

1 **Adaptive divergence in the eyes of *Heliconius* butterflies likely contributes**
2 **to pre- and post-mating isolation.**

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24 **Abstract**

25 When populations experience different sensory conditions, natural selection may favor whole
26 sensory system divergence, from the peripheral structures to the brain. We characterized the
27 outer eye morphology of sympatric *Heliconius* species from different forest types, and their
28 first-generation reciprocal hybrids to test for adaptive visual system divergence and hybrid
29 disruption. In Panama, *Heliconius cydno* occurs in closed forests, whereas *Heliconius*
30 *melpomene* resides in more open areas. Previous work has shown that, among wild
31 individuals, *H. cydno* has larger eyes than *H. melpomene*, and there are heritable, habitat-
32 associated differences in the visual brain structures that exceed neutral divergence
33 expectations. Notably, hybrids have intermediate neural phenotypes, suggesting disruption.
34 To test for similar effects in the visual periphery, we reared both species and their hybrids in
35 common garden conditions. We confirm that *H. cydno* has larger eyes and provide new
36 evidence that this is driven by selection. Hybrid eye morphology is more *H. melpomene*-like
37 despite body size being intermediate, contrasting with neural trait intermediacy. Thus, eye
38 morphology differences between *H. cydno* and *H. melpomene* are consistent with adaptive
39 divergence, and when combined with previous neuroanatomy data, suggest hybrid visual
40 system disruption due to mismatched patterns of intermediacy and dominance in the visual
41 pathway.

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43 **Keywords:** sensory adaptation, selection, visual system, hybrid disruption

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49 **Introduction**

50 Sensory systems mediate the transmission of information between an organism and its
51 surroundings (Stevens, 2013). Natural selection is expected to favor divergent sensory
52 phenotypes across populations exposed to different sensory conditions and/or which exploit
53 different resources, potentially leading to both pre- and post-mating reproductive
54 isolation (Dell'Aglio et al., 2023). Habitat-associated variation in sensory traits is well-
55 documented, particularly for vision (Webster, 2015). However, most studies of visual
56 adaptation between populations have focused on color vision in aquatic organisms (Carleton
57 & Yourick, 2020; Cummings & Endler, 2018), whereas other aspects of visual perception are
58 understudied. For example, we know little about how visual signals are evaluated (Rosenthal,
59 2018) or how adaptive processes operate at different levels within the visual pathway.
60 Moreover, we lack information on the visual capabilities of hybrids despite their potential to
61 contribute to reproductive isolation between species. Here, we characterize the outer eye
62 morphology of *Heliconius* butterflies, examine evidence for adaptive divergence, and
63 consider how this may lead to the disruption of visual systems in hybrids.

64 The compound eyes of insects represent an easily quantifiable sensory structure that
65 directly affects visual perception. The insect compound eye consists of numerous
66 independent photosensitive units, ommatidia, each of which receives visual (light)
67 information and transfers it to the brain. Variation in the total number of ommatidia, their
68 size, and density directly affects visual perception and often correlates with temporal activity
69 (Greiner, 2006; Land, 1997; Stöckl et al., 2017; Warrant, 2004). For example, nocturnal and
70 crepuscular species often have larger eyes and larger facets to enhance photon sensitivity in
71 light-poor environments, as observed across insect taxa (Freelance et al., 2021). Associations
72 between the local environment and the visual systems of diurnal insects are less studied and
73 often included only as a comparison to other nocturnal species (e.g., Frederiksen & Warrant,

74 2008). Nonetheless, the conditions experienced by diurnal insects can vary greatly (Endler,
75 1993), and may represent important adaptations to the local environment.

76 *Heliconius* butterflies inhabit tropical and subtropical regions of the Americas and
77 rely heavily on vision for foraging for both flowers and hostplants (Dell’Aglia et al., 2016;
78 Gilbert, 1982), as well as finding and choosing suitable mates (Crane, 1955; Estrada &
79 Jiggins, 2008; Hausmann et al., 2021; Jiggins et al., 2001; Merrill et al., 2019). In Panama,
80 the closely related species *Heliconius melpomene* and *Heliconius cydno* are broadly
81 sympatric, but occupy different forest types (Estrada & Jiggins, 2002). *H. melpomene*
82 primarily lives in forest edge habitats, whereas *H. cydno* occurs deeper within the forests,
83 with less light and increased habitat complexity (Fig. 1A) (DeVries, 1987; Estrada & Jiggins,
84 2002; Seymoure, 2016). Although patterns of opsin expression suggest few differences in
85 wavelength sensitivity (McCulloch et al., 2017), recent data on brain morphologies of *H.*
86 *melpomene* and *H. cydno* reported heritable differences in the size of the visual neuropils that
87 exceed expected rates of neutral divergence (Montgomery et al., 2021). Using wild caught
88 individuals, Seymoure et al. (2015) similarly found that i) *H. cydno* has larger eyes than *H.*
89 *melpomene* and ii) that *H. cydno* males have larger eyes than *H. cydno* females (intraspecific
90 differences in *H. melpomene* were non-significant). However, these results were based on
91 individuals sampled as adults in their respective habitats and may include effects of
92 environment-induced plasticity. Also, total ommatidia counts were measured for only two
93 individuals for each species and sex, so statistical power to explore different eye morphology
94 traits was limited.

95 Given the evidence for selection acting on the visual processing regions of the brain, a
96 more thorough examination of the visual periphery in these species is warranted. In
97 particular, the role of plasticity and selection, and the potential for the mismatch of
98 components of the visual system in interspecific hybrids have not yet been assessed. To

99 address this, we characterized the outer eye morphology of *H. melpomene* and *H. cydno* to
100 test for patterns of adaptive divergence in the visual system. First, we compared the eye
101 morphology of butterflies (15+ for each species and sex) reared under common garden
102 conditions. We then used a quantitative genetics approach to test if the species-specific eye
103 differences are due to selection using P_{ST} - F_{ST} analysis. Finally, we report patterns of eye
104 morphology in first-generation (F1) hybrids of *H. melpomene* and *H. cydno*.

105

106 **Methods**

107 Butterfly specimens

108 We established outbred stocks of *Heliconius cydno chioneus* (C) and *Heliconius melpomene*
109 *rosina* (M) from butterflies caught in Gamboa and the nearby Soberanía National Park,
110 Panama between 2007-2009 and 2015-2017. We generated reciprocal F1 hybrids between *H.*
111 *cydno* and *H. melpomene* by either crossing a *H. cydno* female with *H. melpomene* a male
112 (CxM), or a *H. melpomene* female with a *H. cydno* male (MxC). All pure individuals and
113 hybrids were reared under common garden conditions in the Smithsonian Tropical Research
114 Institute insectaries in Gamboa, and all specimens were preserved in DMSO/EDTA/NaCl and
115 stored at -80° C as described in Merrill et al. (2019).

116

117 Sample preparation

118 Samples were prepared following previously published methods (Seymour et al., 2015;
119 Wright et al., 2023). In brief, we thawed specimens at room temperature, and dissected out
120 both eyes and the hind legs. The legs were immediately imaged (see below), while the eyes
121 were placed in 20% sodium hydroxide (NaOH) for 18-24 hours to loosen the tissues behind
122 the cuticular cornea. The following day, we cleaned each eye cuticle of excess tissue and

123 mounted it on a microscope slide in Euparal (Carl Roth GmbH). The sample was left to dry
124 overnight before imaging.

125

126 Image analysis

127 We used ImageJ/Fiji (Schindelin et al., 2012) to analyze each mounted cornea for the total
128 number of facets and total corneal area. All slides were imaged at 7.5x on a Leica M80
129 stereomicroscope fitted with a Leica Flexacam C1 camera and the *Leica Application Suite X*
130 (LAS X) software. Each image contained a 1mm scale bar for calibration. Facet counts were
131 measured via image thresholding and the *Analyze particles* function, and corneal surface area
132 was measured with the *Freehand selection* and *Measure* options (full protocol provided as
133 supplementary methods). This semi-automated method differs slightly from the approach
134 used by Seymoure et al. (2015) but gives quantitatively similar results (Fig. S1). To account
135 for differences in body size, we measured hind tibia length using the *Straight line* and
136 *Measure* options. The number of facets (Pearson's r [95% C.I.]: $r(118) = 0.969$ [0.956,
137 0.978]), corneal area ($r(118) = 0.986$ [0.979, 0.990]) and hind tibia length ($r(101) = 0.930$
138 [0.898, 0.952]) on the left vs. right sides of the butterfly were highly correlated. Therefore,
139 for all subsequent analyses, we used only the left eye and left leg unless either was missing,
140 damaged or had poor image quality (i.e., not all facets visible), then the right side was
141 substituted.

142

143 Statistical analysis

144 *Eye morphology.* We used linear models (lm function) in R to explore how facet count and
145 corneal area are influenced by *species* (*H. cydno* vs. *H. melpomene*), *sex* (male vs. female),
146 and *body size* (hind tibia length) as: $\log_{10}(\text{facet count or corneal area}) \sim \text{species} * \text{sex} +$
147 $\log_{10}(\text{tibia length})$. Log₁₀-transformations were used to normalize the residuals around the

148 allometric relationships to meet the assumptions of normality (Thorpe, 1975). We also used
149 linear models to assess i) the relationship between facet count and corneal area as: $\log_{10}(\text{corneal}$
150 $\text{area}) \sim \log_{10}(\text{facet count}) * \text{species} * \text{sex}$ and ii) body size differences (using hind tibia
151 length as a body size proxy): $\log_{10}(\text{tibia length}) \sim \text{species} + \text{sex}$. The significance of fixed
152 effect parameters was determined by likelihood ratio tests via the *drop1* function, and
153 minimum adequate models (MAM) were selected using statistical significance (Crawley,
154 2013; Nakagawa & Cuthill, 2007). We used the *Anova* function in the *car* package (Fox &
155 Weisberg, 2018) to estimate significant fixed effect parameters and report false discovery rate
156 (FDR; Benjamini & Hochberg, 1995) adjusted p-values (*p.adjust* function) to account for
157 multiple testing. Model assumptions were confirmed via visual inspection (residual vs. fitted
158 and normal Q-Q plots). To accurately visualize multiple significant fixed effects, we
159 extracted and plotted the estimated marginal means from each MAM using the *emmeans*
160 function in the *emmeans* package (Lenth et al., 2023).

161 We also explored whether the scaling relationships between eye morphology (facet
162 count and corneal area) and body size (hind tibia length) differed for *H. cydno* and *H.*
163 *melpomene* using major axis regressions via the *sma* function in the *smatr* package (Warton
164 et al., 2012). Following the standard allometric scaling relationship, $\log y = \beta \log x + \alpha$, we
165 tested for shifts in the allometric slope (β). Where a common slope was supported, we
166 subsequently tested for differences in α that would indicate ‘grade-shifts’ (test = “elevation”)
167 and for major axis-shifts along the common slope (test = “shift”).

168
169 *Test of selection.* We next used a quantitative genetics approach to test whether eye
170 morphology differences between *H. cydno* and *H. melpomene* are due to selection. Q_{ST} is a
171 quantitative genetic analogue of F_{ST} that measures additive genetic variation among
172 populations relative to total genetic variance. However, Q_{ST} estimates for quantitative traits

173 are difficult, so Q_{ST} is often replaced with its phenotypic analogue P_{ST} (Leinonen et al.,
174 2013). Comparisons between P_{ST} and F_{ST} can be used as a test of divergent selection, where
175 P_{ST} values that exceed genome-wide F_{ST} suggest greater phenotypic divergence than expected
176 by neutral genetic divergence.

177 We calculated P_{ST} values using the Pst function in the Pstat package (Silva & Silva,
178 2018) for raw, log10-transformed, and body-size corrected eye morphology measurements
179 (i.e., facet count and corneal area). Allometrically scaled body-size correlations (using tibia
180 length) per species and sex were performed via the *allomr* function in the *allomr* package
181 (Schär, 2023). P_{ST} approximation to Q_{ST} depends on heritability, h^2 , and a scalar c that
182 expresses the proportion of the total variance that is presumed to be due to additive genetic
183 effects across populations (Brommer, 2011). Heritability estimates for facet count and
184 corneal area are unknown for these species, so in addition to the default value of 1, we used
185 varying c/h^2 ratios [ranging from 0.33 to 4, following Montgomery et al. (2021)]. Genome-
186 wide F_{ST} values between *H. c. chioneus* and *H. m. rosina* were obtained from Martin et al.
187 (2013), derived from four wild-caught individuals per species using 100-kb genomic
188 windows. We calculated p-values as the proportion of the F_{ST} distribution that was above
189 each P_{ST} value (Leinonen et al., 2013); values above the 95th percentile of the F_{ST} distribution
190 were interpreted as an indication of selection.

191

192 *Hybrid phenotypes.* We re-ran the linear models described above but included the CxM and
193 MxC hybrids as two additional groups within the *species* factor. To test if hybrid body size
194 was intermediate to *H. cydno* and *H. melpomene*, we also re-tested the linear model:
195 $\log_{10}(\text{tibia length}) \sim \text{species} + \text{sex}$. In the case of more than two categories per fixed effect
196 parameter (i.e., *species*), we used post hoc Tukey tests (*glht-multcomp* package (Hothorn et

197 al., 2008)) to obtain parameter estimates and report Bonferroni adjusted p-values for multiple
198 comparisons.

199

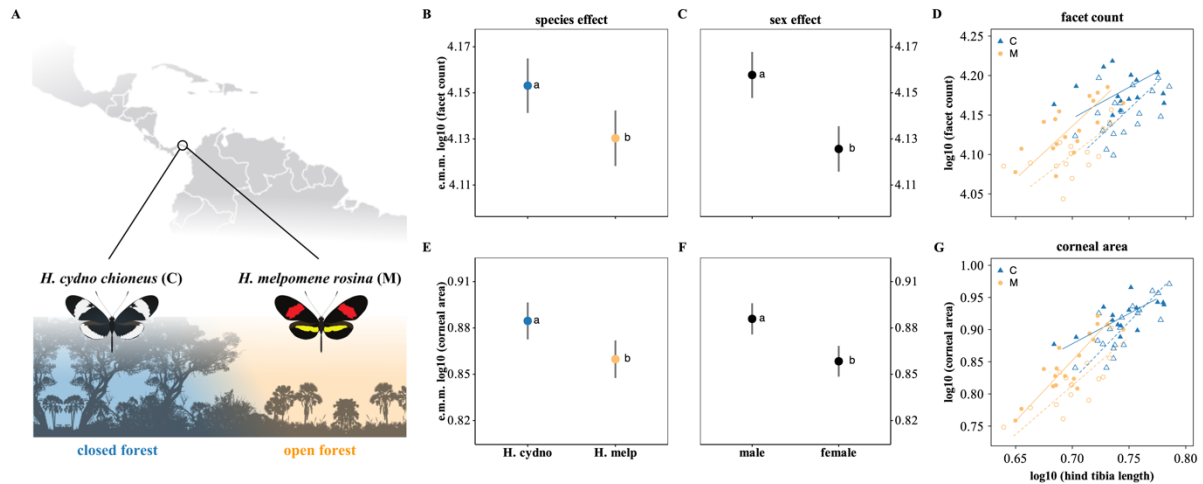
200 **Results**

201 Heritable shifts in eye morphology between species residing in different forest types

202 In total, we sampled 15 male and 19 female *H. cydno* and 18 male and 15 female *H.*
203 *melpomene*, all reared under common garden conditions. Using tibia length as a proxy for
204 body size, we found that *H. cydno* was larger than *H. melpomene* ($F_{1,65} = 58.77$, FDR-p <
205 0.001), but there was no evidence for sexual size dimorphism in either species (FDR-p = 0.7).
206 All three fixed effects, *tibia length*, *species*, and *sex*, were retained in our model examining
207 facet count. After accounting for size (larger butterflies had more facets: $F_{1,63} = 20.44$, FDR-
208 p < 0.001), *H. cydno* had more facets than *H. melpomene* ($F_{1,63} = 7.32$, FDR-p = 0.013; Fig.
209 1B), and males had more facets than females ($F_{1,63} = 27.35$, FDR-p < 0.001; Figs. 1C, S2).
210 The interaction between *species* and *sex* was not significant (FDR-p = 0.7), implying
211 conserved patterns of sexual dimorphism. We found similar results for corneal area (Fig. 1E,
212 F), where all three fixed effects were also retained (Table S1). For all butterflies, larger
213 corneal area was due to an increase in facet number, as evidenced by *facet count* significantly
214 affecting *corneal area* in the model $\log_{10}(\text{corneal area}) \sim \log_{10}(\text{facet count}) * \text{species} * \text{sex}$
215 ($F_{1,64} = 235.38$, FDR-p < 0.001). Importantly, the *facet count* x *species* interaction was not
216 significant in this model (FDR-p = 0.15), suggesting no differences in facet size between
217 species.

218 Given the sex-specific differences in eye morphology reported above, we analyzed
219 the scaling relationships between eye morphology and body size for males and females
220 separately (Fig 1D, G). The only significant difference in slope (β) was when comparing the
221 scaling relationship between corneal area and tibia length for *H. cydno* vs. *H. melpomene*

222 males (FDR- $p = 0.016$; Table 1); all other comparisons were non-significant, confirming
223 common slopes (FDR- $p > 0.23$; Table 1). In isolation, body size and facet count were
224 uncorrelated for *H. cydno* males ($r^2 = 0.004$, $p = 0.8$), but there was no statistical difference in
225 scaling between the species suggesting this is potentially due to increased variance in *H.*
226 *cydno* (Table 1). For all comparisons with a common slope, tests for grade-shifts (α) were
227 non-significant (FDR- $p > 0.4$), but there was a significant shift along the common axis (FDR-
228 $p < 0.001$; Table 1).
229



230

231 **Figure 1. (A)** *Heliconius cydno chioneus* and *Heliconius melpomene rosina* occur sympatrically in Panama but

232 occupy different habitats: *H. cydno* is found in closed forest environments, whereas *H. melpomene* resides in

233 open forests. (B, C, E, F) Estimated marginal means (e.m.m.) of the minimum adequate statistical models for

234 (B, C) facet count and (E, F) corneal area, demonstrating the significant effects of (B, E) *species* and (C, F) *sex*,

235 while accounting for other significant terms (*tibia length* and *sex/species*). The interaction between *species* and

236 *sex* was never significant (FDR- $p > 0.13$). Different letters indicate significant differences ($p < 0.05$), and the

237 error bars represent 95% confidence intervals. (D, G) Major axis regressions of (D) facet count or (G) corneal

238 area and body size, measured as hind tibia length. Double-logarithmic plots are presented to explore the

239 allometric relationships between eye morphology and body size. Males are represented by solid shapes and solid

240 lines, and females are represented by open shapes and dashed lines. C = *H. cydno*; M = *H. melpomene*.

241

242

		β (slope)		α (y intercept)		shift along common axis	
		<i>LR</i>	<i>FDR-p</i>	<i>wald χ^2</i>	<i>FDR-p</i>	<i>wald χ^2</i>	<i>FDR-p</i>
male	facet count	2.458	0.234	0.176	0.650	27.44	<0.001
	corneal area	7.393	0.016	-	-	-	-
female	facet count	0.4435	0.632	0.094	0.760	27.61	<0.001
	corneal area	0.5582	0.632	1.228	0.446	34.22	<0.001

243

244 **Table 1.** Scaling relationships between eye morphology and body size (hind tibia length) for *H. cydno* and *H.*

245 *melpomene* males and females. No values are reported for male corneal area because tests for grade shifts (α)

246 and shifts along the common axis are only appropriate with a common slope.

247

248 Differences in eye morphology are driven by selection

249 Our P_{ST} - F_{ST} analyses suggest that the visual systems of *H. cydno* and *H. melpomene* have

250 likely diverged as the result of selection rather than genetic drift. P_{ST} was significantly higher

251 than F_{ST} for both facet count and corneal area ($p < 0.001$) for all comparisons (Fig. 2; Table

252 S3), where the proportion of phenotypic variance due to additive genetic effects within-

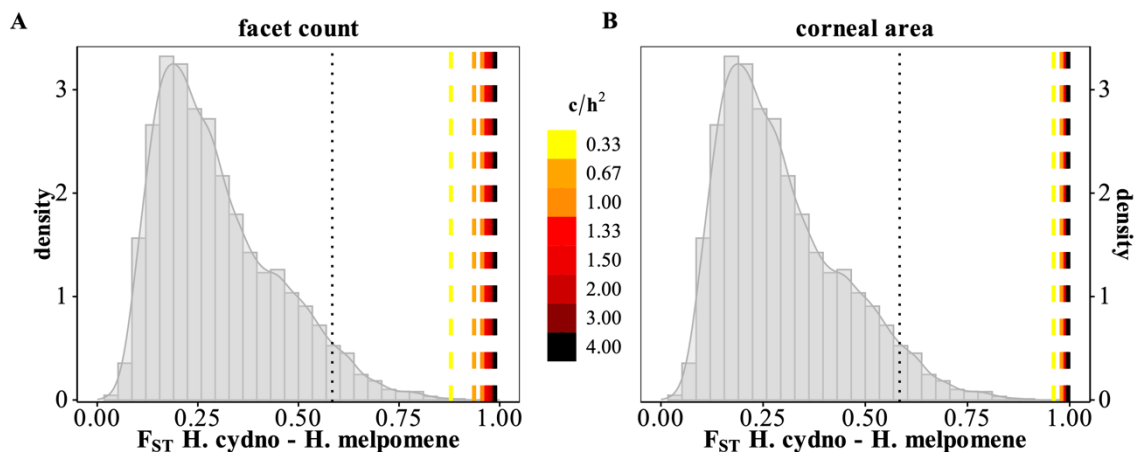
253 populations far exceeds the proportion of phenotypic variance due to additive genetic effects

254 between-populations. Qualitatively similar results were obtained regardless of the phenotypic

255 measurement evaluated (i.e., raw data, log10 transformed, allometrically corrected values;

256 Table S3) and also when each sex was examined separately (Tables S4, S5).

257



258

259 **Figure 2.** Location of the calculated P_{ST} values for (A) facet count and (B) corneal area in the distribution of F_{ST}
260 values between *H. cydno chioneus* and *H. melpomene rosina* (values from Martin et al., 2013). Here, both
261 morphological measurements are allometrically corrected using tibia length as a body-size proxy and presented
262 using varying c/h^2 ratios (see Table S3 for P_{ST} estimates using raw and log10 transformed values). The dotted
263 line represents the 95th percentile of the F_{ST} distribution.

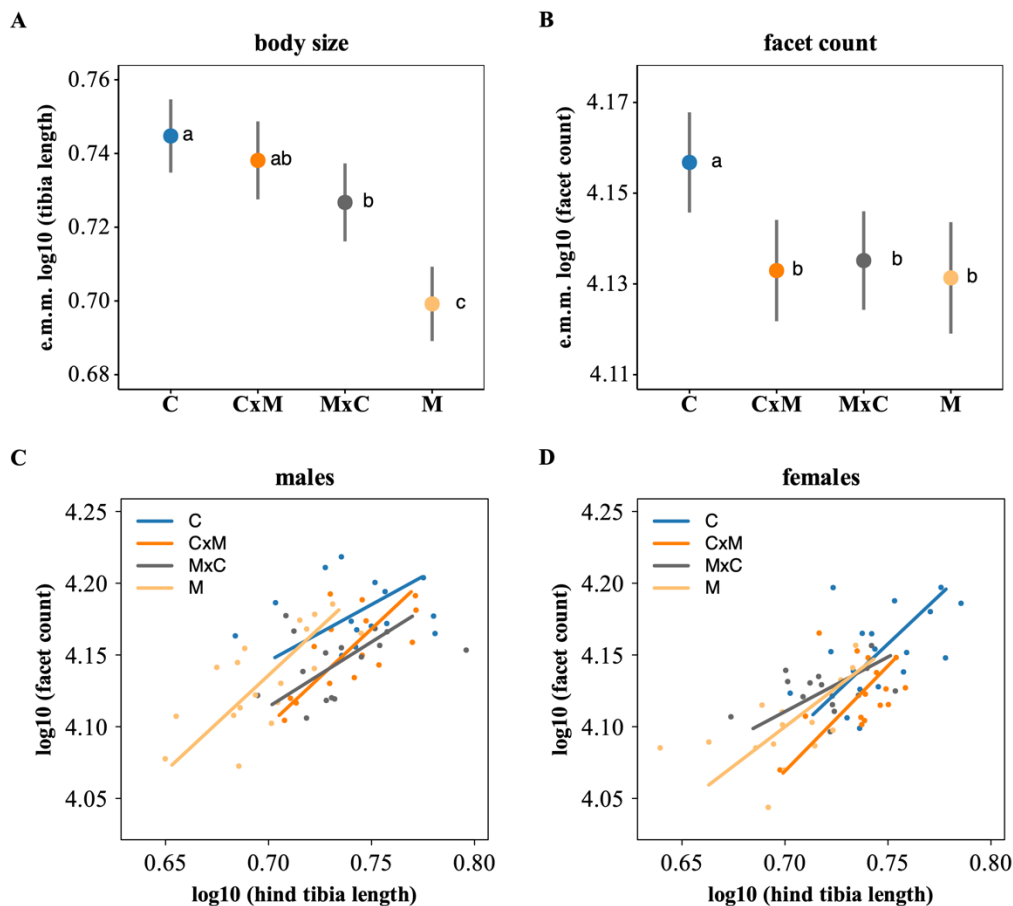
264

265 Hybrid eye morphology is *H. melpomene*-like

266 We examined the eye morphology of 60 F1 hybrids, including 15 male and 15 female CxM
267 individuals, and 15 female and 15 male MxC individuals (Table S2). Tukey post hoc (with
268 Bonferroni correction) revealed patterns of intermediacy in body size (using tibia length as a
269 body size proxy) for both hybrid types compared to the parental species: both CxM and MxC
270 hybrids were larger than *H. melpomene* ($p < 0.001$), MxC was smaller than *H. cydno* ($t = -$
271 3.148, $p = 0.0124$), and CxM did not differ from *H. cydno* ($p = 1.0$; Fig. 3A). However, F1
272 hybrid eye morphology was not intermediate to the parental species: both hybrid types had
273 significantly fewer facets ($p < 0.0035$) than *H. cydno* but did not differ from *H. melpomene* (p
274 $= 1.0$; Fig. 3B). Results for corneal area were similar (Table S2; Fig. S3, S4). These patterns
275 were also evident when exploring the scaling relationships between eye morphology and
276 body size of the F1 hybrids (Fig. 3C, D; Fig. S4).

277

278



279

280 **Figure 3.** (A) Estimated marginal means (e.m.m.) of the minimum adequate statistical model for body size
281 (using tibia length as a proxy) including F1 hybrids, demonstrating the significant effect of *species* (*sex* was
282 non-significant, FDR-p = 0.48). E.m.m for (B) facet count, showing the significant effect of *species*, while
283 accounting for significant *tibia length* and *sex* effects. In both plots, different letters indicate significant
284 differences (Bonferroni adjusted $p < 0.05$), and the error bars represent 95% confidence intervals. (C-D) Major
285 axis regressions of facet count and body size (tibia length) for males and females separately. Double-logarithmic
286 plots are presented to explore the allometric relationships between eye morphology and body size. C = *H.*
287 *cydno*; M = *H. melpomene*; CxM = F1 hybrid of *H. cydno* mother crossed with *H. melpomene* father; MxC = F1
288 hybrid of *H. melpomene* mother crossed with *H. cydno* father.

289

290 **Discussion**

291 When populations are exposed to different sensory conditions, natural selection may favor
292 divergence in sensory traits, which can contribute to speciation. We compared the outer eye
293 morphology of *Heliconius* butterflies that occupy different environments to test for patterns
294 of adaptive visual system divergence. Our results show that *H. cydno*, which occupies more
295 visually complex closed-canopy forests, has larger eyes, and that this is a result of heritable
296 differences in facet number. By combining our phenotypic data with genome wide estimates
297 of F_{ST} , we additionally provide strong evidence that selection has driven the divergence of
298 eye morphology in these butterflies. Finally, we show that F1 hybrid eye morphology is not
299 intermediate to the parental species, contrasting with patterns for body size and neural
300 anatomy. This suggests that visual processing in hybrids may be disrupted by mismatches in
301 different parts of the visual pathway.

302 Our results are consistent with previous work by Seymoure et al. (2015), which
303 reported larger eyes for *H. cydno*, and bigger eyes in *H. cydno* (but not *H. melpomene*) males.
304 However, the individuals sampled for our analyses were raised under common garden
305 conditions, reducing the potential for environmental effects and genotype–environment
306 interactions, which may give a distorted picture of the contribution of genetic variation on
307 which selection can act (Brommer, 2011; Pujol et al., 2008). A potential caveat of our results
308 is that we cut each cuticle four times to mount it on the microscope slides, which may have
309 disrupted the semi-automated counts of individual facets. It is possible that more advanced
310 3D imaging techniques (e.g., Buffry et al., 2023), where cutting is not required, may give
311 slightly higher total counts, though likely at the expense of overall sample size and associated
312 statistical power. Regardless, our facet counts are consistent with prior work (Seymoure et
313 al., 2015) (Fig. S1), and the close correspondence between wild and insectary-reared
314 butterflies further suggests that the differences in eye morphology are largely heritable.

315 Habitat-associated variation in eye morphology has been reported across taxa (e.g.,
316 insects: (Greiner, 2006); mammals: (Veilleux & Lewis, 2011); fish: (Lisney et al., 2020);
317 snakes: (Liu et al., 2012); primates: (Kirk, 2004)), and this variation is generally interpreted
318 as an adaptive response to the local sensory conditions. For example, visual perception in
319 insects is affected by the total number of ommatidia and their size/density (Greiner, 2006;
320 Land, 1997; Warrant, 2004), and nocturnal and crepuscular species often possess larger eyes
321 and larger facets to enhance photon sensitivity in low-light environments (Freelance et al.,
322 2021). However, most studies do not formally evaluate the role of selection, which is a key
323 element to define adaptation (Gould & Vrba, 1982). We are aware of only one study that has
324 attempted to address this topic: Brandon et al. (2015) reported that eye size variation in a
325 wild *Daphnia* population is associated with variation in fitness (reproductive output),
326 suggesting that selection is operating, either directly or indirectly, on eye size variation
327 (though the underlying mechanisms remain unknown).

328 In addition to revealing heritable differences in eye morphology, our results suggest
329 that eye morphology in *H. cydno* and *H. melpomene* have evolved as the result of divergent
330 selection, as opposed to genetic drift. Although our P_{ST} - F_{ST} approach to test for evidence of
331 selection acting on eye morphology is limited by the difficulty in approximating P_{ST} to Q_{ST}
332 (Brommer, 2011), these limitations are largely overcome by rearing our butterflies under
333 common garden conditions (Leinonen et al., 2008, 2013). Moreover, as with most insects, the
334 heritability of facet count and corneal area are unknown for the species used in this study. To
335 account for this, we used a wide range of c/h^2 ratios, including the default assumption of $c =$
336 h^2 (i.e., $c/h^2 = 1$), where the proportion of phenotypic variance due to additive genetic effects
337 is the same for between-population variance and within-population variance (Brommer,
338 2011). In all cases, P_{ST} values were higher than the 95th percentile of the genome wide F_{ST}
339 distribution (Tables S3-S5).

340 In insects, eye size may increase due to an increase in the number of ommatidia, an
341 increase in individual ommatidia size, or both. Our results suggest that larger eye size in *H.*
342 *cydno*, and in males of both species, is predominantly due to an increase in ommatidia
343 number. We did not measure facet diameter directly, but we found no effect of the *facet*
344 *count x species* interaction on *corneal area*, suggesting no interspecific differences in facet
345 size. Seymoure et al. (2015) measured facet diameter from a subset of ommatidia in six
346 anatomical eye regions and reported larger facet diameter in wild *H. cydno* compared to *H.*
347 *melpomene*, with no differences between sexes. However, these results stemmed from an
348 analysis of covariance including other species (*H. sapho* and *H. erato*); there were no direct
349 comparisons between *H. cydno* and *H. melpomene*. Future studies may benefit from
350 exploring facet diameter more directly. Regardless, the larger eyes of *H. cydno*, and male
351 *Heliconius*, appear to be largely due to an increase in ommatidia number. In insects,
352 increased ommatidia number is thought to contribute to higher visual acuity (Land, 1997) -
353 behavioral and morphological data revealing sexual dimorphism in the visual acuity of *H.*
354 *erato* support this prediction (Wright et al., 2023). We note that our allometric analyses
355 suggest part of the variation we observe is associated with body size, within and between
356 species. However, our P_{ST} - F_{ST} analyses account for body size and still suggest a signal of
357 selection. The pattern we observe in hybrids, where grade-shifts are clearly observed between
358 *cydno* and *cydno x melpomene* hybrids (Figure 3C), also suggest a strong genetic component
359 independent of body size. As such, while the behavioural impact of increased eye size likely
360 depends on the raw numbers of facets, we conclude that selection on body size alone does not
361 explain the increase in *cydno* eye size.

362 Multiple non-exclusive selective pressures could be driving the differences we report
363 here. First, species differences may be explained by *H. cydno* occupying more complex
364 closed-forest environments (Estrada & Jiggins, 2002), where more ommatidia are

365 advantageous due to e.g., increased visual acuity (Land, 1997; Wright et al., 2023). More
366 ommatidia in males may also be due to general ecological differences between the sexes, as
367 males actively search for and identify mates (Rutowski, 2000; Yagi & Koyama, 1963).
368 Interestingly, species differences persist for both sexes (Fig. S2) and when exploring the P_{ST} -
369 F_{ST} results for each sex separately, we still observed evidence of selection (Tables S4, S5).
370 Taken together, our results suggest that the selective pressure on males to have more
371 ommatidia acts in both species in concert with selection for more ommatidia in closed-forest
372 environments. Similar patterns may exist across *Heliconius*, but to date, few species have
373 been surveyed for eye morphology.

374 Our results also mirror neuroanatomical comparisons between species across the
375 *cydno-melpomene* clade, where larger visual neuropils are reported for *cydno*-clade species
376 occupying closed-forest environments, as opposed to *H. melpomene* (Montgomery et al.,
377 2021). These neural differences appear to be heritable adaptations, based on similar tests of
378 selection to those reported here. Thus, the combined results on brain morphology
379 (Montgomery et al., 2021) and those presented here suggest whole visual system adaptation,
380 from the sensory periphery to the brain. Similar habitat-associated differences in
381 neuroanatomy have been reported in other Neotropical butterflies (Montgomery & Merrill,
382 2017; Wainwright & Montgomery, 2022), indicating a broader pattern of sensory adaptation
383 in ecologically divergent, but closely related, butterflies.

384 One notable difference in the results obtained for eye and neural traits, however, is in
385 the pattern of variation among F1 interspecific hybrids. The eye morphology of *H. cydno* and
386 *H. melpomene* F1 hybrids tended to be more *H. melpomene*-like. This contrasts with patterns
387 for body size (Fig. 3A) and neuroanatomy, where hybrids are intermediate for at least some
388 traits (Montgomery et al., 2021). The observation that hybrid eye morphology is *melpomene*-
389 like, but hybrid body size tends to be intermediate indicates that these two traits have

390 different underlying genetic architectures (i.e., eye size is not simply genetically correlated
391 with increasing body size). Furthermore, evidence suggests that both eye morphology (this
392 study) and neural anatomy (Montgomery et al., 2021) are under divergent selection and in the
393 predicted direction (i.e., bigger eyes and larger visual neuropils in *H. cydno*). If these
394 observations truly represent adaptations (as our results suggest), then hybrids may have
395 suboptimal visual system functioning, whereby peripheral sensory structures (number of
396 facets) are mismatched to subsequent processing regions (optic lobe neuropils). This would
397 predict that hybrids suffer a fitness deficit due to lower performance in visually oriented
398 tasks, such as foraging and mate detection, but behavioral experiments are required to test
399 these predictions. In conclusion, the adaptive differences in eye structure we observe may
400 contribute to ecologically based pre- and post-mating reproductive barriers.

401

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410

411 **Data availability**

412 The underlying data and R-scripts supporting the findings of this study are available at
413 https://github.com/SpeciationBehaviour/Adaptive_divergence_Heliconius_eyes.

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