

1 **Altitude and life-history shape the evolution of**

2 ***Heliconius* wings**

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13 *Abstract*

14 Phenotypic divergence between closely related species has long interested biologists. Taxa
15 that inhabit a range of environments and have known and diverse, natural histories, can help
16 understand how different selection pressures shape diverging traits. In butterflies, wing
17 colour patterns have been extensively studied, whereas wing shape diversity is less well
18 understood despite its importance for flight. Here we study a measure of wing shape, aspect
19 ratio, and wing size in a large dataset of over 3200 individuals, representing 13 *Heliconius*
20 species, from across the Neotropics. We assess the relative importance of phylogenetic
21 relatedness, natural history and habitat in determining wing shape and size. We find that
22 both larval and adult behavioural ecology affect patterns of adult size dimorphisms. On one
23 hand, species with solitary larvae have larger adult males, in contrast to gregarious
24 *Heliconius* species, and indeed most Lepidoptera. On the other hand, species in the pupal-
25 mating clade are smaller overall than those in the adult-mating clade. Interestingly, while
26 controlling for phylogeny, sex ratios and allometry, we find that species inhabiting higher
27 altitudes have rounder wings and, in one of the two major *Heliconius* clades, are also bigger
28 in size than their lowland relatives. Thus, we reveal novel adaptive wing morphological
29 divergence among *Heliconius* species beyond that imposed by natural selection on
30 aposematic wing colouration. Our study highlights the value of phylogenetic comparative
31 methods in study systems that have diverse and well-studied natural histories to disentangle
32 the selection pressures shaping adaptive phenotypes.

33

34 **Keywords:** *Heliconius*, phenotypic divergence, wing morphology, Lepidoptera,
35 sexual dimorphism, altitude

36

37 *Introduction*

38 Identifying the selective forces driving phenotypic divergence among closely related species
39 lies at the core of evolutionary biology research. Adaptive radiations, in which descendants
40 from a common ancestor rapidly fill a variety of niches, are ideal systems to investigate
41 morphological divergence. The study of adaptive radiations has revealed that evolution often
42 comes up with similar solutions for similar problems (Losos 2010; Marques et al. 2019).
43 Speciose groups that have repeatedly and independently evolved convergent adaptations to
44 life-history strategies and environments are strong systems to study selection drivers
45 (Schluter 2000). Nevertheless, adaptive phenotypic divergence is often complex and
46 multifaceted; with more than a single selective force in action (Maia et al. 2016; Nosil et al.
47 2018). For example in birds, sex differences in plumage colouration are driven by intra-
48 specific sexual selection, while natural selection drives sexes towards more similar
49 colourations (Dunn et al. 2015). Integrative approaches that make use of tractable traits
50 across well-resolved phylogenies are needed to explore the selective forces driving
51 phenotypic evolution.

52

53 Wing colouration has been the focus of considerable research effort and major strides have
54 been made towards understanding how and when evolution leads to complex wing colour
55 patterns, conferring aposematism, camouflage, or a mating advantage (Merrill et al. 2012;
56 Chazot et al. 2016; Nadeau et al. 2016). Indeed, interest in wing patterns as models of
57 evolutionary process dates back to Henry W. Bates who, in his book *The Naturalist on the*
58 *River Amazon*, reflected on the astonishing diversity of butterfly wings he had observed: “It
59 may be said, therefore, on these expanded membranes nature writes, as on a tablet, the
60 story of the modifications of species” (Bates 1863). The dazzling diversity of colour patterns
61 among species has perhaps obscured the less conspicuous phenotypic diversity of wing
62 shapes and sizes (Le Roy et al. 2019), which are more often regarded as the result of sexual
63 selection, flight trade-offs or developmental constraints (Singer 1982; Allen et al. 2011),

64 rather than drivers of local adaptation and species diversification (but see Srygley 2004;
65 Cespedes et al. 2015; Chazot et al. 2016).

66

67 Differences in behaviour between sexes have been identified as one of the main drivers of
68 wing shape and size sexual dimorphism in insects (Rossato et al. 2018b; Le Roy et al.
69 2019). In butterflies, males tend to spend more time looking for mates and patrolling
70 territories, while females focus their energy on searching for suitable host plants for
71 oviposition (Rossato et al. 2018b). The same wing trait can be associated with different life
72 history traits in each sex, resulting in sex-specific selection pressures. For example, in the
73 Nearctic butterfly *Melitaea cinxia*, wing shape only correlates with dispersal in females, as
74 males experience additional selection pressures that counteract selection for dispersal wing
75 phenotypes (Breuker et al. 2007). Sex-specific behaviours can impact both wing shape and
76 size, but differences in ecology and natural history, even across closely related species,
77 could also have large impacts on the strength and direction of these effects (Cespedes et al.
78 2015; Chazot et al. 2016).

79

80 Another important source of phenotypic variation in insect wings is the abiotic environment
81 they inhabit. Air pressure decreases with altitude, which in turn reduces lift forces required
82 for flight. To compensate for this, insects may increase wing area relative to body size to
83 reduce the velocity necessary to sustain flight (Dudley 2002; Dillon et al. 2018). Wing shape
84 in the widespread *Drosophila melanogaster* has been observed to vary adaptively across
85 latitudes and altitudes, with wings getting rounder and larger in montane habitats, possibly
86 to, maintain flight function in lower air pressures (Stalker and Carson 1948; Pitchers et al.
87 2012; Klepsatel et al. 2014). In butterflies, high aspect ratios, i.e. long and narrow wings,
88 reduce drag caused by wing tip vortices, thus lowering the energy required for flight and
89 promoting gliding for longer distances (Le Roy et al. 2019). Variation in wing phenotypes has
90 been detected at the microhabitat level, for example *Morpho* butterfly clades that dwell in the
91 understory have rounder wings than canopy-specialist clades, allowing for increased

92 manoeuvrability (Chazot et al. 2016). An extreme case of environmental effects on wing
93 shape can be found in Lepidoptera inhabiting the windy, barren highlands of the Andes,
94 where an interaction between behavioural sex differences and extreme climatic conditions
95 have led to flightlessness in females of several species (Pyrz et al. 2004).

96

97 *Heliconius* or “longwing” butterflies are an excellent example of colour pattern evolution for
98 aposematic mimicry, where co-occurring subspecies share warning colour patterns to avoid
99 predation, creating multi-species mimicry rings across South America (Merrill et al., 2015).
100 Wing shape and size are part of the mimetic signal (Jones et al. 2013; Mérot et al. 2016;
101 Rossato et al. 2018a). Wing morphology is involved in many aspects of *Heliconius* biology
102 other than mimicry, such as mating or flight mode, but these have been considerably less
103 well studied (but see Rodrigues and Moreira 2004; Srygley 2004; Mendoza-Cuenca and
104 Macías-Ordóñez 2010).

105

106 The wide range of environments that *Heliconius* species inhabit, together with their diverse
107 natural history and well-resolved phylogeny make them a good study system for teasing
108 apart the selective forces driving wing phenotypic divergence. For example, larval
109 gregariousness has evolved independently three times across the phylogeny, with some
110 species laying clutches of up to 200 eggs, while the others lay eggs singly and larvae are
111 often cannibalistic (Beltrán et al. 2007; Jiggins 2016). In gregarious *Heliconius* species,
112 increased female size might be beneficial as the enlarged egg load might necessitate larger
113 wings. Another striking life history trait, pupal-mating, has evolved only once, in one of the
114 two major clades (hereafter the “erato clade”), following the most basal split in the *Heliconius*
115 phylogeny. This mating strategy involves males copulating with females as they emerge
116 from the pupal case, or even before (Deinert et al. 1994; Beltrán et al. 2007). Pupal-mating
117 leads to a whole suite of distinct selection pressures but these are hard to tease apart from
118 the effects of phylogeny due to its single origin (Beltrán et al. 2007; Jiggins 2016). Further
119 ecological differences could arise from relatively high-altitude environmental specialisation of

120 some *Heliconius* species, such as *H. telesiphe* and *H. hierax*, which are only found above
121 1000m. This is in contrast to wide-ranging species such as *H. melpomene* and *H. erato*,
122 which can be found from 0 to 1800 m above sea-level (Rosser et al. 2015; Jiggins 2016).
123 Potential adaptations to altitude are yet to be explored.

124

125 Here we examine variation in wing shape and size across 13 species that span most of the
126 geographical range of the *Heliconius* genus. First, we photographed thousands of wings
127 collected by many *Heliconius* researchers since the 1990s from wild populations across
128 South and Central America, covering a 2100 m elevation range (Fig. 1 A). Wing dimensions
129 for 3242 individuals, obtained from standardised images using an automated pipeline, were
130 then used to address the following questions. (1) Are there size and shape sexual
131 dimorphisms, and if so, do they correlate with known life-history traits? (2) To what extent
132 are species' wing shape and size variation explained by shared ancestry? (3) Are wing
133 shape and size affected by the elevations species inhabit?

134 *Methods*

135 **STUDY COLLECTION**

136 The wild specimens studied here were collected using hand nets between 1998 and 2018 in
137 313 localities across Panama, Colombia, Ecuador, French Guiana, Suriname, and Peru (Fig.
138 1 A), and stored in the Department of Zoology, University of Cambridge (Earthcape
139 database). Collection altitudes ranged from sea level to 2100m above sea level (Fig 1 A).
140 Detached wings were photographed dorsally and ventrally with a DSLR camera with a 100
141 mm macro lens in standardised conditions. All the images are available in the public
142 repository Zenodo (<https://zenodo.org/communities/butterfly/>) and full records with data are
143 stored in the EarthCape database (<https://heliconius.ecdb.io>).

144

145 **WING MEASUREMENTS**

146 Damage to wings was manually scored in all the images and damaged specimens were
147 excluded from our analyses. To obtain wing measurements from the images, we developed
148 custom scripts for Fiji (Schindelin et al., 2012), to automatically crop, extract the right or left
149 forewing, and perform particle size analysis (Fig. 1 B). Butterflies predominantly use their
150 forewings for flight (Wootton 2002; Le Roy et al. 2019) and forewing and hindwing sizes are
151 tightly correlated in this genus (Strauss, 1990), thus we only include forewings here. For
152 wing size, we obtained total wing area (in mm², hereafter “size”).

153

154 For examining wing shape, the custom scripts first fitted an ellipse to the forewings and
155 measured the length of the longest axis and the length of the axis at 90 degrees to the
156 former (Fig. 1 C). Aspect ratio corresponds to the length of the major axis divided by the
157 length of the minor axis, hereafter “shape” (Fig. 1 C). The data were checked for visual
158 outliers on scatter-plots, which were examined, and removed from the analyses if the wing
159 extraction pipeline had failed.

160

161 **STATISTICAL ANALYSES**

162 All analyses were run in R V2.13 (R Development Core Team 2011) and graphics were
163 generated with the package *ggplot2* (Ginestet 2011). Packages are specified below. All R
164 scripts can be found in the public repository Zenodo (Zenodo: tbc), including custom Fiji
165 scripts for wing image analysis.

166

167 *Sexual dimorphism*

168 Species and sexes mean trait values were calculated for the 13 *Heliconius* species in our
169 study that had more than 30 individuals each and for which accurate locality and altitude
170 data were available (SI: Table 1, 16 species were excluded), resulting in a dataset of 3243
171 individuals. Sexual dimorphism in size and shape was estimated as the female increase in
172 mean trait values with respect to males, thus negative values represent larger trait values in
173 males, and vice-versa.

174

175 We modelled variation in size and shape sexual dimorphism with ordinary least squares
176 (OLS) linear regressions, implemented in the 'lm' function. For both models, predictor
177 variables initially included larval gregariousness of the species (following Beltrán et al.
178 2007), mating strategy (pupal-mating vs. adult-mating clade), species mean shape and size,
179 species sex ratio in our dataset, and species sexual wing shape or size dimorphism
180 (respectively). We used backward selection to remove terms according to level of
181 significance, retaining terms at a threshold of $\alpha = 0.05$. We report the overall variation
182 explained by the fitted models (R^2) and the relative contributions of each explanatory
183 variable (partial R^2) (Grömping 2006).

184

185 *Phylogenetic signal*

186 We calculated the phylogenetic signal index Abouheif's Cmean (Abouheif 1999) for mean
187 wing shape, size and species altitude, which is an autocorrelation metric suitable for

188 datasets with a relatively low number of species and that does not infer an underlying
189 evolutionary model (Münkemüller et al. 2012). Observed and expected distribution plots for
190 phylogenetic signal estimates are shown in the Supplementary Materials and were
191 computed with the package *adephylo* (Jombart and Dray 2010). We used a pruned tree with
192 the 13 species under study from the most recent molecular *Heliconius* phylogeny (Kozak et
193 al., 2015). We plotted centred trait means across the phylogeny with the function
194 `barplot.phylo4d()` from the package *phylosignal* (Keck et al. 2016). To test and visualise
195 phylogenetic signal further, we built phylocorrelograms for each trait with the function
196 `phyloCorrelogram()` of the same package, which estimates Moran's I autocorrelation across
197 matrices with varying phylogenetic weights. Then, the degree of correlation (Morans' I) in
198 species trait values can be assessed as phylogenetic distance increases (Keck et al. 2016).

199

200 *Variation across species*

201 Species wing trait means may be correlated due to shared ancestry (Freckleton et al. 2002;
202 Chazot et al. 2016). Therefore, to explore the effects of the environment on the traits under
203 study, models that incorporate expected correlation between species are required. One of
204 the most commonly employed and versatile techniques to do this is phylogenetic
205 generalised least squares (PGLS). Although often ignored, these models assume the
206 presence of phylogenetic signal on the model residuals of the trait under study (here wing
207 shape or size) controlling for all potential covariates, and not just phylogenetic signal on the
208 species mean trait values (Revell 2010; Garamszegi 2014). Thus, we estimated
209 phylogenetic signal as described above (Keck et al. 2016) with the residuals of a generalised
210 least squares (GLS) model that included wing shape or size as response variables and
211 potential allometric, environmental and natural history terms as explanatory variables. We
212 obtained phylogenetic correlograms for trait residuals (presented in the SOM), and centred
213 trait residuals for plotting across the phylogeny as detailed above for trait means (Keck et al.
214 2016).

215

216 Phylogenetic comparative methods assume that species-specific mean trait values are a
217 good representation of the true trait values of the species under study, in other words, that
218 the within-species variation is negligible compared to the across-species variation
219 (Garamszegi 2014). To test this, we first used an ANOVA approach, with species as a factor
220 explaining the variation of mean trait values. We then estimated within-species trait
221 repeatability, or intra-class correlation coefficient (ICC), with a linear mixed model approach
222 that had species as a random effect, specifying a Gaussian distribution and 1000 parametric
223 bootstraps to quantify uncertainty, implemented with the function `rptGaussian()` in *rptR*
224 package (Stoffel et al. 2017). By specifying species as a random effect, the latter approach
225 estimates the proportion of total trait variance accounted for by differences between species.
226 A trait with high repeatability indicates that species-specific trait means are reliable
227 estimates for further analyses (Stoffel et al. 2017). We, nevertheless, accounted for within-
228 species variation in the models described below.

229

230 To examine the effects of altitude on wing shape and size variation between species, we first
231 tested for phylogenetic signal in these traits. Significant phylogenetic signal was detected in
232 the residuals of wing shape and size regression models (SOM Fig. S1, Fig. S2), so we used
233 maximum log-likelihood PGLS regression models with the trait phylogenetic correlation fitted
234 as a correlation term, implemented with the `gls()` function from the *nlme* package (Pinheiro et
235 al. 2007). We assumed a Brownian motion model of trait evolution for both traits, by which
236 variation across species accumulated along all the branches at a rate proportional to the
237 length of the branches (Freckleton et al. 2002). To select the most supported model given
238 the available data, i.e. one that improves model fit while penalising complexity, we used the
239 Akaike information criterion (AIC); (Anderson, 2008; Garamszegi, 2014), where the best
240 models had the lowest AIC values. Thus, to select covariates to include in the model, we
241 used step backward and forward selection based on AIC with the function `stepAIC()`, from
242 the *MASS* package (Ripley, 2011; Zhang, 2016). Maximal PGLS models included species

243 mean altitude and distance from the Equator, sex ratio in our samples interacting with sexual
244 size dimorphism, as well as either wing shape or size to control for potential allometric
245 relationships, which could be different among closely-related taxa (Outomuro and
246 Johansson 2017). Interaction terms between altitude and size/shape were also included in
247 the maximal models when relevant. Minimal PGLS models consisted of the trait under study
248 explained solely by its intercept, without any fixed effects. We weighted PGLS regressions to
249 account for unequal trait variances and sample sizes across species (for sample sizes and
250 standard errors of species' trait means see SOM Table 1). This was achieved by modifying
251 the correlation structure of the model with combined variances obtained with the function
252 `varFixed()` and specified with the argument "weights" (Pinheiro et al. 2007; Paradis 2012;
253 Garamszegi 2014).

254

255 We examined the interactions between fixed effects and phylogenetic correlations and their
256 explanatory power in the selected models by deriving partial R^2 based on maximum
257 likelihood estimates from full and reduced models, implemented in R with:

258

259
$$R2.liik <- 1 - \exp(-2 * (\logLik(mod.f) - \logLik(mod.r)) / n)$$

260

261 where `mod.f` is the full model, `mod.r` is the reduced model with either fixed effects removed
262 or phylogenetic correlation removed, and `n` is the species sample size (Paradis 2012; Ives
263 2018). To visualise the effects of altitude on wing shape and size we plotted the residuals of
264 a PGLS model built as described above without altitude as an explanatory variable, against
265 the species mean altitude. Additionally, to understand the effect of controlling for relevant
266 fixed effects, phylogenetic correlation, intraspecific variance, and sample size, we present
267 the raw wing shape and size species variation across altitudes in the Supplementary
268 Materials.

269

270 *Results*

271 We obtained intact-wing measurements for 3243 individuals of 13 *Heliconius* species from
272 across the phylogeny and from over 300 localities (Fig. 1, Table S1). We have made all of
273 these wing images publicly available at the Zenodo repository.

274

275 **SEXUAL DIMORPHISM**

276 Sexual dimorphism in wing size was found throughout the phylogeny, but in opposing
277 directions in different species (Fig. 2). Mean sizes were significantly different ($P < 0.05$)
278 among sexes in all of the six best sampled species (see Table S1 for t-test summary
279 statistics), indicating that the marginally- or non-significant trends in other species probably
280 reflect a lack of power caused by low numbers of females. The six species with trends
281 toward larger females have gregarious larvae (pink, Fig. 2), whereas the six species with
282 trends toward larger males lay eggs singly (black, Fig. 2). Larval gregariousness alone
283 explained 66.5% of the total natural variation in sexual size dimorphism across species
284 (Table 1; Gaussian LM: $F_{1,11} = 24.9$, $P < 0.001$, $R^2 = 0.67$). There was a significant phylogenetic
285 signal in sexual size dimorphism (Abouheif's $C_{\text{mean}} = 0.28$, $P = 0.03$; SI, Fig. S1). This would
286 be expected from the evolutionary history of gregariousness, as it has evolved
287 independently at least three times across the *Heliconius* phylogeny (Beltrán et al. 2007).
288 However, even when accounting for phylogenetic correlation in the model, larval
289 gregariousness remained a significant predictor of size sexual dimorphism (SI, Table S2).

290

291 Sexual dimorphism in wing shape was found only in two species from the erato clade, the
292 widespread *H. erato* had longer-winged males whereas the high-altitude specialist *H.*
293 *eleuchia* had longer-winged females (T-test, *H. erato*: $t_{739} = -9.52$, $P < 0.0001$, *H. eleuchia*:
294 $t_{49} = 2.21$, $p < 0.05$). Wing shape sexual dimorphism across species could not be explained
295 with the variables here studied and had no phylogenetic signal (Abouheif's $C_{\text{mean}} = 0.02$,
296 $P = 0.2$; SI, Fig. S1).

297

298 **PHYLOGENETIC SIGNAL**

299 The 13 *Heliconius* species studied differed significantly in wing size and shape (ANOVA,
300 Shape: $F_{12, 3230} = 215.8$, $P < 0.0001$, Size: $F_{12, 3230} = 189.3$, $P < 0.0001$). We estimated within-
301 species trait repeatability to assess their reliability as species mean estimates for
302 phylogenetic analyses. Wing shape had higher intra-class repeatability than wing size, with
303 74% and 47% of the total shape and size variance explained by differences between
304 species, respectively (Shape: $R=0.74$, $S.E.=0.09$, $P<0.0001$; Size: $R=0.47$, $S.E.=0.1$,
305 $P<0.0001$). We estimated intra-class repeatability for males and females separately to
306 remove the potential effect of size sexual dimorphism on trait variation, and male size
307 repeatability remained much lower than male wing shape repeatability (Male shape: $R=0.75$,
308 $S.E.=0.08$, $P<0.0001$; Male Size: $R=0.51$, $S.E.=0.1$, $P<0.0001$).

309

310 Mean wing shape showed no phylogenetic signal (Abouheif's $C_{mean}=0.15$, $P=0.1$; SI: Fig.
311 S1, Fig. S2 B), whereas mean wing size showed a strong phylogenetic signal (Fig. 3,
312 Abouheif's $C_{mean}=0.33$, $P=0.01$; SI: Fig. S1, Fig. S3 B). Wing sizes of species in the
313 melpomene clade were on average 14.6% larger than those of species in the erato clade,
314 with *H. timareta* being 56% larger than *H. sara* (Fig. 3 C, *timareta*: mean=605.8 mm²,
315 $s.e.=3.6$; *sara*: mean=386 mm², $s.e.=3.1$). Nevertheless, when controlling for fixed effects on
316 wing shape and size (species mean: size/shape, altitude, latitude, sex ratio, size sexual
317 dimorphism), the residuals of both traits show a phylogenetic signal (SI: Fig. S2 A, C- Shape
318 residuals: Abouheif's $C_{mean}=0.31$, $P<0.05$; Fig. S3 A, C- Size: Abouheif's $C_{mean}=0.21$,
319 $P=0.05$).

320

321 **PATTERNS ACROSS ALTITUDES**

322 To account for the moderate within-species repeatability of wing size, we included intra-
323 specific variance in the error structure of the phylogenetic generalised least squares (PGLS)
324 models for both wing traits. Altitude had an effect on wing size and shape (Table 1). Species

325 wings got rounder with increasing altitudes both when accounting for fixed effects and
326 phylogeny (Table 1, full model Table S2, Fig. 4). These trends were also evident when
327 examining raw mean wing shapes (Fig. S3 A, Gaussian LM: $F_{1,9} = 5.37$, $P < 0.05$, $R^2=0.30$),
328 except in the *H. telesiphe* and *H. clysonymus* highlands clade, which showed significant
329 phylogenetic autocorrelation (Moran's I index: *H. clysonymus* 0.53, *H. telesiphe* 0.49).
330 Altitude had similarly strong effects on wing shape residuals for the erato and melpomene
331 clades (Fig. 4A, Table S2), but there were further clade-specific effects, i.e. evolutionary rate
332 shifts, that could not be accounted for by phylogeny alone in the PGLS models and that are
333 represented by the different intercepts in Fig. 4A.

334

335 Species wing sizes increased with elevation (Table 1, full model Table S2). High altitude
336 species of the erato clade were bigger than their lowland sister species, both with PGLS
337 residuals (Fig. 4B, blue, Gaussian LM: $F_{1,10} = 118$, $R^2=0.97$, $p=0.001$) and with raw wing size
338 (Fig. S3 B, blue, Gaussian LM: $F_{1,10} = 17.1$, $R^2=0.80$, $p=0.03$). When examining the
339 melpomene clade patterns alone, we did not detect a size-altitude relationship (Fig. 4B). At
340 the extremes of the erato clade elevational range were the highland species *H. clysonymus*,
341 which was 39% bigger than the lowland species *H. sara* (Fig. S3 B). The phylogeny term of
342 the wing size model interacted with the fixed effects, and improved the fit and predictive
343 power of the model (Table S4), as expected for a trait with a strong phylogenetic signal
344 (Revell 2010; Ives 2018).

345

346 *Discussion*

347 The fascination of H. W. Bates for butterfly wing colouration has stimulated several
348 generations of research and *Heliconius* wing patterns have proven to be excellent study
349 systems for understanding evolution and speciation. Here we have extended this research
350 by examining wing shape and size variation among more than 3200 individual butterflies,
351 across sexes, clades, and altitudes in 13 species of *Heliconius* butterflies. We have shown
352 that a large proportion of female biased sexual size dimorphism can be explained by the
353 evolution of larval gregariousness, and that male biased sexual size dimorphism is present
354 only in species that lay eggs singly, regardless of their mating strategy. For the first time in
355 this system, we describe wing morphological variation across environmental clines, with
356 species found at higher altitudes consistently having rounder wings. Here we demonstrate
357 that *Heliconius* wing size and shape is affected by a plethora of behavioural and
358 environmental selection pressures, in addition to those imposed by Müllerian mimicry.

359

360 **WING SHAPE VARIATION**

361 Wing shape in butterflies, and other flying animals (Farney and Fleharty 1969; Buler et al.
362 2017), determines flight mode and speed, and is therefore predicted to vary with life-history
363 requirements across sexes and species. Long wings are generally associated with faster
364 gliding flying, whereas round wings with low aspect ratio values favour slow but more
365 manoeuvrable flight motions (Betts and Wootton 1988; Chai and Srygley 1990; Chazot et al.
366 2016; Le Roy et al. 2019). For instance, monarch butterfly populations with longer migrations
367 have more elongated wings than resident populations (Satterfield and Davis 2014), and
368 males of *Morpho* species that dwell in the canopy also have higher aspect ratios to glide
369 faster through open areas (DeVries et al. 2010). In contrast, female *Morpho* butterflies tend
370 to have rounder wings, and shape sex differences are stronger in species with colour
371 dimorphism, as varying crypsis may require specific flight behaviours (Chazot et al. 2016).

372

373 *Heliconius* are not notoriously sexually dimorphic, especially when compared to other
374 butterflies such as *Morpho* (Chazot et al. 2016; Jiggins 2016). However, there are important
375 behavioural differences between the sexes. Females are thought to have different flight
376 habits, as they spend much of their time looking for specific host plants for oviposition
377 (Dell'Aglio et al. 2016), or precisely laying eggs on suitable plants, while males tend to patrol
378 open areas searching for receptive females and visit flowers more often (Joron 2005; Jiggins
379 2016). Thus, it might be predicted that females should have lower aspect ratios, i.e. rounder
380 wings, than males (Jones et al. 2013). However, we only found two species, both of which
381 belong to the pupal-mating clade, with significant, but opposing, sexually dimorphic wing
382 shapes (males: *H. erato* longer, *H. eleuchia* rounder). This result might simply reflect
383 strengthened intra-sexual selection in males of species that can both pupal-mate and adult-
384 mate in the wild. In addition, the relatively low collection numbers of female *Heliconius* could
385 hinder the detection of subtle wing shape differences across the sexes.

386

387 Sexual selection has long been known to affect wing colour pattern in *Heliconius*, as it is
388 used for mate recognition and choice (Merrill et al. 2012). More recently, wing shape has
389 been shown to be part of the mimetic warning signal in *Heliconius* and their co-mimics
390 (Jones et al. 2013), as it determines flight motion and defines the overall appearance of the
391 butterfly (Srygley 1994, 2004). For instance, wing shapes between two different morphs of
392 *H. numata* differed consistently across their overlapping ranges, in parallel with their
393 respective and distantly related *Melipotis* co-mimics (Jones et al. 2013). However, within-
394 morph wing shape variation was observed across the altitudinal range of *H. timareta* in Peru
395 (Mérot et al. 2016), and in the *Heliconius* postman mimicry ring in Brazil significant across-
396 species wing shape differences were also found (Rossato et al. 2018a). These studies
397 highlight that while it is clear that colour pattern and, to some extent, flight are important for
398 mimicry in *Heliconius*, wing shape is also subject to other selection pressures (Mérot et al.
399 2016; Rossato et al. 2018b).

400

401 We also found that species inhabiting higher altitudes have rounder wings, after accounting
402 for phylogeny, sample size and intra-specific variance (Fig. 4 A). Rounder wings aid
403 manoeuvrability and are associated with slower flight in butterflies (Berwaerts et al. 2002; Le
404 Roy et al. 2019) and slower flights are generally associated with a decrease in ambient
405 temperature (Gilchrist et al. 2000). In addition, air pressure, which directly reduces lift forces
406 required to offset body weight during flight (Dillon 2006), decreases approximately 12%
407 across the mean altitudinal range of the species here studied. Thus, the rounder wings in
408 high altitude *Heliconius* species may aid flying in dense cloud forests, where increased
409 manoeuvrability could be beneficial, or compensating for lower air pressure at higher
410 altitude.

411

412 **WING SIZE VARIATION**

413 Wing size showed significant sexual dimorphism in more than half of the species studied
414 here, but some species had larger males and others larger females (Fig. 2). In most
415 butterflies, females are larger than males, presumably because fecundity gains of increased
416 body size are greater for females (Allen et al. 2011). Indeed, the *Heliconius* species in this
417 study that tended to have larger-winged females were those that lay eggs in large clutches
418 (Fig. 2), ranging from 30-200 eggs and whose larvae are highly gregarious (Beltrán et al.
419 2007). Thus, females of these species are likely investing more resources in fecundity than
420 males, which leads to larger body and wing sizes that allow them to carry and lay eggs in
421 clutches throughout adulthood. Selection for larger females is generally constrained by a
422 trade-off between the benefits of increased fecundity at the adult stage and the higher
423 predation risk at the larval stage associated with longer development times (Allen et al.
424 2011). This constraint might be alleviated in the unpalatable larvae of *Heliconius*, as bigger
425 larval and adult size could increase the strength of the warning toxic signal to predators
426 (Jiggins 2016).

427

428 An extensive survey identified that only six percent of lepidopteran species exhibit male-
429 biased sexual size dimorphism, and that these patterns were generally explained by male-
430 male competition (i.e. intrasexual selection), in which larger males had a competitive
431 advantage (Stillwell et al. 2010; Allen et al. 2011). In contrast, nearly half of the *Heliconius*
432 species studied here have male-biased sexual size dimorphism, and all of these lay eggs
433 singly and have solitary larvae (Fig. 2). Male-male competition is high for *Heliconius* species,
434 as females rarely re-mate despite their very long reproductive life-spans (Merrill et al. 2015).
435 In addition, large reproductive investments in the form of nuptial gifts from males can, in
436 principle, explain male-biased sexual size dimorphisms, as is the case in the polyandrous
437 butterfly *Pieris napi* whose male spermatophore contains the amount of nitrogen equivalent
438 to 70 eggs (Karlsson 1998; Allen et al. 2011). Male *Heliconius* spermatophores are not only
439 nutrient-rich, but also loaded with anti-aphrodisiac pheromones that prevent re-mating of
440 fertilised females (Schulz et al. 2008; Merrill et al. 2015). Therefore, it seems likely therefore
441 that in species that lay eggs singly, sexual selection favouring larger males exceeds
442 selection pressures for the large female size needed to carry multiple mature eggs. To our
443 knowledge, *Heliconius* is the first example of a butterfly genus in which both female- and
444 male-biased size dimorphism are found and can be explained by contrasting reproductive
445 strategies.

446

447 We found a strong phylogenetic signal for wing size, with species from the erato clade being
448 on average 12% smaller than those in the melpomene clade (Fig. 3). There are many
449 ecological factors that could explain this pattern, and all could have contributing effects that
450 are hard to disentangle (Fig. 3). Firstly, the erato clade is characterised by facultative pupal-
451 mating (Beltrán et al. 2007; Jiggins 2016), by which males fight for pupae, guard them, and
452 mate with females as they are emerging from the pupal case (Deinert et al. 1994; Jiggins
453 2016). This may increase male intra-sexual competition where smaller males that
454 outcompete others for a spot on the female pupal case more successfully inseminate

455 emerging females compared to larger, less agile males, and remove the potential choice of
456 females for larger males (Deinert et al. 1994).

457

458 Secondly, pupal-mating seems to have far-reaching impacts on species life-histories (Boggs
459 1981). Pupal-maters are largely monandrous, with an extensive survey only finding 3 out of
460 251 wild *H. erato* with signs of re-mating in their reproductive tract, i.e. two or more
461 spermatophores (Walters et al. 2015). In contrast, species in the melpomene or adult-mating
462 clade are polyandrous, which leads to selection favouring large spermatophores (Boggs
463 1981), as seen in the polyandrous *Pieris napi* (Karlsson 1998). These can take up a
464 remarkable proportion of the male abdomen, and provide mated females with abundant
465 nutritional resources and defences that prevent them from re-mating with other males
466 (Cardoso et al. 2009; Cardoso and Silva 2015). This could decrease selection pressure for
467 larger males in the pupal-mating clade, as nuptial gifts need not be so large or
468 nutrient/defence rich, leading to smaller male and female offspring. However, recent studies
469 question the relative frequency of pupal-mating in the wild vs. adult mating encounters
470 (Thurman et al. 2018), and others have found that the level of female emergence
471 asynchrony determines the reproductive strategy of males (Mendoza-Cuenca and Macías-
472 Ordóñez 2010). Furthermore, the single origin of pupal-mating in *Heliconius* (Fig. 2) makes it
473 challenging to infer the impacts of this mating strategy on wing morphology, as the
474 behaviour is confounded by phylogeny.

475

476 Finally, host-plant use varies considerably, with the erato clade generally having a more
477 restricted and specialised diet than the melpomene clade, which feed on most lineages of
478 *Passiflora* (Kozak et al. 2015; Jiggins 2016; de Castro et al. 2018). For example, size and
479 female fecundity in *H. erato phyllis* has been shown to vary with host plant-use and seasonal
480 availability across its extensive Brazilian range (Rodrigues and Moreira 2002, 2004). Thus,
481 adult size could be largely a result of the quality host plants consumed at the larval stage.

482

483 In the erato clade larger species are found at higher altitudes (Fig. 4 B), unlike the
484 melpomene clade species here studied. For example, the melpomene clade high-altitude
485 specialist *H. hierax* is smaller than many of its lowland relatives and, interestingly, clusters
486 with the other high-altitude specialists from the smaller erato clade (Fig. 4 B, species 8). Two
487 major environmental factors are known to affect insect size across altitudinal clines. One is
488 temperature, such that at lower temperatures, development times are longer and insects
489 grow larger (Chown and Gaston 2010). This perhaps explains cases of Bergmann's rule
490 among ectotherms, where larger species are found in colder climates (Shelomi 2012;
491 Classen et al. 2017). The generality of Bergmann's rule for insects has been widely studied
492 and debated, both across latitudinal and altitudinal clines, and it is highly dependent on
493 sampling design and the level of biological organization studied (Shelomi 2012). Additionally,
494 wing beat frequency tends to be lower at low temperatures, so larger wings are required to
495 compensate and gain the extra lift required for flight, as seen in *Drosophila robusta*
496 (Azevedo et al. 2006; Dillon 2006). A second factor likely to contribute to altitude related
497 differences in wing size is air pressure changes and the correlated lower oxygen availability,
498 which affects flight motion and kinematics as well as many physiological processes. High-
499 altitude insects can minimise the impacts of lower air pressure by having larger wings,
500 because this lowers the velocity required to induce flight (Dudley 2002). To assess the
501 relative impacts of these and other environmental factors on *Heliconius* wing sizes and their
502 relation to body sizes would require common-garden rearing and flight assays in controlled
503 conditions.

504

505 **HERITABILITY**

506 Our study demonstrates that multiple selective forces influence *Heliconius* wing size and
507 shape. One important issue in phenotypically diverging populations is distinguishing
508 between highly condition dependent or phenotypically plastic traits as opposed to heritable,
509 conserved traits. In this study we found that 74% of the variation in wing shape could be
510 explained by species identity, in contrast to 47% of the variation in wing size. High intra-

511 class repeatability is often considered an indication of high trait heritability (Nakagawa and
512 Schielzeth 2013). Additionally, wing shape is predicted to be phenotypically more
513 constrained than size, because subtle shape variation can have large impacts on flight
514 motion and kinematics (Jones et al. 2013; Le Roy et al. 2019).

515

516 In *Drosophila*, the genetic architecture of wing shape appears to be complex and differs
517 (Gilchrist and Partridge 2001), and is independent of that of wing size (Carreira et al. 2011).
518 Variability of wing size dramatically reduced when flies were reared in controlled conditions
519 compared to wild populations, but both wing shape and size had a detectable heritable
520 component (Bitner-Mathé and Klaczko 1999; Klaczko 1999). The patterns of variation in size
521 across altitudes or latitudes are often not due to phenotypic plasticity, as many studies have
522 shown their retention when populations are reared in common-garden conditions (Chown
523 and Gaston 2010). In Monarch butterflies, for example, common-garden reared individuals
524 from wild populations that had different migratory habits showed a strong genetic component
525 for both wing shape and size (Altizer and Davis 2010). We have shown that different
526 selection pressures affect wing shape and size and that the strength of these potentially
527 varies across sexes and environmental clines (Altizer and Davis 2010).

528

529 **CONCLUSIONS**

530 Here we have demonstrated how an understanding of natural and evolutionary history can
531 help to disentangle the putative agents of selection on an adaptive trait. Wing trait
532 differences across sexes, clades and environments give insight into the selective forces
533 driving phenotypic divergence in *Heliconius*, beyond the effects of natural selection imposed
534 by Müllerian mimicry. Our study highlights the complexity of selection pressures affecting
535 seemingly simple traits and the need for a thorough understanding of life history differences
536 among species.

537 *Acknowledgements*

538 We would like to thank all the *Heliconius* researchers and field assistants that have
539 contributed to the wing collection used in this study. We also thank Simon H. Martin and
540 Stephen H. Montgomery for useful comments on early versions of this manuscript. G.M.K.
541 was supported by a Natural Environment Research Council Doctoral Training Partnership.
542 J.E.S. was supported by the Scurfield Memorial Bursary (University of Sheffield). Funding
543 was provided to C.B. by the Spanish Agency for International Development Cooperation
544 (AECID, grant number 2018SPE0000400194).

545

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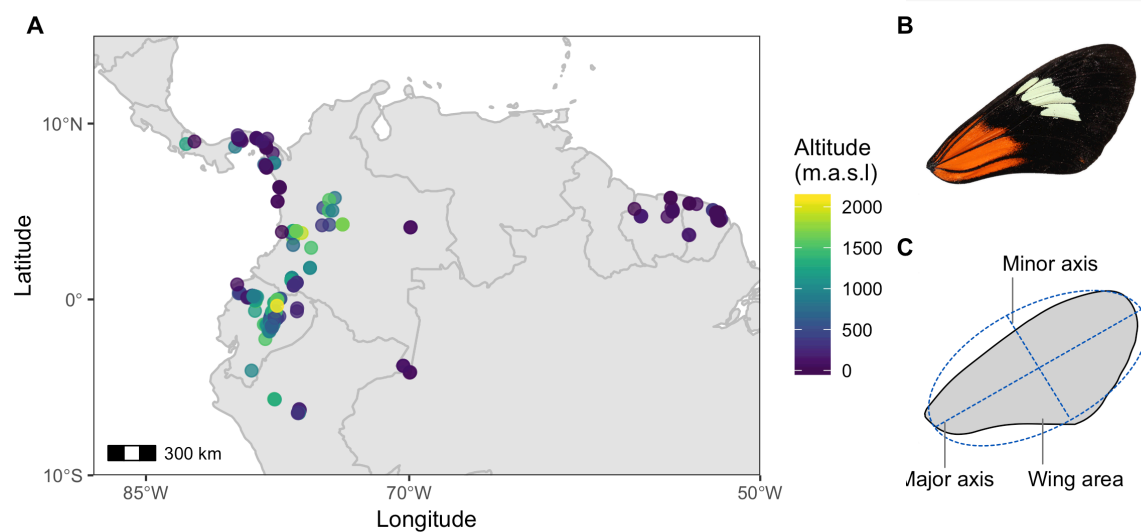
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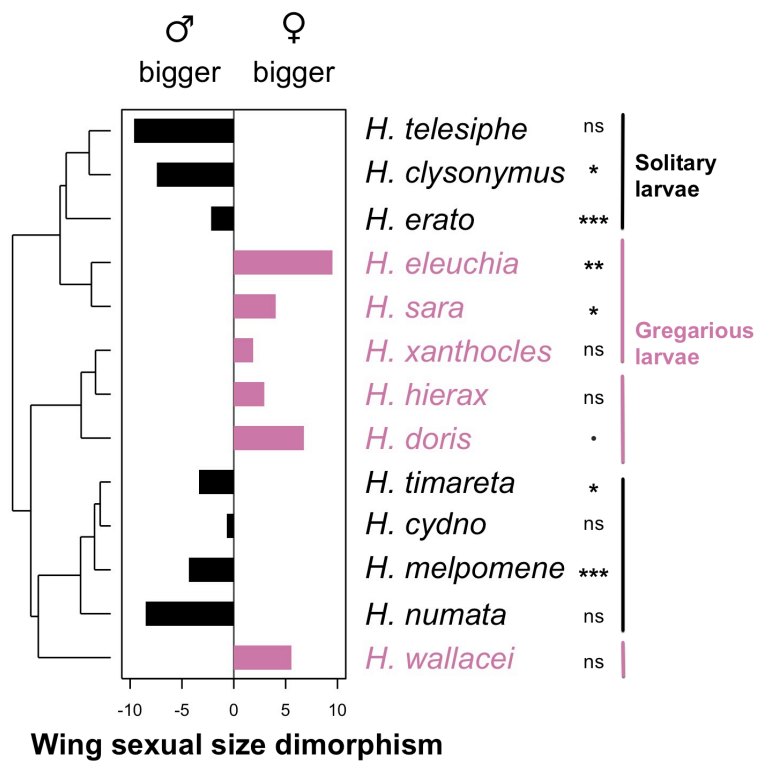
755 **Figures**



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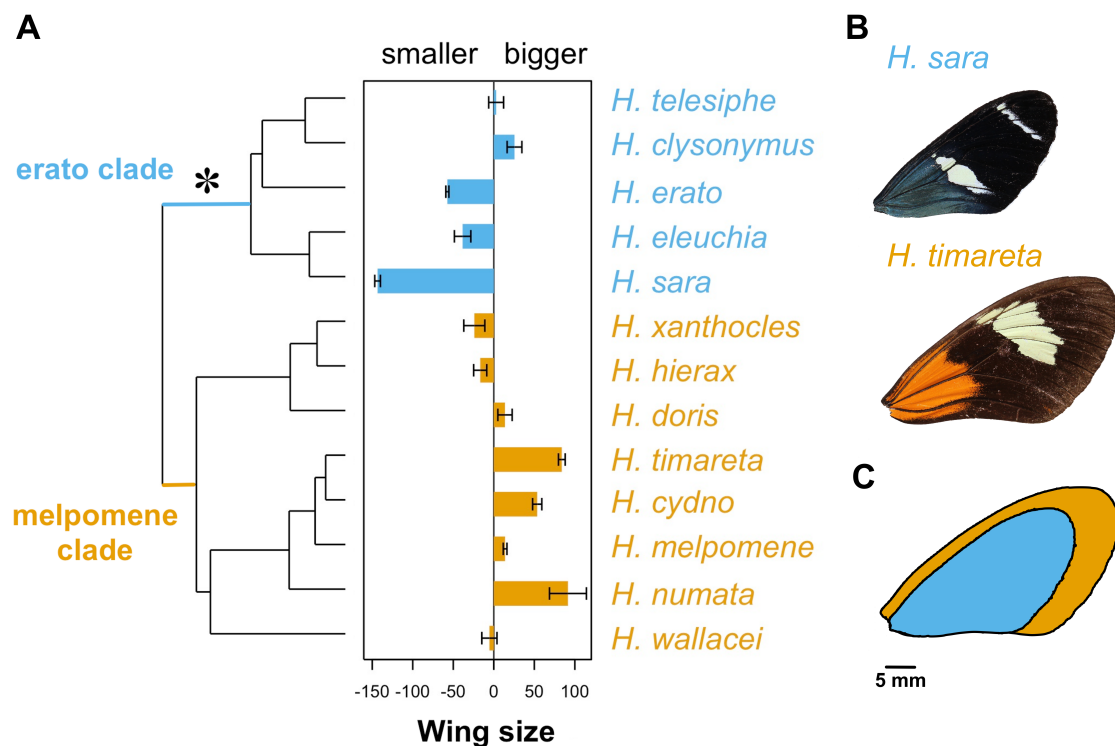
757 **Figure 1.** Localities and forewing measurements. (A) Map of exact locations (n=313) across
758 South America from where the samples used for our analyses were collected. Points are
759 coloured by altitude. (B) Representative of a right forewing image of *H. melpomene malleti*.
760 (C) Measurements taken from each wing by fitting an ellipse with Fiji custom scripts.

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Figure 2. Wing size sexual dimorphism variation across the phylogeny. Wing size was measured as total right or left forewing area (in mm²). Bar plot represents sexual size dimorphism calculated as percentage difference in female vs. male size (positive means bigger females). Species with gregarious larvae are coloured in pink, and those with solitary larvae are coloured in black. Stars represent significance levels of two sample t-tests between female and male wing sizes for each species ($\bullet < 0.1$, $* < 0.05$, $** < 0.01$, $*** < 0.001$), for full t-tests output see Table S1.

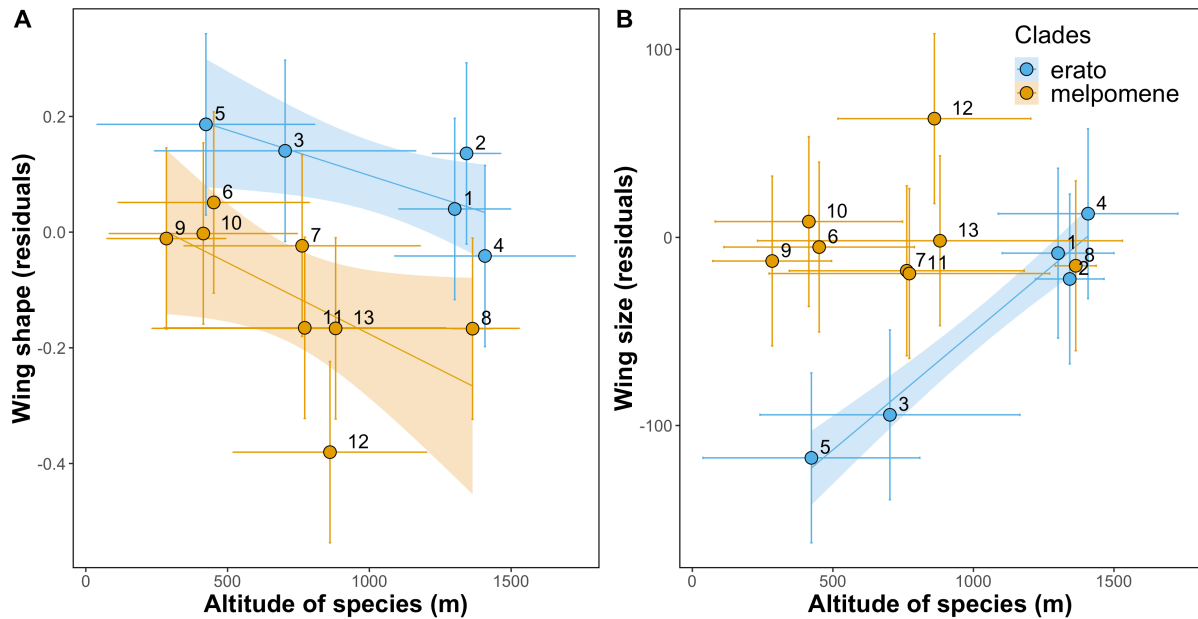


772 **Figure 3.** Male wing size differences across the phylogeny. (A) Bar plot represents centred
 773 mean wing size per species (positive values represent species with bigger wings than the
 774 mean *Heliconius* wing). Wing size, x-axis, is the difference in wing size from the mean (in
 775 mm²). Error bars represent standard errors. The star represents the origin of pupal-mating.
 776 Species from the erato clade are in blue, and those from the melpomene clade are in orange.
 777 (B) Representatives of *H. timareta* and *H. sara* closest to the mean wing size of the species
 778 are shown (606.25 mm² and 386.6 mm², respectively). (C) Images from (B) superimposed to
 779 compare visually the mean size difference between the two species.
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Figure 4. Species variation in wing shape (A) and wing size (B). Residual plots show the effect of altitude (meters above sea level) on wing shape (aspect ratio) and wing size (area mm²) residuals of PGLS model controlling for selected fixed effects (Table S2), intraspecific variance, sample size, and phylogenetic correlation. Points represent species mean values. Horizontal and vertical lines show standard error for species mean altitude and mean trait residuals, respectively. Lines show best linear fit for significant effects and are coloured by clade (blue: erato clade, orange: melpomene clade). Shaded areas show confidence bands at 1 standard error. Numbers correspond to *Heliconius* species: 1 *H. telesiphe*, 2 *H. clysonymus*, 3 *H. erato*, 4 *H. eleuchia*, 5 *H. sara*, 6 *H. doris*, 7 *H. xanthocles*, 8 *H. hierax*, 9 *H. wallacei*, 10 *H. numata*, 11 *H. melpomene*, 12 *H. timareta*, 13 *H. cydno*.

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799 **Table 1.** Summary of model outputs derived from the analyses of size sexual dimorphism
 800 (positive values represent species with bigger females, negative represent species with bigger
 801 males), and shape and size phylogenetic generalised least squares. For the latter, summary
 802 statistics are presented for the overall model and the explanatory variable of interest, altitude.
 803 Full model tables for PGLS can be found in the Supplementary Materials (Table S2).

Response variable (wing trait)	Trait repeatability (R)	Model type	Corr. structure	Fixed effects	Est.	S.E.	t-value	predictor p-value	d.f. (res. d.f.)	Adj. R ²
Sexual size dimorphism	NA	lm	Gaussian	(Intercept) Solitary larvae	5.12 -10.3	1.28 1.74	4.00 -5.9	0.002*** 0.0001***	1 (11)	0.76
Shape	0.74 (p=0)	PGLS (nmle)	Phylogeny, intra-sp variance, sample size	(Intercept) Altitude	0.15 -2.2E-4	0.53 7.9E-5	0.27 -2.75	0.79 0.028*	13 (7)	NA
Size	0.47 (p=0)	PGLS (nmle)	Phylogeny, intra-sp variance, sample size	(Intercept) Altitude	-705.4 1.3	392.1 0.49	-1.79 2.63	0.13 0.047*	13 (5)	NA

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