Light environment influences mating behaviours during the early stages of divergence in tropical butterflies

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ABSTRACT

Species divergence is facilitated when traits under divergent selection also act as mating cues. Fluctuations in sensory conditions can alter signal perception independently of adaptation to contrasting sensory environments, but how this fine scale variation affects behavioural isolation has received less attention, especially in terrestrial organisms. The warning patterns of *Heliconius* butterflies are under selection for aposematism and act as mating cues. Using computer vision, we extracted behavioural data from 1481 hours of video footage for 387 individuals. We show that the putative hybrid species *H. heurippa* and its close relative *H. timareta linaresi* differ in their response to divergent warning patterns, and that these differences are strengthened with increased local illuminance. Trials with live individuals reveal low-level assortative mating that are sufficiently explained by differences in visual attraction. Finally, results from hybrid butterflies are consistent with linkage between a major warning pattern gene and the corresponding behaviour, though the differences in behaviour we observe are unlikely to cause

rapid reproductive isolation as predicted under a model of hybrid trait speciation. Overall, our results highlight that the role of ecological mating cues for behavioural isolation may depend on the immediate sensory conditions during which they are displayed to conspecifics.

Keywords: *Heliconius* – Ecological Speciation – Sensory Environment – Magic traits – Hybrid Trait Speciation – Computer vision

BACKGROUND

During ecological speciation, barriers to gene flow evolve as a result of ecologically-based divergent selection [1]. These barriers are generally expected to build up gradually over time [2], with premating isolation evolving early alongside ecological differences [1,3,4]. The evolution of premating isolation is facilitated when traits under ecological selection act as mating cues (sometimes known as 'magic traits' [5,6]), as this allows divergent natural selection acting on ecological traits to be transferred to mating behaviours. However, the strength of behavioural barriers may be influenced by the immediate, and perhaps rapidly changing, sensory conditions, but this has received relatively little empirical attention (but see [7–13]). One reason may be that robustly detecting these effects during the early stages of divergence, when they may be most relevant, presents a substantial empirical challenge.

It is well established that the sensory environment can alter signal detection and perception [14,15]. Colour perception depends not only on an object's reflectance spectrum, but also the illumination, available light spectrum and background, all of which may change within seconds and over very short distances [16]. This can affect within-population preferences. For example, red colouration in male *Habronattus* spiders is only an efficient mating signal if

presented in broad-spectrum sunlight [17]. Other sensory modalities can be also affected: Female swordtail fish are strongly attracted to the chemical signals of conspecific males in clean water, but not in polluted water [7]. Similarly, urban noise can disrupt the transmission of bird songs and interfere with acoustic-based mate choice [9].

The sensory environment may affect the evolution of reproductive isolation in two main ways. First, adaptations to meet the ecological needs of different sensory environments can influence female preferences, subsequently driving divergence in male signals, and leading to reproductive isolation through sensory drive [18]. For example, in two closely related *Pundamilia* cichlid fishes, adaptation of the visual system to the local environment is associated with divergent female mate preference for male colouration [19]. Second, prevailing environmental conditions may alter the efficacy of signals used in mate choice [14], so that individual mating preferences may act as an important reproductive barrier under some sensory conditions, but not others ('context-sensitive preferences' [20]). If the strength of preferences depends on the sensory environment, then this will influence their contribution to reproductive isolation. However, compared to the role of sensory adaptation, the immediate influence of local sensory conditions on mating behaviours, and how this may relate to the evolution and maintenance of new species, has been less-studied, especially in terrestrial organisms.

By affecting context-sensitive preferences, the sensory conditions during signalling may also influence the strength of genetic associations (*i.e.* linkage disequilibrium, LD) between mating and ecological traits, which are typically required for speciation to proceed when gene flow persists [21]. Specifically, when ecological traits act as mating cues, LD (between ecological and preference loci) will arise as a natural consequence of mating preferences, but this will be proportional to the strength of preference [22], which may be influenced by the sensory

75 environment. Regardless, unless preferences are very strong, more robust coupling between
76 mating and ecological components of reproductive isolation likely requires genetic architectures
77 that impede recombination (or one-allele mechanisms, see [23]). These include physical linkage,
78 which may be further strengthened by genomic rearrangements like inversions, or – in the
79 extreme – pleiotropy, where distinct traits are controlled by the same allele [24]. To date, studies
80 have reported physical linkage between behavioural and ecological components of reproductive
81 isolation for a few animal taxa, including pea aphids [25], fish [26] and butterflies [27].

82 *Heliconius* butterflies are known for their bright warning patterns, which are often under
83 selection for Müllerian mimicry [28]. Closely related *Heliconius* species frequently – but not
84 always – have very different wing patterns, which additionally act as mating cues (e.g. [29–32]).
85 This contributes to assortative mating because males almost invariably prefer wing patterns
86 resembling their own, and warning patterns in *Heliconius* are considered one of the best examples
87 of 'magic traits' in nature [6,29,33]. Variation in warning pattern is largely controlled by a few
88 genes; the genetics of the corresponding visual mate preference are less well known, though
89 recent work implicates a handful of genes associated with neural signalling in tight linkage to the
90 colour pattern gene *optix* [27,34].

91 There is substantial evidence that colour pattern alleles have introgressed between
92 otherwise separately evolving *Heliconius* lineages (e.g. [35]). In particular, taxa within the
93 *heurippa-timareta* group, found in the eastern slopes of the South-American Andes, have
94 acquired red colour pattern elements from local races of *H. melpomene* via introgression of *optix*
95 alleles [36]. This has frequently led to near perfect mimicry between local races of *H. timareta*
96 and *H. melpomene*; elsewhere, however, the resulting colour patterns are not shared with local
97 *Heliconius* species. In particular, *H. heurippa* has a unique red-yellow banded forewing pattern

98 (Fig. 1A). Its close relative *H. timareta linaresi*, which is assumed to represent the ancestral wing
99 colour pattern of the *heurippa-timareta* group [37], only displays a yellow band (Fig. 1A) and
100 also does not have an obvious co-mimic. These two populations are geographically adjacent (Fig.
101 1A) and presumably share a contact zone. Despite their nominal status as separate species, *H. t.*
102 *linaresi* and *H. heurippa* likely represent an early stage of divergence; hybrids between *H. t.*
103 *linaresi* and *H. heurippa* are fully viable and fertile [38] and any post-mating isolation is
104 probably limited to selection acting against immigrant warning patterns.

105 The red pattern element of *H. heurippa* is presumably maintained by strong frequency-
106 dependent selection imposed by predators [39]. However, its effectiveness as intraspecific signal
107 may depend on how it is perceived by conspecifics under natural sensory conditions. In this way,
108 premating reproductive barriers may depend on interactions between the signal, environment and
109 receiver. *H. melpomene* are broadly separated from *H. heurippa-timareta* (along with closely
110 related *H. cydno*) taxa across a gradient of open to closed forest and decreasing light intensity
111 [32,40]. These contrasting sensory environments are predicted to alter how colour patterns are
112 perceived by butterfly visual systems [41]. While *H. heurippa* and *H. t. linaresi* are not
113 ecologically isolated in this way, their forest habitats are highly heterogenous and *Heliconius* are
114 known to settle with their wings open in more brightly illuminated 'light patches' [42]. Whether
115 changes in illuminance affect male behaviours has not been investigated, but such an effect
116 would suggest that the efficacy of wing patterns as premating reproductive barriers may depend
117 on fluctuations in sensory conditions as females move through the environment.

118 To test whether warning patterns contribute to premating isolation between *H. heurippa*
119 and *H. t. linaresi*, we used a novel computer vision pipeline to extract behavioural data from
120 video footage, alongside choice trails to determine levels of assortative mating. Our data,

121 including ~17,000 behavioural interactions for 387 individuals, allowed us to test (i) whether the
122 two species show differences in visual attraction towards con- and hetero-specific patterns and
123 (ii) whether these differences segregate with the colour patterns, consistent with physical linkage
124 between the behavioural and colour patterning genes. Measuring illuminance in real-time at each
125 female pattern also allowed us to ask (iii) whether these behavioural differences are influenced by
126 fluctuating local light conditions.
127

128 **MATERIAL AND METHODS**

129 **(a) Butterfly collection, maintenance and crossing design.** We maintained outbred stocks of *H.*
130 *timareta linaresi* and *H. heurippa*, established from wild individuals (Table S1), between January
131 2016 and September 2019 at the Universidad del Rosario insectaries in La Vega, Colombia
132 (4°59' N, 74°20' W, elevation 1257 m). We generated F_1 hybrids and backcross hybrids to each
133 species (BL, backcross to *H. t. linaresi* and BH, backcross to *H. heurippa*) (Table S2). All
134 butterflies were supplied with ~10% sugar solution and *Lantana*, *Psiguria* and/or *Gurania spp.*.
135 Females were kept individually, with *Passiflora* for oviposition, whereas males were kept in
136 groups. Eggs were collected every other day and the caterpillars were raised until eclosion in
137 plastic cups, and fed on fresh *Passiflora* leaves.
138

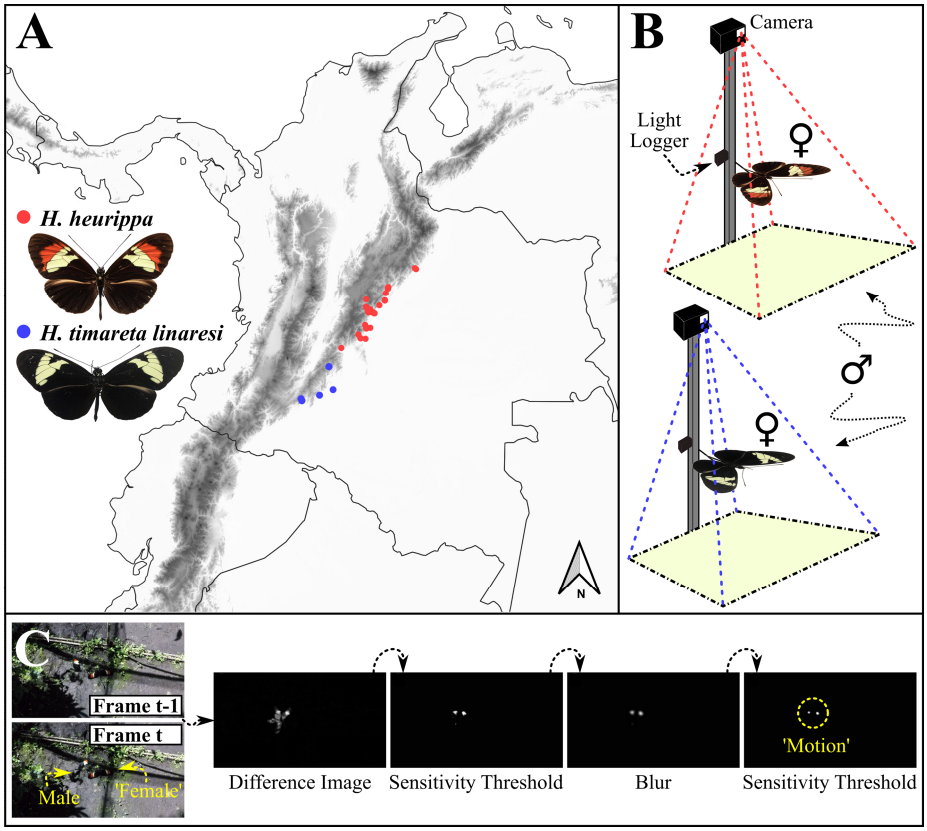

Figure 1: A) Locations in Colombia where *H. heurippa* (red) and *H. timareta linaresi* (blue) are known to occur. B) Video recording setup. Mounted females were presented simultaneously: Each was filmed and an associated light logger recorded illuminance every second. 'Tripod' was not casting shade on light logger or mounted females at any time. C) *Post-hoc* motion detection pipeline. A difference image is formed between each frame and its predecessor (identifying pixels with a change in value). Thresholding, blurring and again thresholding pick out significant local changes ('signals'). 'Signal' frames (and surrounding frames) were extracted.

(b) Trials with mounted females. To test for differences in visual mating behaviours, we assayed males in choice trials with dead mounted *H. heurippa* and *H. t. linaresi* females presented simultaneously in an exposed 4x4x2m insectary, in which light conditions varied due to changes in cloud coverage and patterns of shade cast by vegetation at certain daytimes. Virgin females were frozen with their wings spread on the day of eclosion and kept at -20°C for >168h. They were then dried and subsequently washed in hexane to remove residual cuticular hydrocarbons and other pheromones, and mounted onto a small piece of wire. Throughout the

experiment, we used 21 *H. heurippa* and 23 *H. t. linaresi* mounted females, randomly choosing a pair each day. Mounted females were individually attached to a horizontal wire (~70cm above ground) at one of six locations within the experimental cage. This location was changed every hour (hereafter 'trial'), and the new location was chosen randomly (but female types were never in the same location twice during the same day). A *GoPro Hero 5 Black*™ (GoPro, San Matteo, CA) camera (settings and equipment in Table S3) was installed at each position, and ~50cm above the respective mounted female (Fig. 1B). At same height as the mounted female at ~30cm distance, we attached a *HOBO*™ *UA-002-64* logger (sensor facing up) to measure illuminance [*lux*] every second (Fig. 1B). Cameras and light loggers were synced using *GoPro Quik*™ and *HOBOware*™ software, respectively, allowing us to match video frames with light measurements.

Most of the virgin naive males matured in one separate communal cage before being introduced into the experimental cage >4 days after eclosion, where they were tested in mixed groups (median group size = 22). Males were numbered and received a unique code of dots on the dorsal side of the wings, allowing identification from videos. Each male was tested over multiple days (median = 12d). We recorded an average of 3.01h of material on each of the 246 recording days; conducting behavioural trials across different seasons and at different daytimes allowed us to capture a variety of light conditions.

(**c**) **Computer vision and video analyses.** We used a custom motion-detection pipeline, which *post-hoc* discarded video footage with no activity. The detection of frames with motion ('signals') was based on difference imaging and consecutive steps of blurring and thresholding, as implemented in the *OpenCV* library available for C++ (Fig. 1C). Not all of the frames of male motion sequences made it over the threshold, so we determined the 'signal'-frames and the

178 surrounding frames one sec before and after a signal in R [43]. Reduced videos were created with
179 the *OpenCV* library in C++. (Code is accessible at:
180 github.com/SpeciationBehaviour/visual_preference_heurippa_linaresi). Video material was
181 processed on an *HP*™ Desktop computer (i7-7700 CPU, 4 cores), at runtimes ~80% of video
182 duration. All videos were processed under the same threshold (145) and blur (30) settings.
183 Remaining footage was curated manually at 66.6% speed using the *MPCHC*™ *player*. We
184 recorded three behaviours: 'approach' (male is changing its flight direction towards the mounted
185 female, resulting in a curve or circling motion), 'courtship' (sustained hovering above mounted
186 female) and 'sitting' (male sits down on mounted female). Behaviour types were combined for
187 subsequent analyses.
188
189 **(d) Tetrad experiments with live females**. We performed 'tetrad' trials with virgin males and
190 females to test for assortative mating. For each trial, sexually mature *H. heurippa* and *H. t.*
191 *linaresi* males (one of each) were allowed to acclimatize for 15 mins in a 2x4x2m insectary, at
192 which point *H. heurippa* and *H. t. linaresi* virgin females (one of each) were introduced. Once the
193 first mating occurred, the experiment was stopped.
194
195 **(e) Statistical analyses**. We measured illuminance at both mounted females for 83% of recorded
196 behaviours. Illuminance, measured in *lux*, is the intensity of light falling onto a surface. We
197 log10-transformed *lux* measures (log-illuminance) and scaled (and centred) each set of log-
198 illuminance measurements, making the choice of logarithm base irrelevant for the analyses.
199 Analyses were conducted in R [43] (supplemental R Markdown and
200 https://github.com/SpeciationBehaviour/visual_preference_heurippa_linaresi). Posteriors will be
201 described with a 95% equal-tailed credible interval and the mean as point estimate. We analysed

202 data from trials with mounted females using logistic regression with the *brms* package [44], an
203 interface to the Bayesian software Stan [45]. Male behaviours directed towards the *H. t. linaresi*
204 or *H. heurippa* mounted females were fitted as binary Bernoulli-distributed response variable (*i.e.*
205 0 and 1, respectively); estimates from the model can be understood as a proportion of interactions
206 with the mounted *H. heurippa* female, with higher values indicating stronger relative attraction to
207 the *H. heurippa* 'female' and lower values indicating stronger relative attraction to the *H. t.*
208 *linaresi* 'female'. Models initially included all possible nested variations of the fully saturated
209 model explaining effects of 1) male type, 2) log-illuminance at the *H. heurippa* 'female', 3) log-
210 illuminance at the *H. t. linaresi* 'female', and their interactions. Individual ID and trial were fitted
211 as random effects.

212 To test for 'species' differences, we initially fitted models for the 'pure' males (male type
213 = '*H. heurippa*' or '*H. t. linaresi*'). Segregation of the red bar in BL hybrids (controlled by alleles
214 at *optix* [46]) allowed us to test for linkage between colour and preference loci (see [27,47]) (here
215 male type = 'red' (*Bb* genotype) or 'no red' (*bb* genotype) and brood was additionally fitted as a
216 random effect). To determine which terms to retain [48], we calculated the widely applicable
217 information criterion (WAIC) and the leave-one-out information criterion (LOOIC) for each set
218 of models, using the *loo* package [49], and WAIC weights using the *brms* package [44]. Weakly
219 informative priors (centred around the value for no preference) were set for coefficients
220 corresponding to the different male types, which gave small prior probabilities for extreme values
221 very close to 0 or 1. For all other coefficients, default (non-informative) priors were applied. We
222 also fitted 'categorical illuminance' models adopting the best fitting model structure determined
223 for each dataset, where values =<median were 'poorly lit', and values >median were 'brightly lit'
224 (Fig. S1). Posteriors of the estimated marginal means (EMMs) were calculated using the

225 *emmeans* package [50]. From this, we retrieved posteriors of contrasts and calculated the
226 posterior probability (PP) that EMMs differ.
227 For the tetrad data, we fitted observed counts of each mating outcome (type of male and
228 female involved) as Poisson-distributed response variable. We included the specific male-female
229 combination of the mating outcome as fixed factor. Transforming the resulting model estimates
230 into proportions effectively makes this a multinomial model [51]. Non-informative default priors
231 were applied throughout. PPs were calculated with the *brms* package [44]. We compared the
232 observed frequency of each mating combination to the predicted frequencies based on our
233 measurements of visual preference from the mounted female experiment. Predictions were
234 derived by multiplying the frequency that a male type was involved in *any* mating combination
235 by its respective EMMs from the models fitted to the mounted female data. Predictions were
236 based either on the overall EMM for each type, or the interaction term EMMs from the
237 categorical model. Posterior distributions for each prediction were calculated using the *binom*
238 package under the default prior [52].
239
240 **RESULTS**
241 **(a) Species comparisons: i) Divergent visual attraction behaviours in *H. timareta linaresi***
242 **and *H. heurippa* males are dependent on the light environment.**
243 Over 1.5 years we collected 1481 hours of footage. Our computer vision pipeline reduced this to
244 66 hours requiring human curation (*i.e.* 4.5% of the total footage recorded), including 16,995
245 behavioural 'interactions' from 387 males (~43.9 per male). These data allowed us to determine
246 the effects of male type and illuminance on relative visual attraction to the *H. heurippa* mounted
247 'female' (hereafter 'preference'). The best fitting model for the pure *H. heurippa* and *H. t.*
248 *linaresi* males retained male type, log-illuminance at the *H. heurippa* 'female' and their

interaction (Table S4: model #1). Overall, our results suggest that the local light environment influences the strength of visual attraction.

Across the entire dataset, illuminance at the mounted *H. heurippa* female increased the difference in preference between the male types, as evidenced by the interaction between male type and log-illuminance at the *H. heurippa* 'female' (Fig. 2A & S3, PP simple slope *H. heurippa* > *H. t. linaresi* = 0.996). This was largely driven by an increase in log-illuminance at the *H. heurippa* 'female' leading to a stronger conspecific preference in *H. heurippa* males (PP simple slope > 0 = 0.993); there was only limited support for an effect on *H. t. linaresi* males (PP simple slope < 0 = 0.768). Overall, *H. heurippa* males showed a higher proportion of interactions with the *H. heurippa* pattern than *H. t. linaresi* males. Although supported with high credibility (PP relative visual attraction *H. heurippa* > *H. t. linaresi* = 0.969), this difference in preference was relatively small (0.07, CrI: 0.00 - 0.14) and characterised by considerable within-population variation (Fig. 2B). Nevertheless, this effect nearly doubled when the *H. heurippa* 'female' was in brighter light (0.13, CrI: 0.05 - 0.22; Fig 2D) and was absent when the *H. heurippa* 'female' was poorly lit (0.01, CrI: -0.07 - 0.09; Fig 2C).

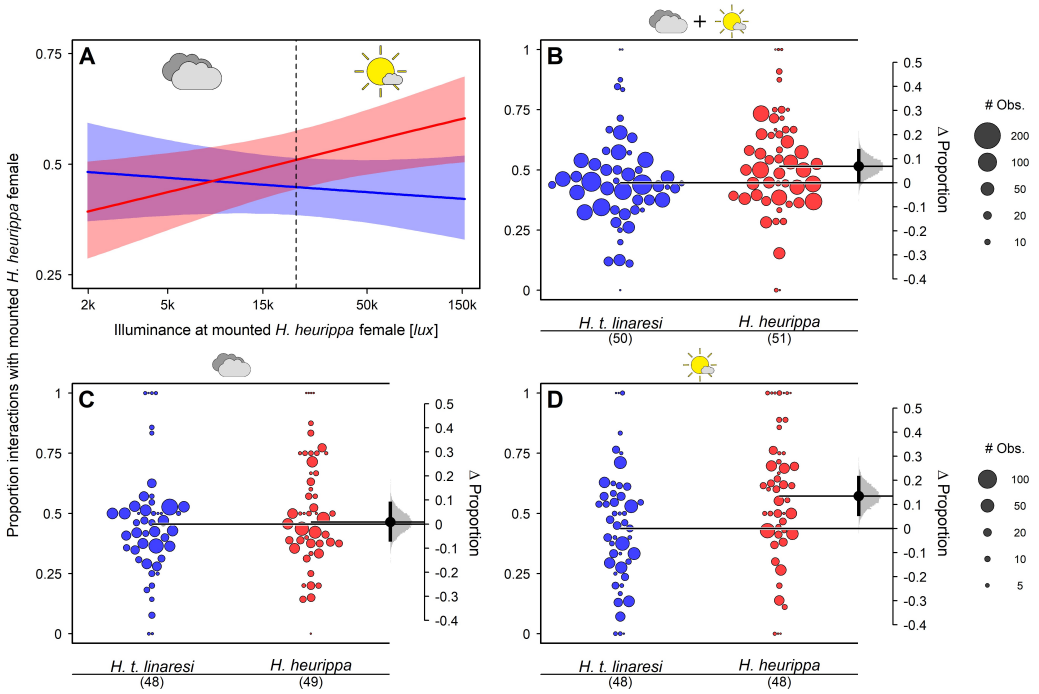

Figure 2: The effect of illuminance on relative attraction towards *H. heurippa* 'females' (*i.e.* proportion of interactions with the *H. heurippa* 'female' as opposed to the *H. t. linaresi* 'female'). **A**: Relative attraction towards *H. heurippa* 'females' of *H. t. linaresi* (blue) and *H. heurippa* males (red) under changing illuminance at the *H. heurippa* 'female'. Illuminance on the x-axis is log-scaled. Coloured area around each regression line represents the 95% credible interval (CrI). Dashed vertical line represents the median log-illuminance used as a cutoff to define the poorly and brightly lit conditions (below). Relative attraction towards *H. heurippa* 'females' for *H. t. linaresi* males (blue) and *H. heurippa* males (red): (**B**) across light environments; (**C**) for poorly lit *H. heurippa* 'female'; (**D**) for brightly lit *H. heurippa* 'female'. Gardner-Altman plots in B-D show the difference between the two male types: Horizontal lines project from the means of the posteriors for each male type (means and CrIs in Table S5). The mean and the 95% CrI for the posterior of the difference between the male types are shown on the right. Each point represents a single individual and its size is scaled to the number of observations. Custom swarmplot was used to distribute the dots horizontally.

(b) Species comparisons: ii) *H. heurippa* males mate more often with conspecific females in choice trials.

To test for assortative mating we also conducted 'tetrad' mate choice trials between *H. heurippa* and *H. t. linaresi*. During 89 tetrad trials we observed 50 con- and 39 heterospecific matings (PP for positive assortative mating = 0.88). This trend was driven by a higher proportion of conspecific matings involving *H. heurippa* males (0.405, CrI: 0.305 - 0.508) than heterospecific

matings involving *H. heurippa* males (0.270, CrI: 0.183 - 0.366]) (PP = 0.94). *H. t. linaresi* males did not participate more frequently in con- rather than heterospecific matings (PP = 0.43) (Fig. 3). In general, *H. heurippa* males mated more often than *H. t. linaresi* males (60 vs. 29 times). Our results closely match predictions derived from the mounted females experiment with brightly lit *H. heurippa* 'female' (horizontal purple bars in Fig. 3), and, to some extent, those derived from all of the illuminance conditions combined (Table S6).

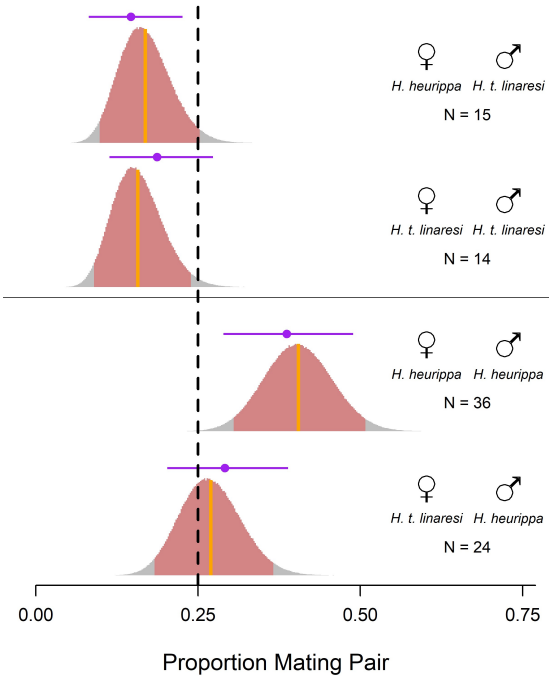

Figure 3: *H. heurippa* males show a preference for live, conspecific females in the tetrad experiments. Dashed vertical line indicates expected proportion under no assortative mating. Posterior distributions for each mating pair combination are displayed as histograms (red = 95% CrI, orange line = mean). Predictions based on the visual attraction data from the mounted females experiment with brightly lit *H. heurippa* 'female' are displayed with their 95% CrI as horizontal purple lines.

(c) Hybrid comparisons: Patterns of behaviour in backcross hybrids are consistent with linkage between colour and preference loci.

Estimates of preference suggest that first generation hybrid (F_1) males behave like *H. heurippa* males (Fig. S4D), perhaps suggesting that *H. heurippa* alleles for attraction to red are dominant;

however, challenging this, BH males seem to display a preference similar to *H. t. linaresi* males (Fig. S4E). For BL males, the model including all possible interactions was the best fit (Table S7, model #1). As for the 'pure' males, the six best fitting models included the interaction between type (*i.e.* red (*Bb*) and non-red (*bb*) wing pattern) and log-illuminance at the *H. heurippa* 'female' (91% of cumulative WAIC weight for an effect of this interaction term).

As for males from the parental populations, the difference between the two genotypes was higher when the *H. heurippa* 'female' was in bright light (Fig. 4CD). There was a small effect of the three-way interaction with illuminance measures at the *H. t. linaresi* 'female' - the difference was slightly higher when only the *H. heurippa* mounted female was brightly lit (Fig. 4C, 0.11, CrI: 0.02 - 0.21), as opposed to when both were (Fig. 4D, 0.07, CrI: 0.00 - 0.13). A brightly lit *H. t. linaresi* mounted female led to a slightly reversed pattern (*i.e. bb* > *Bb*), but only when the *H. heurippa* mounted female was poorly lit (Fig. 4B, -0.03, CrI: -0.13 - 0.07). If both mounted females where poorly lit, there was no difference in preference (Fig. 4A, 0.01, CrI: -0.05 - 0.08). Across the entire dataset, illuminance at the mounted *H. heurippa* female increased the differences in preference between the two BL genotypes (Fig. S2B, PP simple slope *Bb* > *bb* = 0.999). While the slope for *Bb* males is only slightly positive (PP = 0.859), the slope for *bb* males is strongly negative (PP = 0.999). In contrast to the pure males, we also observed an effect of illuminance at the *H. t. linaresi* 'female'. Specifically, *bb* males showed a stronger preference for the *H. heurippa* 'female' when the *H. t. linaresi* 'female' was bright (Fig. S2D & S3, PP = 0.999). Overall, *Bb* males were more likely to interact with the *H. heurippa* 'female' than *bb* males (Fig. S5). This difference was supported with moderately high probability (PP relative visual attraction *Bb* > *bb* = 0.897). Although these represent small effects in absolute terms (0.03, CrI: -0.02 - 0.08]), this difference accounts for ~50% of the difference in means of the parental populations, consistent with linkage between colour and preference loci.

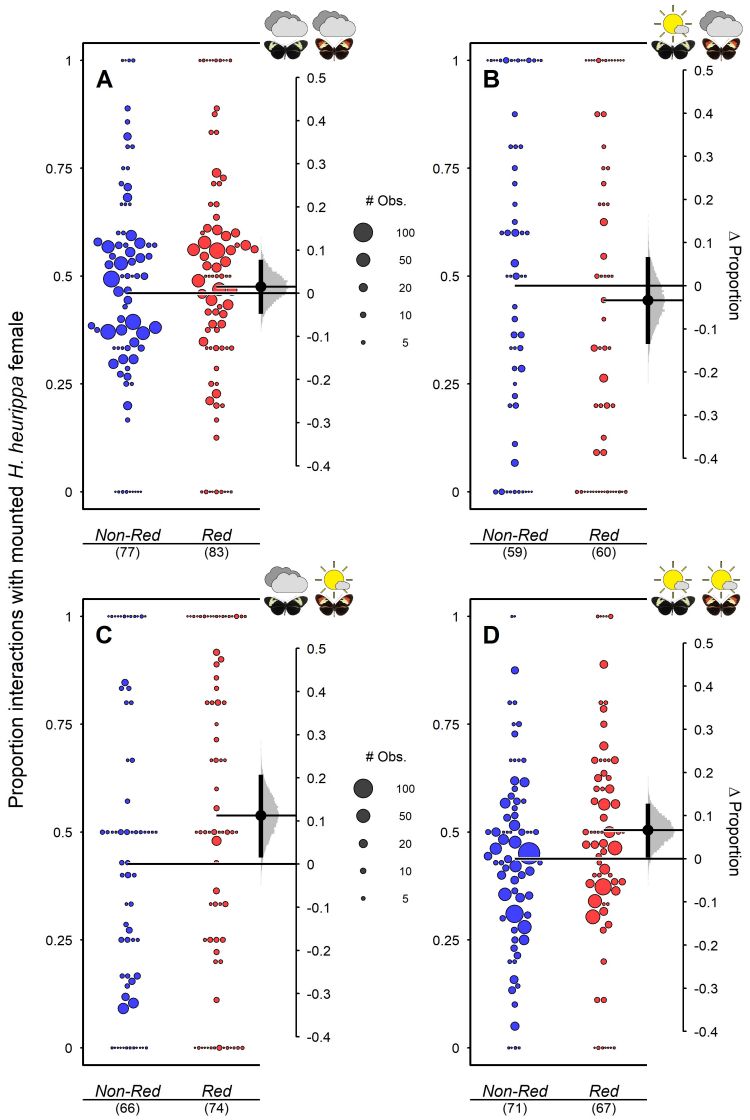

Figure 4: The effect of illuminance on relative attraction towards *H. heurippa* 'females' (i.e. the proportion of interactions with the *H. heurippa* 'female' as opposed to the *H. t. linaresi* 'female') for BL hybrid males (no red/*bb* = blue dots; red/*Bb* = red dots): (**A**) with poorly lit *H. t. linaresi* and *H. heurippa* 'females'; (**B**) brightly lit *H. t. linaresi* and poorly lit *H. heurippa* 'females'; (**C**) poorly lit *H. t. linaresi* and brightly lit *H. heurippa* 'females'; and (**D**) brightly-lit *H. t. linaresi* and *H. heurippa* 'females' (means and CrIs in Table S5). Gardner-Altman plots are as in Figures 1B-D.

DISCUSSION

By collecting ~1500h of mate choice data we have shown that the light environment can influence visual mating behaviours during the early stages of divergence in *Heliconius* butterflies. Although our data are characterised by considerable individual variation, we observed significant

337 differences in behaviours of *H. heurippa* and *H. timareta linaresi* males, and this difference is
338 stronger when the female patterns are more brightly lit. Experiments with live males and females
339 revealed a degree of assortative mating, and the differences in visual attraction behaviours that
340 we observe are sufficient to explain this. We have also shown that differences in visual attraction
341 are associated with the presence of the red forewing band in interspecific hybrids under bright
342 light conditions, perhaps suggesting physical linkage between an ecologically relevant colour
343 pattern gene and those for the corresponding behaviour.

344 Studies of speciation often focus on already diverged groups, which are maintained by
345 multiple reproductive barriers [53,54], making it difficult to draw conclusions about the role of
346 the individual barriers and their ecological context. At the other extreme, barriers acting at the
347 early stages of divergence may be of small effect and, especially in the case of behavioural
348 phenotypes, require very large datasets to draw robust conclusions, which may not always be
349 feasible. Behavioural researchers increasingly use computer vision [55], and software for
350 automated tracking and individual identification is available (e.g. [56,57]). However, applying
351 these techniques to footage with heterogenous backgrounds, variable light environments and
352 arenas larger than the camera's field of view remains a challenge. To overcome these limitations,
353 we combined computer vision, allowing *post-hoc* motion detection, with subsequent human
354 curation. This permitted a simple low-cost solution, and importantly allowed us to capture frames
355 before and after motion is detected from many hundreds of hours of video footage.

356 This large volume of data revealed that increased illuminance at the red mounted *H.*
357 *heurippa* female increased the frequency of interactions between *H. heurippa* males with *H.*
358 *heurippa* females (relative to *H. t. linaresi* females). *H. heurippa* males were no more likely than
359 *H. t. linaresi* males to interact with the *H. heurippa* female when she was poorly lit (lower 50%

360 quantile of illuminance values; Fig. 2C), but were 1.3 times (*i.e.* 0.57/0.44, Table S5) more likely
361 to interact with the *H. heurippa* female in brighter light (upper 50% quantile of illuminance
362 values; Fig. 2D). Similar effects of the light environment have been observed in aquatic
363 organisms. For example, the attractiveness of red colour during courtship is dependent on the
364 spectrum of available light, influenced by water depth and turbidity, and recognition of con-
365 specifics may fail entirely under certain light environments (e.g. [10–12,19]). However, far fewer
366 studies have directly tested how changing light environments influence mating preference
367 behaviours that contribute to population divergence in terrestrial organisms (but see [17,58,59]).

368 The mechanism underlying the differences in behaviour we observe under contrasting
369 lighting conditions isn't immediately clear. Insects, including butterflies [60], have frequently
370 evolved colour constancy across light environments [61,62], and although this may only partially
371 succeed [60,61], we generally expect individuals to be able to distinguish *H. heurippa* and *H. t.*
372 *linaresi* patterns under different brightness conditions. Alternatively, male attraction to female
373 colour patterns might to some degree be 'wavelength-specific', where triggering of behaviours
374 depends greatly on an object's emission of specific wavelengths and their intensity [62]. Under
375 this scenario, a colour cue might only trigger a behaviour when presented at intense illuminance
376 including wavelengths from the relevant part of the spectrum [62–64]. The differences in
377 illuminance we measured may correlate with spectral differences; under shaded conditions, the
378 available light may be reflected from the vegetation or from outside the path of direct sunlight,
379 and consequently lack red wavelengths (but be rich in greenish or bluish light) [16,17]. Indeed,
380 previous work modelling *Heliconius* vision suggests that red patterns are more conspicuous to
381 *Heliconius* when presented in bright sunlight [41] (though whether this affects *Heliconius*
382 behaviours has not previously been tested).

383 Regardless of the proximate mechanism, our data indicate that prevailing light conditions
384 can influence visual mating behaviours in *Heliconius*. Unlike many other examples, often
385 involving anthropogenic induced changes in aquatic environments [10–12], the effects we see
386 here potentially act across very short time and spatial scales. This reflects the forest environment
387 where the light environment can vary rapidly across both time and space. As such, male response
388 may depend less on the broader environment (perhaps influenced by forest type), than on the
389 movement of females across smaller spatial scales (for example between dimly and brightly lit
390 patches). *Heliconius* butterflies are known to bask in the sun with their wings open, particularly
391 during the morning hours [42]. This may primarily be for thermoregulation, though other species
392 are known to display in environments where they are most conspicuous, and *Heliconius* might
393 follow a similar strategy. *Anolis* lizards, for example, occupy micro-habitats in which their
394 dewlap colour is most conspicuous [65]. Visual modelling indicates *Heliconius* red patterns are
395 more conspicuous to avian predators when presented in bright sunlight, suggesting it may also
396 enhance aposematism [41]. Whatever the ultimate reason for aggregating in sun-exposed patches,
397 our data suggest that these behaviours could enhance the strength of divergent mating
398 preferences.

399 Whether the differences in visual attraction behaviours that we observe translate to
400 assortative mating, and therefore contribute to reproductive isolation, is an important question.
401 Previous studies testing for assortative mating between *Heliconius* species in tetrad experiments
402 similar to ours report low frequencies of interspecific mating [29,66,67]. This was not the case in
403 our experiments. Nevertheless, the large number of trials in our study allowed us to detect
404 positive assortative mating, albeit at low levels, though this was much stronger for the 60 trials in
405 which *H. heurippa* males mated (Fig. 3). Unfortunately, we do not have illuminance data for

406 these experiments, and this would be difficult to measure given the movement of females.
407 Considering that the live females may have been basking in the sun, that overall activity of
408 *Heliconius* is highest when it is sunny [68] and that mating in *Heliconius* seems to occur more
409 frequently on sunny than on cloudy days (M. Linares, pers. obs.), it is likely that a large
410 proportion of the trials with mating outcomes included sun-exposed females. When accounting
411 for the more frequent involvement of *H. heurippa* males in mating, the mating rates between *H.*
412 *heurippa* and *H. t. linaresi* in our tetrad experiments closely match estimates derived from our
413 experiments with dead mounted females in bright light. Although we cannot rule out a role for
414 cues acting across other sensory modalities, differences in visual attraction alone are sufficient to
415 explain the levels of assortative mating we observe.

416 Considering our data for hybrids, it is overall difficult to ascertain patterns of dominance
417 for visual attraction. This is perhaps not surprising given that the differences in behaviour
418 between populations are subtle and are shaped by considerable variation. However, segregation
419 of the *optix* alleles, which control red pattern elements in *Heliconius* [46], in the backcross to *H.*
420 *t. linaresi* did allow us to test for linkage between the warning pattern cue and the corresponding
421 behaviour. Once again, we observed illuminance-induced shifts in visual attraction, so that the
422 two types of backcross to *H. t. linaresi* males (*i.e.* red/*Bb* vs. non-red/*bb*) differed in behaviour,
423 but only under higher illuminance conditions. These results are consistent with physical linkage
424 between behavioural loci and *optix*, as has been shown elsewhere – but with much greater effects
425 – between the closely related species *H. cydno* and *H. melpomene* [27]. Physical linkage will help
426 to maintain key genetic associations (*i.e.* LD) between loci underlying ecologically relevant traits
427 and those for premating isolation, like mating preferences, facilitating speciation with gene flow
428 in general [21,23], and hybrid trait speciation [69] in particular. Although the differences between

429 backcross to *H. t. linaresi* genotypes account for ~50% of that observed in the two parental taxa,
430 in absolute terms, the effects are unlikely to permit sufficient power for a formal QTL study and
431 caution should be exercised when interpreting our results. Our results are consistent with a simple
432 genetic mechanism by which behavioural alleles were acquired alongside red colour pattern
433 alleles through introgression from *H. melpomene* into the '*heurippa*/*timareta*/*cydno*' lineage
434 [69,70]. However, it seems unlikely that the behavioural differences we observe here would
435 rapidly lead to strong reproductive isolation, as predicted under a model of hybrid trait speciation
436 [66,69].

437 In conclusion, the behavioural differences we observe for *H. heurippa* and *H. t. linaresi*
438 are similar in strength to those reported elsewhere for other *Heliconius* taxa at the early stages of
439 divergence (e.g. [30,53]). Alone, these may represent only weak barriers to gene flow; however,
440 by augmenting divergent ecological selection acting on the warning pattern cue, they may
441 facilitate the accumulation of additional barriers as speciation proceeds. In addition, our results
442 suggest that the degree to which *Heliconius* warning pattern contribute to premating isolation
443 may depend on local illuminance, which can change rapidly in both time and space. Without
444 more stable differences in the sensory environment, perhaps facilitated by shifts in habitat use,
445 these fluctuations may constrain speciation. However, *Heliconius* are known to aggregate and
446 display their warning patterns in sun-exposed patches within the broader forest environment, and
447 our results suggest that this context would enhance premating isolation. Traits predominantly
448 shaped by ecology frequently act as mating cues, which by coupling divergent natural selection to
449 premating isolation can promote speciation with gene flow [6]; our results highlight that this
450 effect may depend on the sensory conditions during which these ecological mating cues are
451 displayed to conspecifics.
452

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Supplementary material: Light environment influences mating behaviours during the early stages of divergence in tropical butterflies

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1) Supplementary Figures

Figure S1: Examples from processed videos showing ‘poorly lit’, median and ‘brightly lit’ *H. heurippa* ‘female’ (left) and a *H. t. linaresi* ‘female’ as a comparison at same illuminance (right). Median illuminance at the *H. heurippa* ‘female’ was calculated from illuminance data at the *H. heurippa* ‘female’ when ‘pure’ males showed a response to either model. This median value was used as threshold to build the categorical model for ‘pure’ males. The exact same procedure was used for BL hybrid males. The specific *lux* values for ‘poorly lit’ and ‘brightly lit’ conditions (6’200 and 132’268 lux) shown here were randomly chosen from the bottom and the top quartile of the distribution. The presented frames were picked from different recording days. Additionally to the mounted female, each frame shows a conspecific male approaching/courting the ‘female’ and dots used to identify individual males are visible on their wings. A change in hue of the red patch on *H. heurippa* seems to be visible under different illuminance conditions, but it should not be forgotten that 1) human vision is vastly different from butterfly vision and 2) that this is a representation produced by the GoPro camera (GoPro, San Matteo, CA), which is post-processing colour-composition of frames.

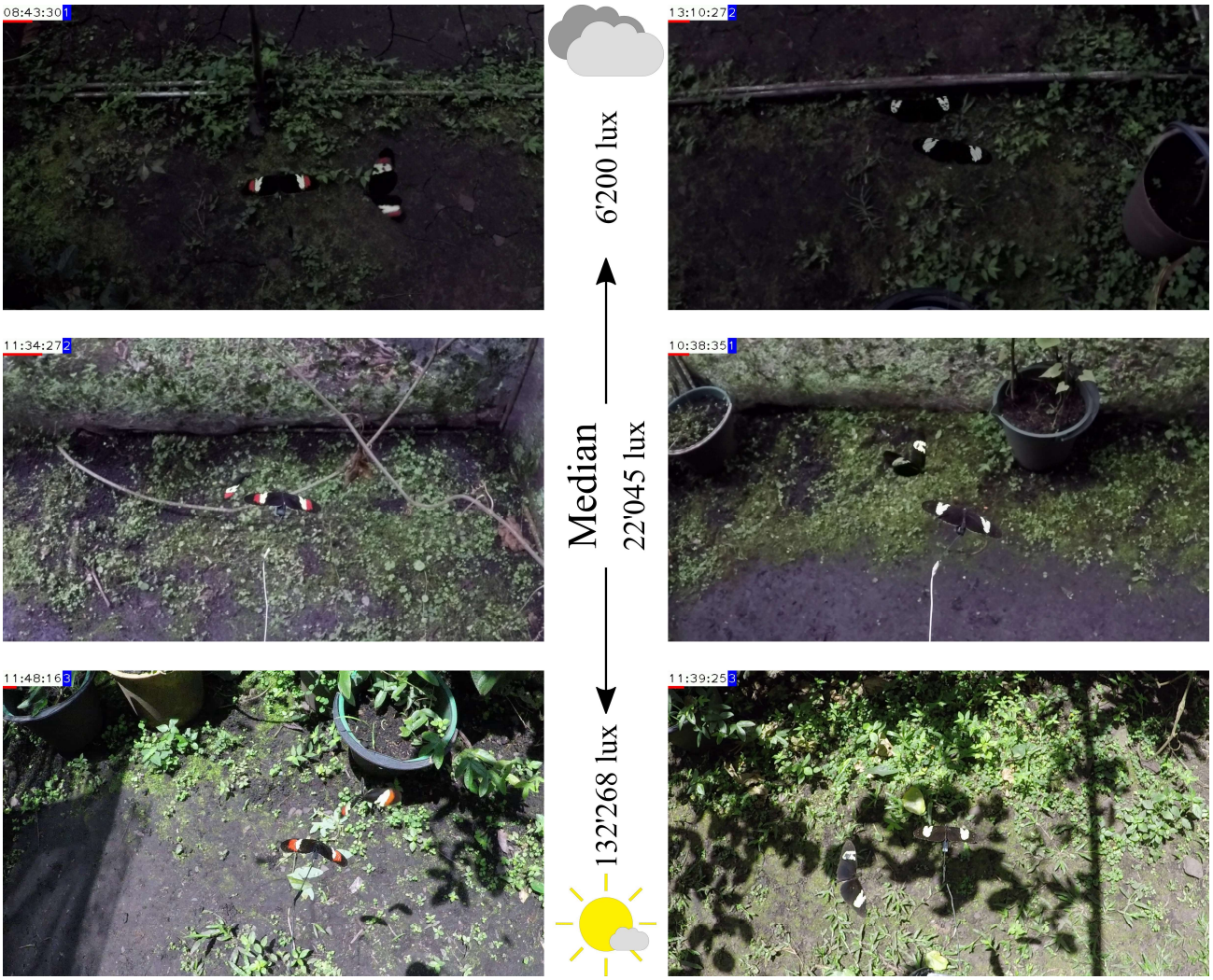


Figure S2: All interaction terms involving male type from fully saturated models (*i.e.* Table S2, model #3 for *H. heurippa* and *H. t. linaresi* males and Table S4, model #1 for backcross to *H. t. linaresi* males). **A+B**: Two-way interactions between illuminance at the *H. heurippa* mounted female and male type. Proportion of interactions with the *H. heurippa* female of *H. heurippa* (red) and *H. t. linaresi* (blue) males (A; resembles Fig. 2A, but comes from a different underlying model); and differently coloured backcross to *H. t. linaresi* males (B, red males (red), males without red (blue)). **C+D**: The same for the two-way interactions between illuminance at the *H. t. linaresi* mounted female and male type **E-H**: Three-way interactions between male type and the two illuminance measures. Male type is indicated on top of each graph. Colouration of each region in the graph depends on predicted preference for the *H. heurippa* mounted female at the given light conditions (scale depicted on right). White contour lines show Kernel densities weighted by preference score, which essentially gives the same information as the colour gradient ('peaks' are where the landscape is most red). All illuminance-axes are logarithmically scaled. Dashed lines in G and H show median illuminance measures at the respective axis, used to categorize conditions into 'poorly' and 'brightly' lit for the categorical models.

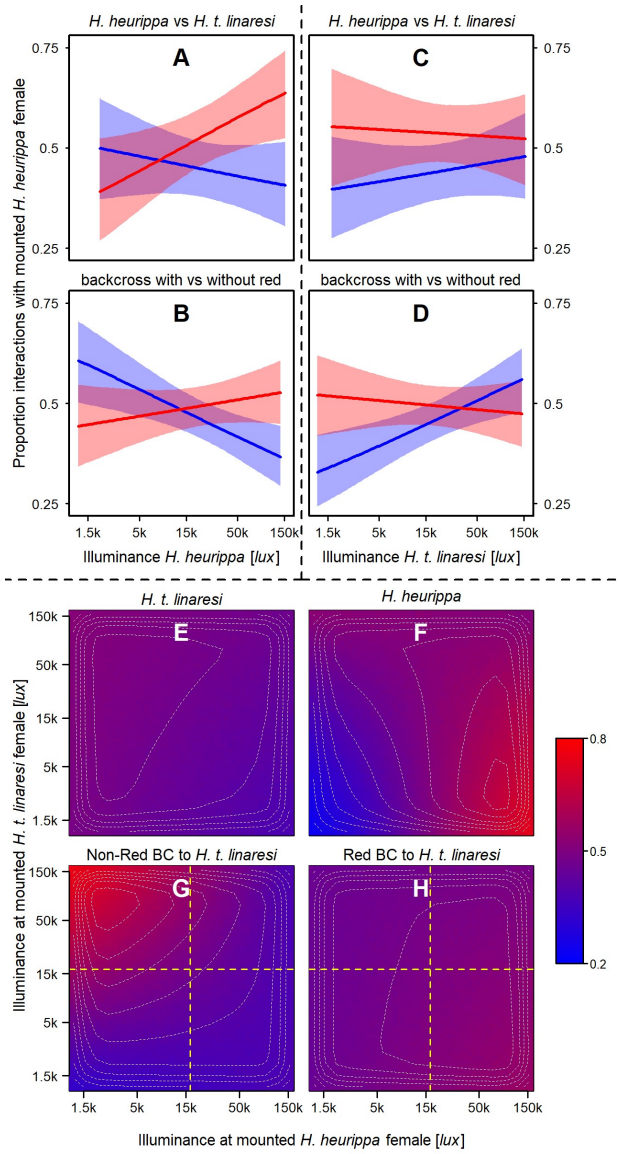


Figure S3: **A-D**: Raw data points of *H. t. linaresi*, *H. heurippa*, non-red and red backcross to *H. t. linaresi* males (from top to bottom) in a two-dimensional illuminance space. Horizontal dimension shows illuminance at the mounted *H. heurippa* female, vertical dimension shows illuminance at the mounted *H. t. linaresi* female. Blue dots indicate responses of males to the mounted *H. t. linaresi* female and red dots indicate responses to the mounted *H. heurippa* female. Kernel densities are shown for each set of data (blue lines for responses to *H. t. linaresi*, red lines for responses to *H. heurippa*). **E-H**: the two-dimensional illuminance space was then divided into 15 squares for each male type and local preferences within each square were calculated. Graphs directly relate to graphs A-D. The higher the preference for the mounted *H. heurippa* female in a square, the more red the square; the higher the preference for the *H. t. linaresi* female, the more blue (see scale on the right). Squares without response are white. These local preferences have to be interpreted in combination with the local sample sizes (as shown in left column), since they often rely on very few observations. All axes are logarithmically scaled.

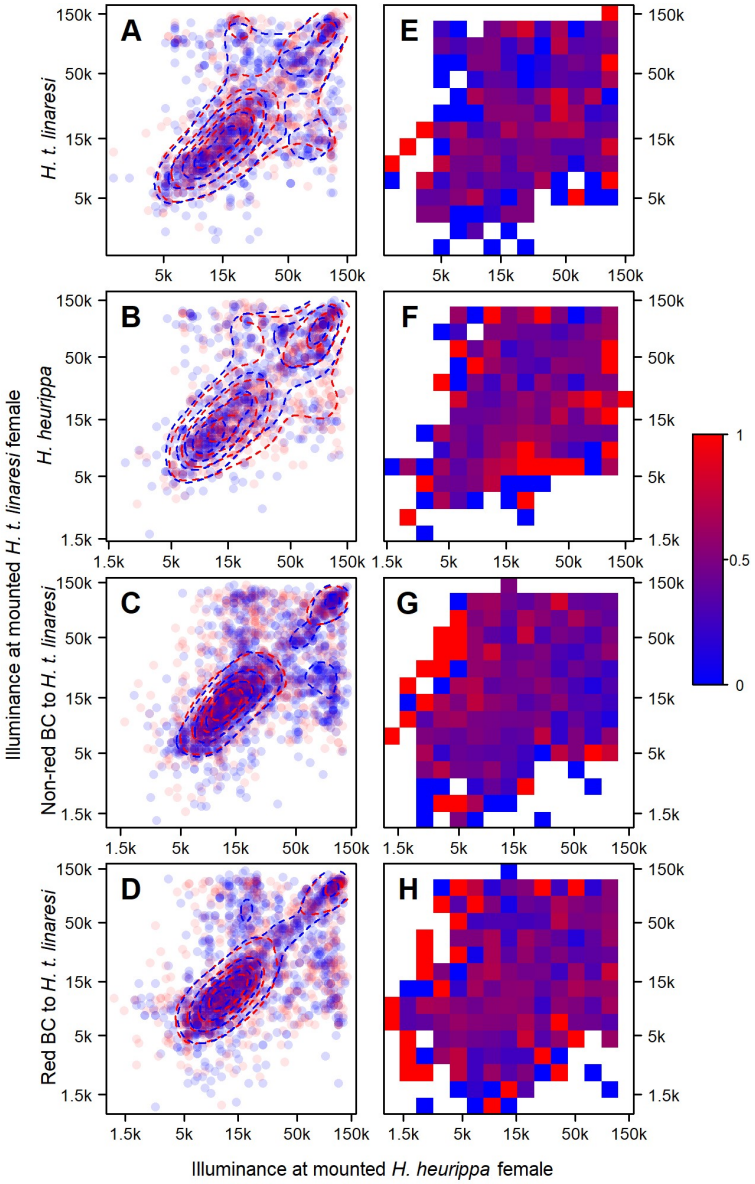
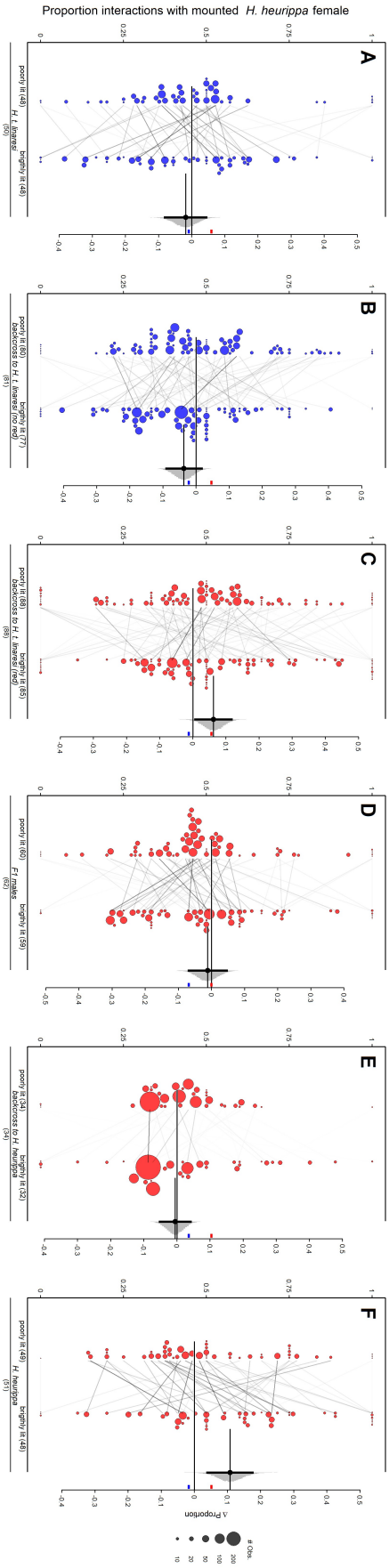
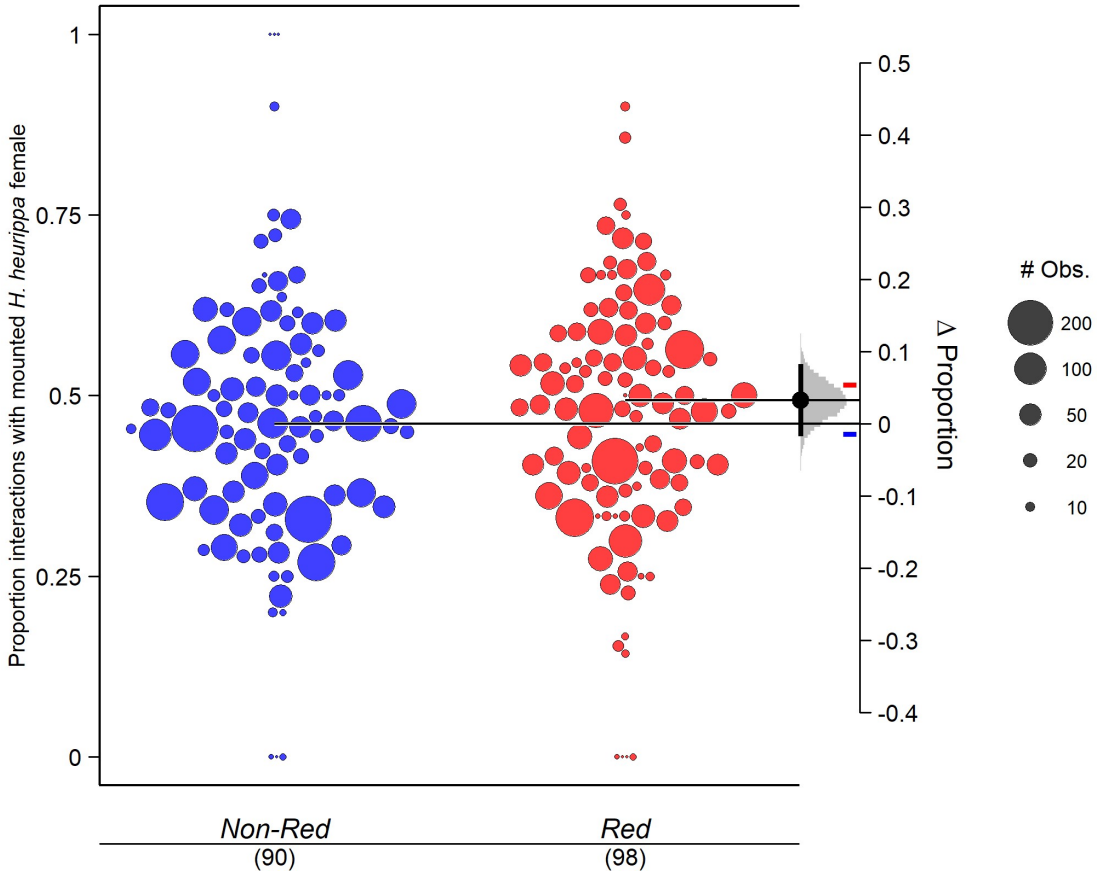


Figure S4: Individual trajectories from categorical models. Visual preferences of pure males and hybrid males are displayed by type. Within each type, data is split depending on illuminance around the *H. heurippa* mounted female, into poorly and brightly lit. Whenever an individual appeared under both light conditions, it is connected by a reaction norm line in this plot. Transparency of reaction norm lines within each panel is scaled by the maximum number of observations available for one individual within this panel. For *H. t. linaresi*, *H. heurippa* and BL males, posterior means for each EMM are calculated from the same underlying categorical models as used for Fig. 2 and Fig. 4, respectively. For F_1 and BH males, a categorical model was fit including male type, the illuminance category around the *H. heurippa* 'female' (determined again by the median measurement) and their interaction. EMMs were then extracted using the same logic as for the other types. The difference between two groups (in this case between poorly and brightly lit conditions within each type) is shown by the same Gardner-Altman plot type as used for Fig. 2 and Fig. 4. Small red and blue line located at the right side of each panel show estimators for *H. t. linaresi* and *H. heurippa* males from Fig. 2B as a reference. [*Figure follows on next page*]


Proportion interactions with mounted H. heurippa female
A
poorly lit (48)
H. f. linaresi
(35)
brightly lit (48)
B
poorly lit (80)
backcross to H. f. linaresi (no red)
(61)
brightly lit (77)
C
poorly lit (88)
backcross to H. f. linaresi (red)
(66)
brightly lit (85)
D
poorly lit (60)
F1 males
(62)
brightly lit (59)
E
poorly lit (34)
backcross to H. heurippa
(34)
brightly lit (32)
F
poorly lit (49)
H. heurippa
(31)
brightly lit (49)
Δ Proportion
Obs.
10
20
50
100
200

720

Figure S5: Relative attraction towards *H. heurippa* 'females' (i.e. the proportion of interactions with the mounted *H. heurippa* 'female' as opposed to the *H. t. linaresi* 'female') for non-red (*bb*) BL males (blue dots) and red (*Bb*) BL males (red dots). Data and estimators are across all light environments. Note that only preference data with associated illuminance data were included in the underlying statistical models (a few of the raw data points displayed here therefore were not considered by the underlying model). The difference between the two groups is shown with the same logic as used for Fig. 2 and Fig. 4. Small red and blue line located at the right side of each panel show estimators for *H. t. linaresi* and *H. heurippa* males from Fig. 2B as a reference.



2) Supplementary Tables

Table S1: Collection locations of *H. t. linaresi* and *H. heurippa*.

Taxon	Location
Heliconius timareta linaresi	Guayabal (2°41'04"N, 74°53'17"W)
Heliconius heurippa	Lejanías (03°34'0"N, 74°04'20.4"W) Buenavista (4°10'30"N, 73°40'41"W) Santa María (04°53'28.2"N, 73°15'11.4"W)

Table S2: Information on different experimental hybrid lines. Left column shows F_1 broods from which lines originated. For the third line, we summarized two F_1 crosses and their subsequent crosses into one hybrid line. These two F_1 crosses had the same *H. heurippa* father, but a different *H. t. linaresi* mother. Individual counts in brackets show how many males of each brood were tested in the experiment. 32 other F_1 males from 7 broods were tested in the experiment and are not mentioned here, as their brood was not involved in generating backcrosses (13 individuals/5 broods of those being from a cross between *H. t. linaresi* mother and *H. heurippa* father and 19 individuals/2 broods being from the reciprocal F_1 cross with *H. heurippa* mother and *H. t. linaresi* father). *H. heurippa* is abbreviated as *heu* and *H. t. linaresi* as *lin* in the table.

F_1 brood	**Backcross to *lin* with male F_1**	**Backcross to *lin* with female F_1**	**Backcross to *heu* with male F_1**
mother: *lin*, father: *heu*	mother: *lin*, father: F_1	mother: F_1, father: *lin*	mother: *heu*, father: F_1
C18_002 (12 indiv.)	7 broods (45 indiv.)	2 broods (9 indiv.)	2 broods (20 indiv.)
C18_020 (6 indiv.)	3 broods (83 indiv.)	1 brood (15 indiv.)	1 brood (9 indiv.)
C18_034 & C18_036 (half-sib.) **(13 indiv.)**	4 broods (18 indiv.)	2 broods (10 indiv.)	3 broods (6 indiv.)
C19_014 (-)	1 brood (8 indiv.)	-	-

Table S3: *GoPro Hero 5 Black*™ (GoPro, San Matteo, CA) camera settings and equipment. All auto-settings (except were indicated) were disabled.

Settings	
Resolution	1920 x 1080 pixels
Frame rate	60 fps
Shutter speed	1/480
Field of view	*Narrow*
Colour	*GoPro*
Sharpness	*High*
ISO	400
White balance	*Auto*
Equipment	• 128 GB *SanDisk™ Extreme* SD card • *Neewer™ UV* filter (reduced overexposure of yellow bands) • *Xlayer™ 210033* powerbank (charging while recording)

Table S4: 19 possible fixed-effect structures for the pure males of *H.* heurippa and *H. t.* linaresi (fixed effect 'type'). 'I_H' and 'I_L' stand for log-illuminance measures at the *H.* heurippa and the *H. t.* linaresi mounted females, respectively. A '*' sign in a model formula indicates that all involved variables as well as all possible interactions between them are included. All models include trial and male ID as random effects. Including trial (and in many cases also individual ID) as a random effect has often been disregarded in previous analyses. However, we found strong correlation among individuals' behaviour during trials (see supplementary R Markdown), likely due to the unique combination of males, mounted females and their position in the experimental cage during a given trial. The latter may have also affected sensitivity of our motion-detection software. We therefore considered it inevitable to correct for this. For each model we calculated a LOOIC and WAIC value, which mostly agree. Based on the WAIC value, differences between best model and other models are calculated (ΔWAIC) as well as a model-weight score.

#	Fixed Effects Term	LOOIC	WAIC	ΔWAIC	$\text{Weight}_{\text{WAIC}}$
1	~ type * I_H	3773.40	3768.52	0.00	0.29
2	~ type * I_H + I_L	3774.63	3769.68	1.16	0.16
3	~ type * I_H * I_L	3775.20	3770.08	1.56	0.13
4	~ type * I_H + I_H * I_L	3775.99	3771.04	2.52	0.08
5	~ type * I_H + type * I_L	3776.53	3771.52	3.01	0.06
6	~ type * I_H + type * I_L + I_H * I_L	3776.62	3771.56	3.04	0.06
7	~ type	3777.82	3773.07	4.55	0.03
8	~ type + I_L	3778.10	3773.20	4.68	0.03
9	~ type + I_H	3778.11	3773.33	4.81	0.03
10	~ I_L	3778.50	3773.62	5.10	0.02
11	~ type * I_L	3778.83	3773.95	5.43	0.02
12	~ 1	3779.24	3774.44	5.92	0.02
13	~ I_H	3779.45	3774.58	6.06	0.01
14	~ type + I_H + I_L	3779.75	3774.87	6.35	0.01
15	~ I_H + I_L	3780.58	3775.58	7.06	0.01
16	~ type + I_H * I_L	3780.77	3775.80	7.28	0.01
17	~ type * I_L + I_H	3780.82	3775.99	7.47	0.01
18	~ type * I_L + I_H * I_L	3781.61	3776.60	8.09	0.01
19	~ I_H * I_L	3781.85	3776.78	8.26	0.00

Table S5: Posterior means and 95% equal-tailed credible intervals for male types from Fig. 2, Fig. S5 and Fig. 4. Minus ("-") in the second column stands for a poorly lit mounted female, a plus ("+") for a brightly lit one.

Male type	Light environment	Mean	Lower	Upper
H. t. linaresi (left in Fig. 2B)	Across all	0.447	0.383	0.513
H. heurippa (right in Fig. 2B)	Across all	0.515	0.449	0.582
H. t. linaresi (left in Fig. 2C)	*H. heurippa* -	0.464	0.391	0.540
H. heurippa (right in Fig. 2C)	*H. heurippa* -	0.456	0.385	0.527
H. t. linaresi (left in Fig. 2D)	*H. heurippa* +	0.437	0.362	0.514
H. heurippa (right in Fig. 2D)	*H. heurippa* +	0.572	0.493	0.650
BL without red (left in Fig. S5)	Across all	0.461	0.414	0.508
BL with red (right in Fig. S5)	Across all	0.493	0.444	0.543
BL without red (left in Fig. 4A)	*H. heurippa* - / *H. t. linaresi* -	0.460	0.406	0.515
BL with red (right in Fig. 4A)	*H. heurippa* - / *H. t. linaresi* -	0.475	0.419	0.531
BL without red (left in Fig. 4B)	*H. heurippa* - / *H. t. linaresi* +	0.477	0.401	0.554
BL with red (right in Fig. 4B)	*H. heurippa* - / *H. t. linaresi* +	0.443	0.359	0.528
BL without red (left in Fig. 4C)	*H. heurippa* + / *H. t. linaresi* -	0.426	0.350	0.505
BL with red (right in Fig. 4C)	*H. heurippa* + / *H. t. linaresi* -	0.538	0.461	0.616
BL without red (left in Fig. 4D)	*H. heurippa* + / *H. t. linaresi* +	0.438	0.384	0.491
BL with red (right in Fig. 4D)	*H. heurippa* + / *H. t. linaresi* +	0.504	0.446	0.563

Table S6: Posterior means and 95% equal-tailed credible intervals for estimates of proportions of each mating outcome from tetrad experiments. First row is based on empirically measured data, the other rows are stochastic predictions based on mounted female data from all light environments, from a poorly lit *H. heurippa* mounted female and a brightly lit one, respectively.

Estimates based on	***H. heurippa*** ♀ ***H. t. linaresi*** ♂	***H. t. linaresi*** ♀ ***H. t. linaresi*** ♂	***H. heurippa*** ♀ ***H. heurippa*** ♂	***H. t. linaresi*** ♀ ***H. heurippa*** ♂
tetrad experiment	0.168 [0.099; 0.253]	0.157 [0.09; 0.239]	0.405 [0.305; 0.508]	0.27 [0.183; 0.366]
mounted females: across all	0.149 [0.084; 0.23]	0.184 [0.111; 0.27]	0.349 [0.255; 0.45]	0.329 [0.236; 0.429]
mounted females: poorly lit *H. heurippa*	0.152 [0.086; 0.233]	0.181 [0.109; 0.266]	0.315 [0.224; 0.414]	0.363 [0.267; 0.465]
mounted females: brightly lit *H. heurippa*	0.146 [0.082; 0.226]	0.187 [0.114; 0.273]	0.387 [0.289; 0.489]	0.291 [0.203; 0.389]

Table S7: 19 possible fixed-effect structures for the BL males (fixed effect 'type' refers here to wing colour phenotype of hybrids). 'I_H' and 'I_L' stand for log-illuminance measures at the *H. heurippa* and the *H. t. linaresi* mounted females, respectively. A '*' sign in a model formula indicates that all involved variables as well as all possible interactions between them are included. All models include trial, male ID and brood as random effects. Each model structure has a LOOIC and WAIC value, which mostly agree. Based on the WAIC value, differences between best model and other models are calculated (ΔWAIC) as well as a model-weight score.

#	Fixed Effects Term	LOOIC	WAIC	ΔWAIC	$Weight_{WAIC}$
1	~ type * I_H * I_L	6815.06	6811.69	0.00	0.38
2	~ type * I_H + type * I_L + I_H * I_L	6815.98	6812.60	0.91	0.24
3	~ type * I_H + type * I_L	6817.15	6813.84	2.15	0.13
4	~ type * I_H + I_H * I_L	6817.82	6814.43	2.74	0.10
5	~ type * I_H	6820.15	6816.86	5.17	0.03
6	~ type * I_H + I_L	6820.78	6817.48	5.79	0.02
7	~ type + I_H * I_L	6821.33	6817.96	6.27	0.02
8	~ I_H * I_L	6822.03	6818.67	6.98	0.01
9	~ type	6821.94	6818.68	6.99	0.01
10	~ type * I_L + I_H * I_L	6822.28	6818.95	7.27	0.01
11	~ 1	6822.46	6819.18	7.50	0.01
12	~ type + I_H	6822.96	6819.70	8.01	0.01
13	~ type + I_L	6823.18	6819.94	8.26	0.01
14	~ I_H	6823.50	6820.19	8.50	0.01
15	~ I_L	6823.80	6820.52	8.84	0.00
16	~ type + I_H + I_L	6824.36	6821.03	9.35	0.00
17	~ type * I_L	6825.21	6821.92	10.23	0.00
18	~ I_H + I_L	6825.20	6821.93	10.24	0.00
19	~ type * I_L + I_H	6825.55	6822.23	10.54	0.00