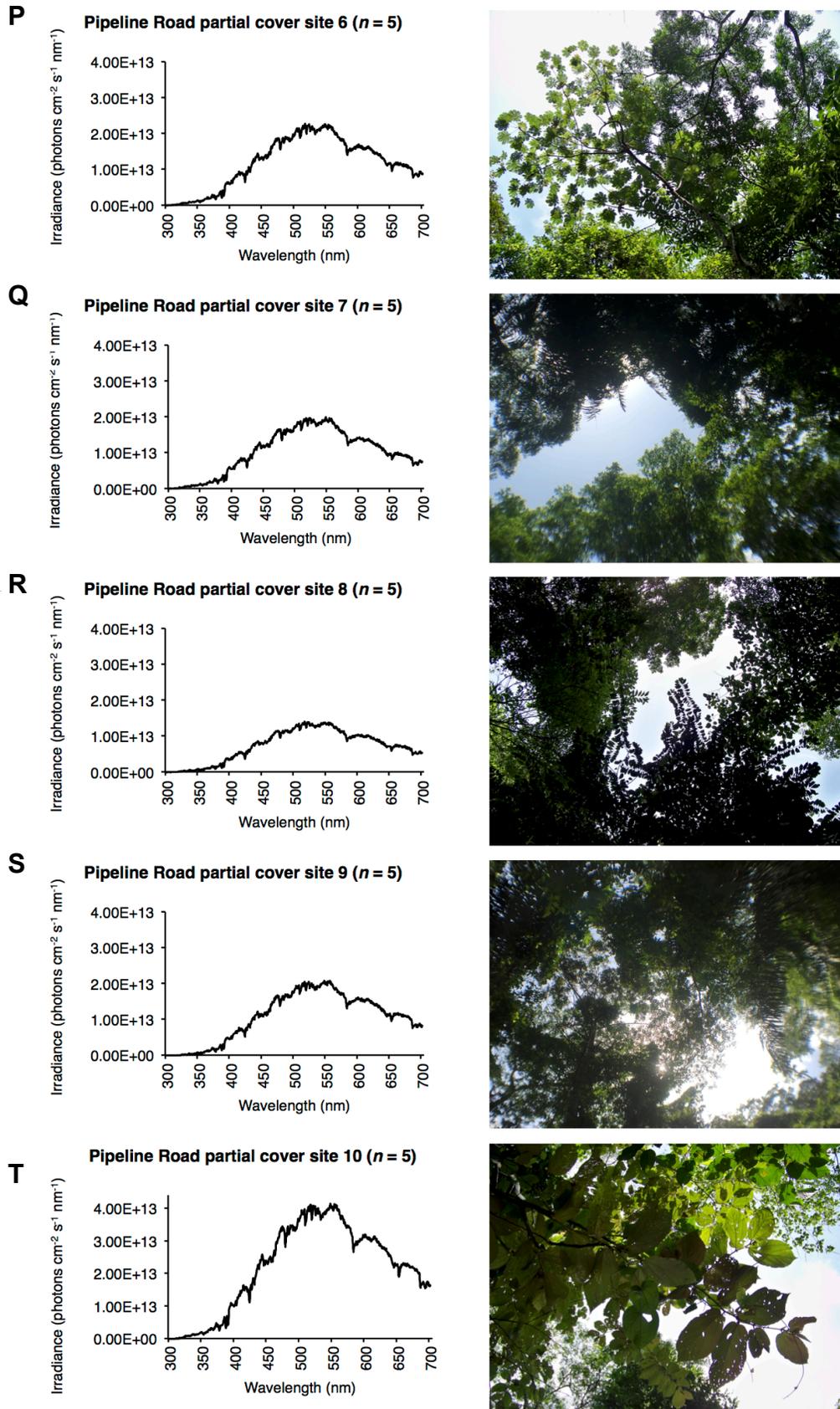
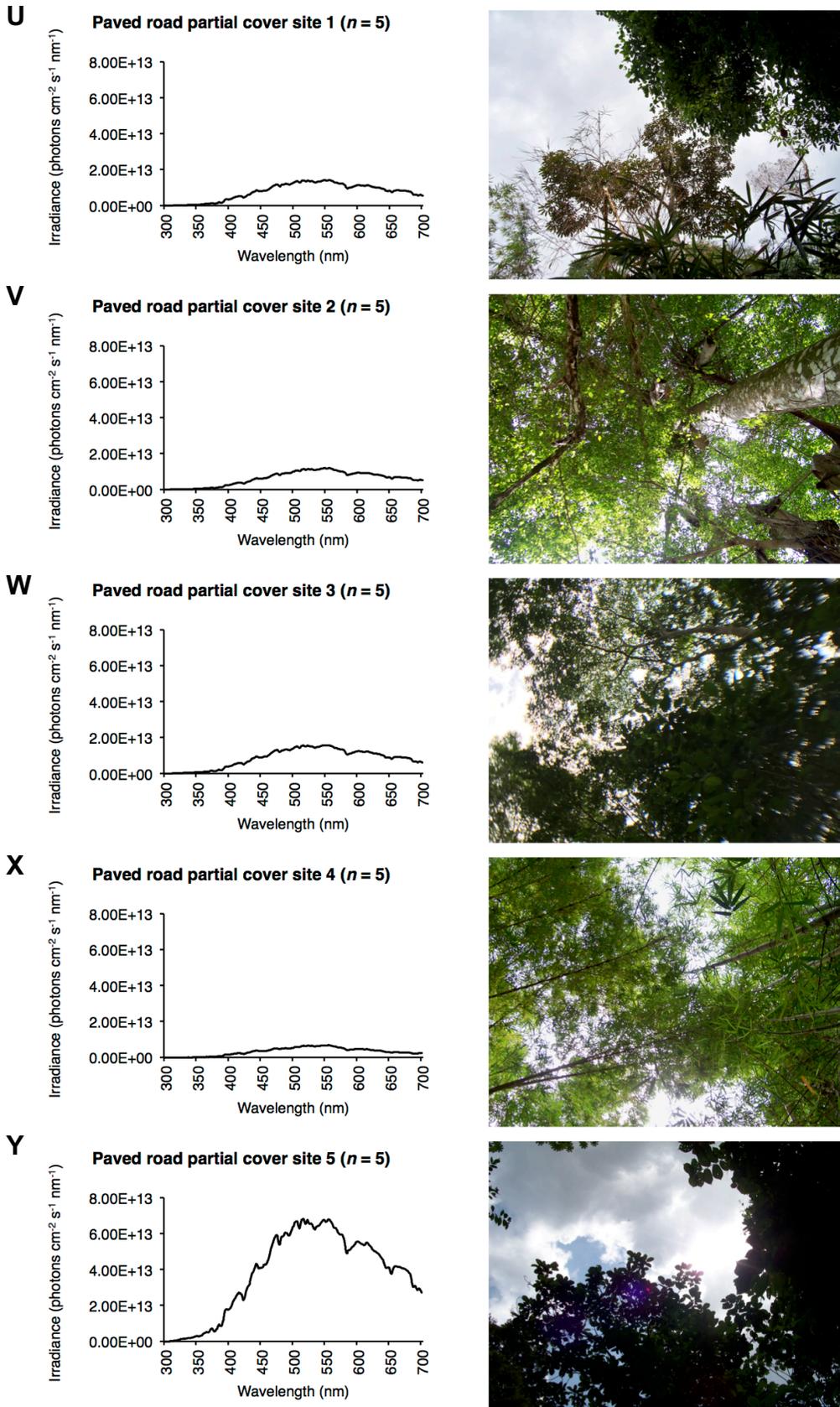


Fig. S3



**Fig. S3**



## Fig. S3

### **Fig. S3. Habitat types.**

Irradiance spectra with photos of corresponding foliage cover, taken from the four major habitat types used in the predation study: forest cover (A-E); forest edge (F-J); Pipeline Road (unpaved road with partial forest cover), (K-T); and paved road with partial forest cover (U-Y). Five different sites were measured (repeated five times) for forest cover, forest edge, and paved road, whereas ten different sites were measured (repeated five times) for Pipeline Road because this was the dominant habitat type used in the study.

**Fig. S3**

Examples of four habitat types:



Forest cover



Forest edge

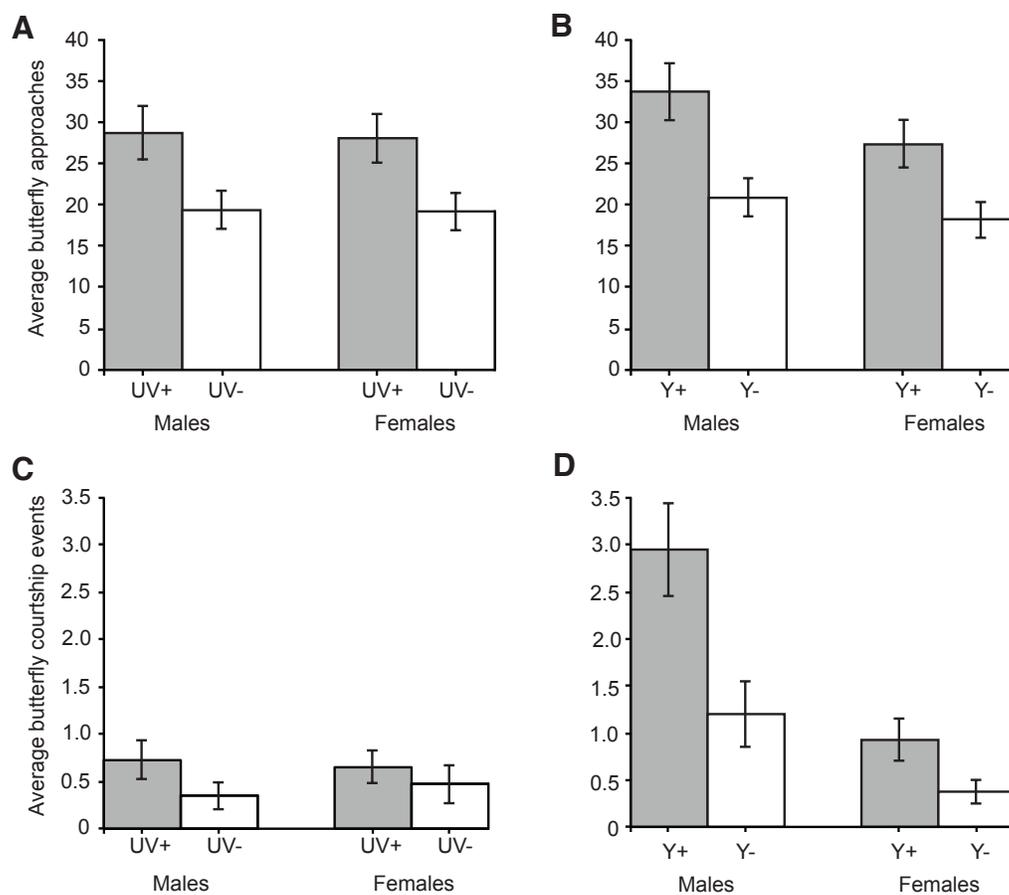


Pipeline Road



Paved road

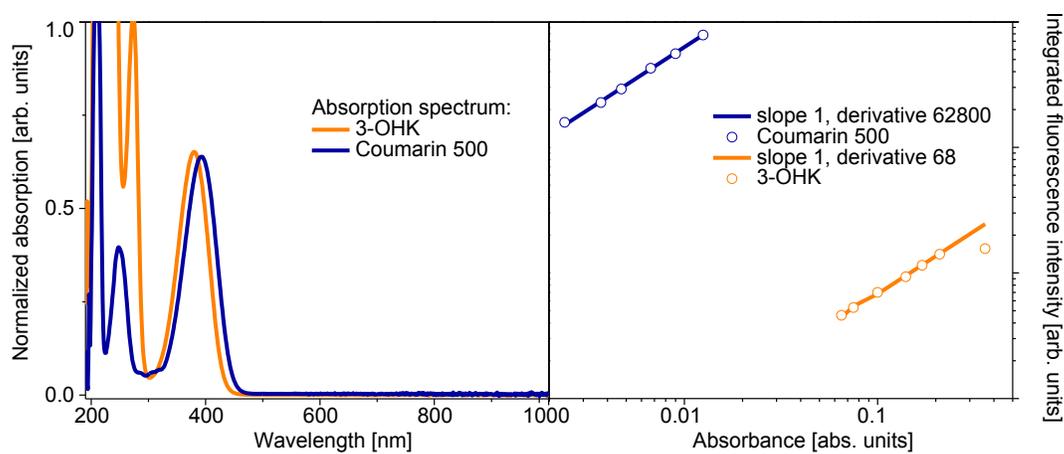
Fig. S4



**Fig. S4. Male and female *H. erato* approach and courtship behavior.**

Male and female *H. erato* butterflies approach and court UV- and Y- manipulated artificial butterfly models at varying rates (A-D). All behaviors directed toward UV models are in the left column, and behaviors directed toward Y models are in the right column. Shown are the mean approach and courtship values  $\pm$  s.e.m. (n=80 butterflies: 40 males and 40 females).

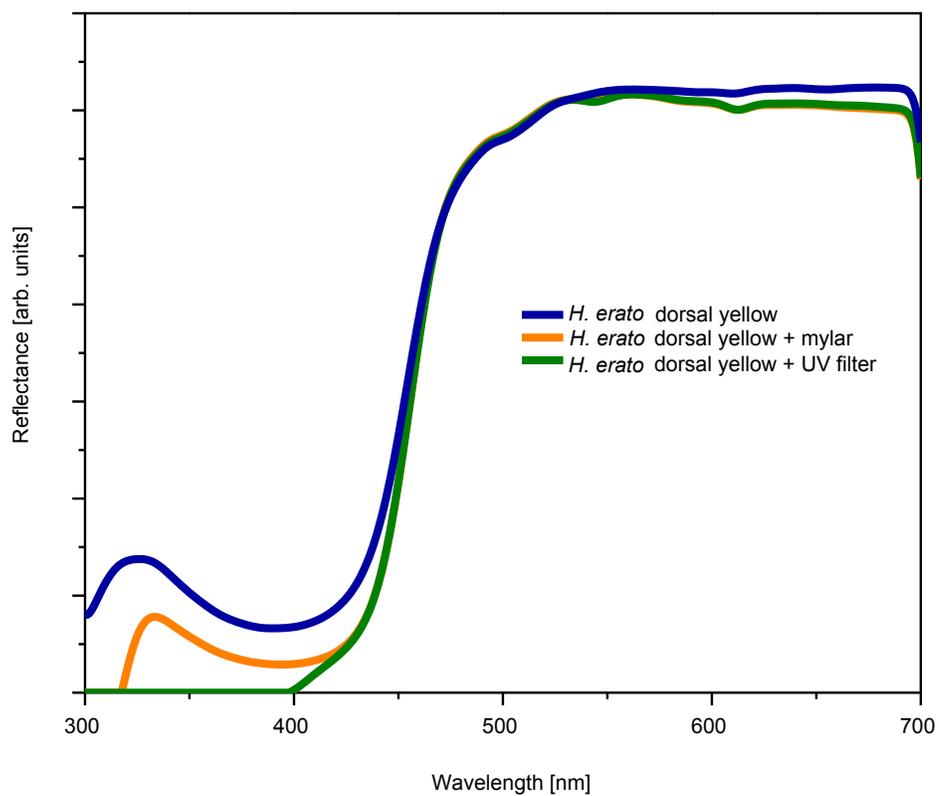
Fig. S5



**Fig. S5. Experimental data used to determine the quantum yield of 3-OHK in methanol.**

(A) Absorption spectrum of 3-OHK pigment and Coumarin 500. Both dye and pigment have a very similar absorption spectrum making Coumarin 500 a good choice as a reference in quantum yield measurements. (B) Quantum yield determination using Coumarin 500 dye (blue curve) and 3-OHK pigment (orange curve). Coumarin 500 quantum yield is 0.46.

Fig. S6



**Fig. S6. Reflectance spectrum of *H. erato* dorsal yellow hind wing with and without neutral density or UV-cutoff filters as measured using daylight-simulating illumination.**

The neutral density filter (Mylar) has an identical spectrum to the UV-cutoff filter in the visible range (above 400 nm) indicating that UV-induced fluorescence has no impact on the reflectance spectrum of 3-OHK yellow.