

1 What shapes the continuum of
2 reproductive isolation?
3 Lessons from *Heliconius* butterflies.

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5 Mérot C.^{1,2}, Salazar C.³, Merrill R. M.^{4,5}, Jiggins C.^{4,5}, Joron M.^{1,6}

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9 ¹ ISYEB UMR 7205, Muséum National d'Histoire Naturelle, 45 rue Buffon, Paris

10 ² IBIS, Université Laval, 1030 Avenue de la Médecine, Québec, Canada

11 ³ Biology Program, Faculty of Natural Sciences and Mathematics. Universidad del Rosario.
12 Carrera. 24 No 63C-69, Bogota D.C., 111221. Colombia.

13 ⁴ Department of Zoology, University of Cambridge, Downing Street, Cambridge, CB2 3EJ,
14 United Kingdom

15 ⁵ Smithsonian Tropical Research Institute, MRC 0580-12, Unit 9100 Box 0948, DPO AA
16 34002-9998

17 ⁶ Centre d'Ecologie Fonctionnelle et Evolutive, UMR 5175 CNRS - Université de Montpellier
18 - Université Paul Valéry Montpellier - EPHE, 1919 route de Mende, 34293 Montpellier, France

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25

26 **Abstract**

27 The process by which species evolve can be illuminated by investigating barriers that limit gene
28 flow between taxa. Recent radiations, such as *Heliconius* butterflies, offer the opportunity to
29 compare isolation between pairs of taxa at different stages of ecological, geographic and
30 phylogenetic divergence. We carry out a comparative analysis of existing and novel data in
31 order to quantify the strength and direction of isolating barriers within a well-studied clade of
32 *Heliconius*. Our results highlight that increased divergence is associated with the accumulation
33 of stronger and more numerous barriers to gene flow. Wing pattern is both under natural
34 selection for Müllerian mimicry and involved in mate choice, and therefore underlies several
35 isolating barriers. However, pairs which share a similar wing pattern, also display strong
36 reproductive isolation mediated by traits other than wing pattern. This suggests that, while wing
37 pattern is a key factor for early stages of divergence, it is not essential at a higher level.
38 Additional factors including habitat isolation, hybrid sterility and chemically-mediated mate
39 choice are associated with complete speciation. Therefore, although most previous work has
40 emphasised the role of wing pattern, our comparative results highlight that speciation is a
41 multidimensional process, whose completion is stabilized by many factors.

42

43 **Introduction**

44 Studies of speciation have long contrasted allopatric and sympatric speciation, speciation
45 through sexual versus natural selection, and ecological versus non-ecological speciation.
46 However, these contrasts do not always reflect the diversity of processes involved in divergence
47 and the challenge is to reach an integrated understanding of speciation [1-4]. Species divergence
48 involves multiple different traits and processes that can lead to reproductive isolation [5]. These
49 include adaptation to local environmental conditions, pre-mating isolation as well as post-
50 mating effects that reduce the fitness of hybrids. To untangle the evolutionary processes at play,
51 it is useful to quantify the relative importance of the factors reducing gene flow between
52 diverging populations [6].

53

54 Speciation is a continuous process and we can typically only observe the results of divergence
55 at a specific stage, not the process in its entirety. For instance, incompatibilities between extant
56 species may not reveal the ecological and evolutionary forces initially causing phenotypic
57 divergence [7]. Conversely, ecotypes or subspecies at early divergence may shed light on
58 factors favouring early divergence but speciation is not a necessary outcome [8, 9] and the
59 challenge of speciation with gene flow might not be its initiation but its progression and
60 completion [10]. In that context, a useful way to study speciation as a continuous process is to
61 compare multiple pairs of incipient or closely-related species which vary in their extent of
62 divergence, possibly depicting stages along the so-called speciation continuum [9, 11-14].

63

64 With a large diversity of recently diverged species and sub-species, the radiation of *Heliconius*
65 butterflies is an excellent system for studying speciation [15]. Within *Heliconius*, the two sister
66 clades, melpomene clade and cydno clade each contains a large number of local representatives
67 across the Neotropics (Fig 1). They provide replicate pairs of sister-taxa distributed along the

68 speciation continuum, notably spanning the “grey zone of speciation” [14], providing an
69 opportunity to assess the factor shaping reproductive isolation along the speciation process.

70

71 *H. melpomene* is considered a single taxonomic species but comprises populations with
72 significant genetic differentiation between western and eastern populations on either side of the
73 Andes [16, 17]. The cydno clade includes four taxonomic species, *H. cydno*, *H. pachinus*, *H.*
74 *timareta* and *H. heurippa*. Across their range, representatives of the cydno clade are typically
75 broadly sympatric with *H. melpomene* and hybridize at low frequency [18-20], resulting in
76 persistent inter-specific admixture across the genome [21].

77

78 Within this clade, the modalities of reproductive isolation have been examined across numerous
79 studies considering taxa at various levels of divergence, falling into three main categories. First,
80 pairs of taxa at low divergence, such as sympatric forms of *H. cydno alithea* (Ecuador) [22, 23]
81 and parapatric subspecies of *H. timareta* (Colombia) [24, 25] or *H. melpomene* (Peru) [26].
82 Secondly, at intermediate divergence, populations belonging to the same clade (either cydno or
83 melpomene) but characterized by significant genetic clustering. Within the cydno clade, those
84 correspond to separate species, such as *H. cydno galanthus* and *H. pachinus* (Costa Rica) [23,
85 27] or *H. cydno cordula* and *H. heurippa* (Colombia) [28, 29]. Within the melpomene clade,
86 this includes allopatric subspecies of *H. melpomene* belonging to the eastern and western
87 lineages (Panama/French Guiana)[30]. Thirdly, comparison of highly-divergent pairs,
88 involving a population of *H. melpomene* and a representative of the cydno-group, found in
89 sympatry, parapatry or allopatry (Panama, Colombia, Peru, French Guiana) [28-36].

90

91 Emphasis has been given to behavioural pre-mating isolation, found to be strong in most pairs
92 of taxa [23, 26, 30, 34]. However, other components of divergence such as habitat preference
93 [37], hybrid fertility [29, 32], hybrid survival in the wild [36] and hybrid mating success [33]
94 have also received some attention. Here, to provide an extensive comparison across the whole
95 clade, we conduct a joint re-analysis of those published data with new data and quantify the
96 contribution to reproductive isolation of each isolating component.

97

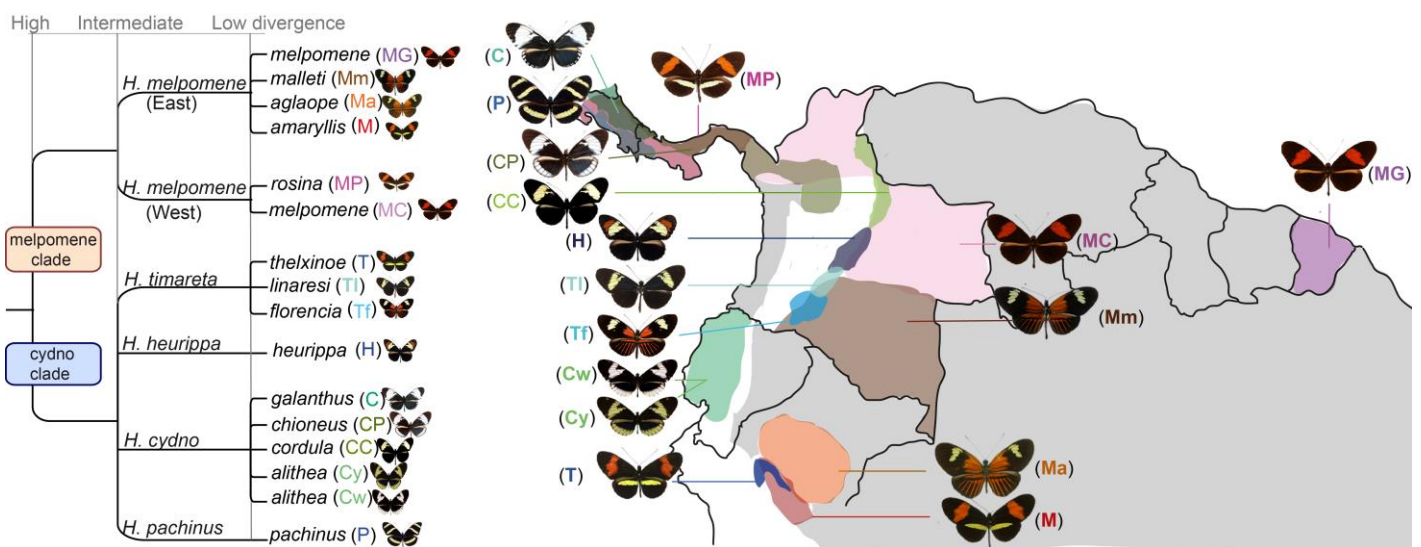
98 Most studies focus on pairs of species diverging in wing colour pattern. Wing pattern has been
99 termed a ‘magic trait’ causing speciation, because disruptive selection and assortative mating
100 operate directly on the same trait, wing pattern, thereby coupling two key forms of reproductive
101 isolation [30, 36, 38-40]. First, *Heliconius* wing patterns are warning signals under strong
102 natural selection for Müllerian mimicry [41-44]. Individuals not fitting one of the warning
103 patterns recognised by predators suffer a higher risk of predation and there is evidence for
104 selection against immigrant and hybrid wing patterns [36, 41, 42, 45]. Second, wing patterns
105 are also involved in mate-recognition in *Heliconius*, and males typically preferentially court
106 females displaying their own colour pattern [22, 26, 30, 33]. The loci controlling colour pattern
107 appear to be tightly linked to mate preference loci, which may help maintain the association
108 between signal and preference [23, 35]. Consequently, wing pattern divergence causes
109 reproductive isolation both through hybrid unfitness and assortative mating, and in *Heliconius*,
110 speciation is indeed frequently associated with a colour pattern shift [38, 46, 47].

111

112 Cases of mimicry between sibling species were unknown in *Heliconius* until the discovery of
 113 new cryptic subspecies of *H. timareta* in sympatry with its co-mimic *H. melpomene* in
 114 Colombia, Ecuador and Peru [20, 31, 48, 49]. Less is known about the mechanisms responsible
 115 for reproductive isolation between these species pairs with similar wing patterns (co-mimics),
 116 but this will be important in understanding the role of mimicry shifts in reproductive isolation.
 117 Indeed, wing-pattern similarity may be predicted to favour hybrid matings as well as increase
 118 the survival of hybrid adults, and so may weaken both pre-mating and post-mating isolation
 119 between taxa.

120

121 In this study, we investigate the mechanisms involved in the build-up of reproductive isolation
 122 by means of a large-scale, comparative analysis on this clade of *Heliconius* butterflies. We
 123 combine new data with data collected from the existing *Heliconius* literature. The numerous
 124 studies of *Heliconius* taxon-pairs at various levels of divergence and in various geographic
 125 contexts allow us to evaluate the relative importance of different isolating barriers and their
 126 appearance at various stages of divergence. We have applied a unified framework for the
 127 quantification of isolating barriers that facilitates these comparisons [6]. By contrasting co-
 128 mimetic vs. non-mimetic pairs of species, we also specifically address the importance of wing-
 129 pattern as a ‘magic trait’ for reproductive isolation in *Heliconius*.



131 **Figure 1: Geographic range and relationships of the taxa included in this study.**

132 *H. m. melpomene* and *H. m. malleti* have a wide range through South America but we chose to represent
 133 only their range in the country where they were studied. Grey areas represent areas harbouring other
 134 subspecies of *H. cydno/timareta* and *H. melpomene* which we did not include in our analyses. Phylogeny
 135 is adapted from [16, 17]. Range localisation is adapted from [52].

136

137

138 **Material and methods**

139 ***Species studied***

140 We considered published data from all representatives of the cydno clade, *H. cydno*, *H.*
141 *pachinus*, *H. timareta*, *H. heurippa* and from the two *H. melpomene* lineages (Fig. 1; see all the
142 references in Table S1 for each pair). New data is provided for the pair of co-mimics *H. t.*
143 *thelxinoe*/*H. m. amaryllis*, *H. t. florencía*/*H. m. malleti* and three Colombian non-mimetic pairs
144 *H. heurippa*, *H. c. cordula* and *H. m. melpomene* in supplementary material.

145 ***General framework: quantifying the strength of reproductive isolation (RI)***

146 We quantified the strength of reproductive isolation (RI) for each isolating barrier following [6]
147 inspired by [50, 51]. Briefly, the index *RI* offers a linear quantification of RI associated with
148 the presence of a given barrier relatively to expectations in the absence of all barriers. It allows
149 a direct link to gene flow: *RI* = 1 when isolation prevents gene flow, whereas *RI* = 0 if the
150 probability of gene flow does not differ from expectations without this barrier. Confidence
151 intervals for the index can be drawn from confidence interval on the data.

152

153 The strength of RI provided by each pre-mating barrier is estimated with the expression:

$$154 \quad RI_{\text{barrier}1} = 1 - 2 \times \frac{H_1}{H_1 + C_1}$$

155 where H_1 is the frequency of heterospecific mating and C_1 the frequency of conspecific mating
156 (when barrier 1 is the only barrier acting).

157 This is equivalent to:

$$158 \quad RI_{\text{barrier}1} = 1 - \frac{\text{Observed hybridization}_{(\text{with only barrier}1)}}{\text{Expected hybridization}_{(\text{with only barrier}1)}}$$

159

160 The strength of RI associated with each post-mating barrier is evaluated with the same
161 expression where H is the fitness of hybrids and C the fitness of pure individuals. RI was
162 calculated separately for both directions of crosses (AxB and BxA; female given first). We
163 summarize hereafter how each barrier was investigated. Detailed methods and calculations are
164 given in supplementary material and Table S1.

165

166 ***Spatial isolation (at small and large scale)***

167 Since some species ranges are poorly known, the geographic overlap was not evaluated
168 quantitatively. Instead, we provided a qualitative estimate of the level of parapatry, based on
169 the literature [52] in Table S1 and Fig. 1.

170

171 Although taxa may overlap in range at a broad geographic scale, this does not necessarily entail
172 equal encounter rates, for instance if relative frequencies differ between microhabitats. For four
173 pairs of species collected in several locations equally distributed along the transition zone (Fig.
174 S1), we use raw collection data (assuming equal collecting efforts on both species) as a proxy
175 for natural encounter rates, and draw an estimate of the expected number of heterospecific vs
176 conspecific matings which we use to calculate reproductive isolation due to spatial distribution,

177 RI_{spatial} .

178 **Behavioural pre-mating isolating barriers**

179 *Heliconius* males usually patrol the habitat, approach females and perform courtship
180 characterized by sustained hovering and intense wing flapping over the female. Females can
181 accept or reject mating [53]. Most studies have investigated male attraction by visual cues (on
182 models), male preference towards live females, and realised mating. Those three facets of mate
183 choice were analysed separately to dissect their respective contribution to sexual isolation.
184 Realised mating, which reflect the multiple aspects of mate choice by both sexes leading to a
185 mating event, was used for the whole comparison between barriers.

186 **Model experiments: male attraction on visual cues**

187 In all studies, male preference for different visual cues has been estimated by presenting a male
188 or a group of males with a model made with dead female wings dissected and by recording
189 courtship towards each model.

190 **Experiments with live females (LF exp): male preference behaviour based on all cues**

191 In all studies, individually-marked males were monitored for courtship during a short time
192 interval when presented with a heterospecific and a conspecific freshly emerged, virgin female.

193 **Mating experiments: full male-female interaction (M+F)**

194 To investigate mating achievement, most studies have simulated a natural situation, either with
195 a no-choice experiment in which a virgin female (conspecific or hetero-specific) is presented
196 to a one male or a group of ten mature males for 48h, or with a tetrad experiment, where four
197 individuals, one male and one female of each species, were kept until the first mating occurred.

198 **Post-mating isolating barriers**

199 **Hatch rate and hybrid sterility**

200 In all studies, mated females of crosses and pure females were kept in individual cages with
201 various fresh shoots of several *Passiflora* species. Eggs were collected on a regular schedule,
202 stored individually in small plastic cups, identified and checked daily for hatching. For each
203 female, fertility was defined as the hatch rate, the percentage of egg hatched over the total
204 number of eggs laid.

205 **Hybrid larval fitness**

206 Hybrid survival was recorded only for four pairs (Table S1). In all cases, larvae were raised in
207 individual plastic containers for the first instar. Then, they were gathered by family group in a
208 larger box and fed ad libitum on young shoot of *Passiflora* sp. Survival rate was calculated for
209 each family as the proportion of larvae growing until imago.

210 **Hybrid adult fitness**

211 Survival in the wild was tested experimentally only in Panama with paper models of *H. m.*
212 *rosina/H. c. chioneus* and their F₁ hybrids [36].

213
214 Hybrid ability of mating has been investigated with no-choice experiment or live-virgin female
215 experiment in six pairs (Table S1). For Costa Rican *H. cydno* x *H. pachinus* hybrids, mating
216 success is estimated from experiments with F₁ wing models.

217 Results

218 *Pre-mating spatial isolation*

219 The highly-divergent species pairs considered here are either sympatric or parapatric but
220 overlap on a large portion of their range. For four of those, local distribution was finely
221 quantified in the area of overlap (Fig. S1). As the probability of encounters between species
222 depends on coexistence within microhabitats, we found that micro-spatial distribution
223 contributes significantly to RI in both mimetic and non-mimetic pairs ($RI_{spatial}=0.7-0.8$, Fig. 2,
224 Table 1). For instance, *H. c. chioneus* occupies tall forest habitats where its co-mimic *H. sapho*
225 is abundant, whereas *H. m. rosina* is more frequent in disturbed and edge habitats where *H.*
226 *erato* is abundant [37, 54]. Similarly, with increasing altitude, *H. t. thelxinoe*, *H. t. florencia* or
227 *H. heurippa* progressively replace the local *H. melpomene* representative, and are also
228 associated with closed forested habitat.

229

230 At intermediate divergence, population pairs are mostly geographic replacements of one
231 another, and do not show any (known) geographic overlap.

232

233 At low divergence, *H. cydno alithea* alternative morphs have a patchy distribution, associated
234 with their local co-mimics [41], while pairs of populations within *H. melpomene* or *H. timareta*
235 are geographical races, with narrow hybrid zones where both morphs and hybrids are found.

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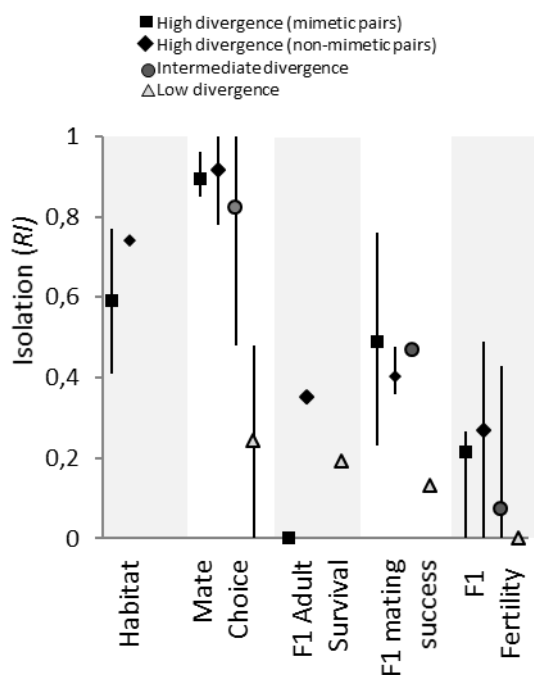


Figure 2: Mean strength of reproductive isolation for each relevant isolating barrier
RI associated with each barrier averaged by stage of divergence. The bar range from minimal to maximal values. All detailed values of *RI* are displayed in Table 1

237

238

239 **Table 1: Strength of reproductive isolation associated with each barrier to gene flow**
 240 *RI* ranges from 0 (non-significant barrier), to 1 (full isolation). For each pair of species, the two lines
 241 correspond to the two possible directions of heterospecific mating with the female given first. Barriers
 242 that could not be estimated are not shown. We indicated by a dash barriers that could not be estimated
 243 but are likely non-significant.

#1 #2	♀ X ♂	Spatial	Mating	F ₁ egg	F ₁ larva	F ₁ adult	F ₁ mating with #1	F ₁ mating with #2	F ₁ fertility	
High divergence	<i>H. c. chioneus</i> (CP) <i>H. m. rosina</i> (MP)	CPxMP MPxCP	0.73 0.73	1 1	0 0		0.35 0.35	0.20 0.52	0.31 0.13	
	<i>H. c. cordula</i> (CC) <i>H. m. melpomene</i> (MC)	CCxMC MCxCC	- -	0.82 0.88	0 0	0 0			0.33 0.22	
	<i>H. heurippa</i> (H) <i>H. m. melpomene</i> (MC)	HxMC MCxH	0.75 0.75	0.93 0.90	0 0	0 0		0.44 0.75	0.29 0.20	0.33 0
	<i>H. c. chioneus</i> (CP) <i>H. m. melpomene</i> (MG)	CPxMG MGxCP		0.78 1	0 0				0.49 0.33	
	<i>H. t. thelxinoe</i> (T) <i>H. m. amaryllis</i> (M)	TxM MxT	0.77 0.77	0.86 0.85	0 0	0 0	- -	0.46 0.87	0 0	0.32 0.08
	<i>H. t. florencina</i> (Tf) <i>H. m. malleti</i> (Mm)	TfxMm MmxTf	0.75 0.75	0.90 0.96	0 0		- -	0.52	1	0.33 0.19
Intermediate	<i>H. heurippa</i> (H) <i>H. c. cordula</i> (CC)	CCxH HxCC		0.56 0.98	0 0	0 0			0 0	
	<i>H. c. galanthus</i> (C) <i>H. pachinus</i> (P)	CxP PxP		0.91 1	- -	- -	0 0	0.94 0.94	- -	
	<i>H. m. rosina</i> (MP) <i>H. m. melpomene</i> (MG)	MGxMP MPxMG		1 0.48	0 0	- -			0 0.43	
Low	<i>H. c. alithea</i> white/yellow (Cw/Cy)	CyxCw CwxCy		0.26 0.07	- -	- -	0.19 0.19	- -	0.26 0.26	- -
	<i>H. t. florencina</i> (Tf) <i>H. t. linaresi</i> (Tl)	TfxTl TfxTl		0.02 0.48	0 0	- -			0 0	
	<i>H. m. amaryllis</i> (M) <i>H. m. aglaope</i> (Ma)	MaxM MxMa		0.40 0	- -	- -			- -	

245 **Behavioural pre-mating isolating barriers (Fig.3)**

246 Colour pattern model experiments: male preference based on colour

247 At high divergence, isolation due to male preference based on visual cues is strong for pairs
248 with different colour patterns. It is generally higher in the direction involving *melpomene* males
249 ($RI_{colour} = 0.75-0.94$, except for the Colombian *H. c. cordula* and *H. m. melpomene* pair reaching
250 only 0.28) than in the other direction (*H. melpomene* X cydno clade males, $RI_{colour} = 0.35-0.5$).
251 Colour preference is lower between *H. heurippa* and *H. m. melpomene* than between other pairs
252 diverging in colour pattern ($RI_{colour} = 0.07/0.2$). This might be due to the intermediate pattern of
253 *H. heurippa*, which includes the red band of *H. m. melpomene*. In the co-mimetic pairs (*H. t.*
254 *thelxinoe* and *H. m. amaryllis* ; *H. t. florencia* and *H. m. malleti*) males do not discriminate
255 between models, as expected given the high visual similarity of the two species.

256
257 At intermediate divergence, colour preference remains an isolating factor although its strength
258 varies depending on the pair considered. RI_{colour} estimates can reach 0.85/0.98 between *H. c.*
259 *galanthus* and *H. pachinus* but only 0.17/0.56 between *H. heurippa* and *H. c. cordula*. It is zero
260 between the allopatric *H. m. rosina* and *H. m. melpomene*, probably because of the red forewing
261 band shared by the two subspecies.

262
263 At low divergence, between *H. t. florencia* and *H. t. linaresi*, some preference is observed,
264 leading to an estimated RI_{colour} of 0.27/0.35.

266 Experiments with live females: male preference based on all cues

267 At high divergence, male preference for conspecifics is stronger than in the experiments with
268 models, suggesting that a wider range of proximal cues are available, such as chemical signals
269 or behavioural cues, and influence male courtship decision leading to a higher RI (RI_{male}
270 $choice = 0.64-1$).

271
272 The use of proximal vs. long-range visual cues by males seems to depend on the direction of
273 the hetero-specific interaction: *H. melpomene* males appear to choose based on visual cues
274 while cydno-clade males show an accentuated choice with live females, possibly reflecting their
275 response to chemical information (Mérot *et al*, 2015). *H. melpomene* males indeed respond to
276 wing models with a very strong choice, and appear to show little discrimination when presented
277 with *H. timareta* females showing the same wing pattern, suggesting that additional short-range
278 cues do not play a strong role in *H. melpomene* males courtship decision. By contrast, *H. cydno*
279 or *H. heurippa* males show some discrimination against *H. melpomene* models, but it is weaker
280 than for *H. melpomene* males [30, 35], and choice is generally enhanced by real-females cues.
281 Moreover, in the mimetic Peruvian pair, *H. t. thelxinoe* males strongly prefer conspecific
282 females over heterospecific females using close range chemical cues.

283
284 At intermediate and at low divergence, a limited amount of reproductive isolation due to male
285 courtship behaviour is sometimes observed ($RI_{male\ choice} = 0.5-0.78$ and 0-0.4, respectively)
286 although the strength of isolation is generally weaker and more asymmetric than at high
287 divergence.

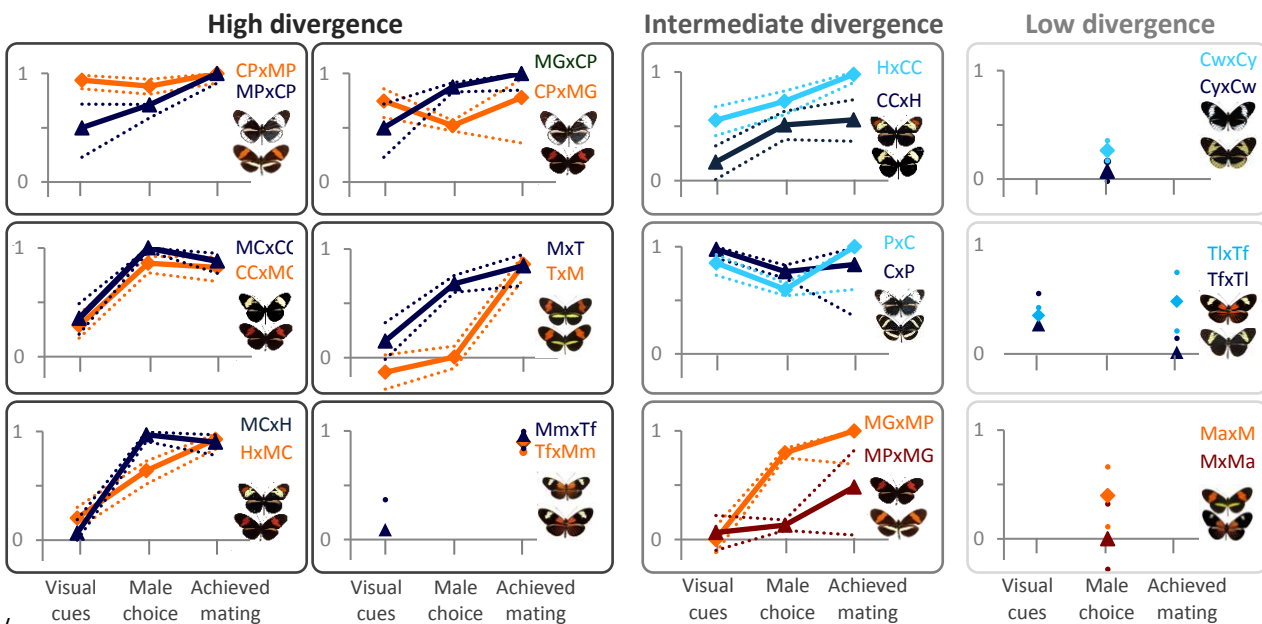
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289 **Mating experiments: full male-female interaction**

290 At high divergence, the total index of sexual isolation is high for all pairs and in both directions
 291 of crosses ($RI_{mating}=0.78-1$, Table 1). RI estimated using realised mating is higher than when
 292 estimated based on visual cues or short range cues from virgin females, suggesting that female
 293 response and contact interactions (beyond male courtship) also contribute to pre-mating
 294 isolation, especially for the mimetic pairs (preventing $T \times M$ heterospecific mating for instance).
 295

296 At intermediate divergence, isolation is generally high, though asymmetric, such as between *H.*
 297 *c. cordula* and *H. heurippa* ($RI_{mating}=0.56/0.98$) or between Panamanian and Guianan allopatric
 298 populations of *H. melpomene* ($RI_{mating}=0.65/1$). RI estimated on total mating is again higher
 299 than RI estimated on experiments with models, suggesting that close-range cues and male-
 300 female interactions may also be relevant at intermediate divergence.
 301

302 By contrast, at low divergence between the parapatric races *H. t. florencia* and *H. t. linaresi*,
 303 reproductive isolation is much lower. It is observed only in one direction ($T \times M$, $RI_{mating}=0.48$)
 304 and largely explained by colour pattern preference.
 305
 306



307

308 **Figure 3: Level of RI associated with each behavioural pre-mating barrier to gene flow**

309 For each pair of species, the two colours correspond to the two possible directions of heterospecific
 310 mating with the female given first. Dotted lines are the confidence intervals.

311

312

313

314 *Post-mating isolating barriers*

315 Hatch rate of F₁ hybrids: cytoplasmic incompatibilities

316 At high divergence, F₁ hybrids show no significant reduction of hatch rate (Table S1, S2).

317 Larval fitness of F₁ broods: host-plant adaptation

318 Oviposition preferences for different *Passiflora* hosts generally constitute an axis of
319 differentiation between the melpomene and the cydno clade, *H. melpomene* being generally
320 more specialised than its local cydno-clade counterpart [20, 31, 55] with some exception in
321 Colombia where *H. melpomene* has a diverse range of oviposition plants [56].

322

323 Hybrid larval survival has only been tested in three pairs at high divergence but shows no
324 significant reduction of survival, leading to a null contribution to reproductive isolation. This
325 suggests neither hybrid viability breakdown related to genetic incompatibilities nor incapacity
326 to metabolize the host-plant are acting in these pairs. For the experiment on hybrids between
327 *H. c. cordula*/*H. m. melpomene* and *H. heurippa*/*H. m. melpomene* (Table S5), this result
328 corresponds to expectations since the hybrids were fed on a common host-plant (*P. oesterdii*).
329 However, this may be surprising for the Peruvian pair *H. t. thelxinoe*/*H. m. amaryllis*, which
330 was fed on the maternal host-plant (Table S2).

331

332 Testing survival in experimental conditions with unlimited access to food, fewer parasites and
333 no competition might have underestimated the importance of efficient host-plant use in hybrid
334 growth. We can note for instance, that, in semi-natural conditions, early stage *H. melpomene*
335 larvae from central America had a higher survival rate on *P. menispermifolia* than on other
336 *Passiflora* species [55] while in insectaries, similar growth rates have been achieved for various
337 species of *Passiflora* (Smiley 1978). In Peru, several preliminary attempts of feeding *H. m.*
338 *amaryllis* larvae and some hybrids (back-crosses towards *H. m. amaryllis*) with *P. edulis* or *P.*
339 *granadilla* (well-accepted by *H. t. thelxinoe*) led to higher mortality rate.

340

341 F₁ adult survival

342 Adult mortality due to predation was estimated only for the hybrids between *H. c. chioneus* x
343 *H. m. rosina*. Its contribution to isolation was significant with $RI = 0.35$, but lower than that
344 due to pre-mating barriers.

345

346 In the co-mimetic pairs, F₁ hybrids are visually similar to the parents and predation is not
347 expected to participate in reproductive isolation.

348

349 In other cases, F₁ hybrids may also be similar to one parent (*H. c. galanthus* X *pachinus* hybrids
350 being like *H. c. galanthus* [23], *H. heurippa* X *H. m. melpomene* hybrids being similar to *H. m.*
351 *melpomene* [29], and heterozygotes at the K locus determining white or yellow morph of the
352 polymorphic taxa *H. cydno alithea* are white (Chamberlain *et al*, 2009)), which introduces
353 asymmetry in isolation because they are expected to survive better in one habitat. For instance,
354 mark-resight experiments on *H. cydno alithea* (Kapan, 2001) let us estimate predation against

355 white morphs in areas dominated by the yellow mimic, suggesting a mean $RI_{adult\ survival}$ due to
356 predation against F₁ hybrids around 0.19 (0.38 in areas dominated by yellow, 0 in areas
357 dominated by white).
358

359 F₁ mating ability: sexual selection against F₁ hybrid

360 At high divergence, in non-mimetic as well as co-mimetic pairs, mate discrimination against F₁
361 hybrids appears as an additional isolating barrier although its strength is highly variable and
362 asymmetric, depending on which parental partner is tested ($RI_{F_1\ success}=0-0.87$, Table 1, Table
363 S3-4, S6-7).

364

365 At intermediate divergence, mating discrimination against F₁ hybrids is also observed for *H. c.*
366 *galanthus* x *H. pachinus*. Those hybrids are white like *H. c. galanthus* and F₁ wing models get
367 as much approaches from *H. c. galanthus* males as the pure species, whereas they are
368 discriminated against by *H. pachinus* males, resulting in asymmetric isolation
369 ($RI_{F_1\ success}=0/0.94$).
370

370

371 Fertility of F₁ broods: hybrid incompatibilities

372 At high divergence, the estimated isolating strength of hybrid sterility is intermediate compare
373 to other factors, ranging from 0.33 to 0.49 in one direction and from 0 to 0.33 in the other
374 direction (Table 1, Fig. 2). F₁ hybrid males are fully fertile except for *H. c. chioneus* (Panama)
375 X *H. m. melpomene* (French Guiana) males which show a slight reduction in fertility [32].
376 Female F₁ fertility is more complex. All studies involving crosses between a *H.*
377 *cydno/heurippa/timareta* mother and a *melpomene* father found complete sterility of female F₁
378 (Table S2)[29, 32]. In the other direction of crosses, i.e. a *melpomene* mother x
379 *cydno/timareta/heurippa* father, F₁ fertility is highly variable. At the extremes, all *H. m.*
380 *melpomene* X *H. heurippa* females tested were fully fertile [29] whereas *H. m. melpomene*
381 (French Guiana) X *H. c. chioneus* (Panama) females were all sterile [32]. For most other pairs,
382 partial fertility was reported [24, 32] (Table S8) with intriguing non-uniform pattern. For
383 instance, in *H. melpomene* X *H. timareta* hybrids, some hybrid females had a lower fertility,
384 compared with pure females, while others were completely sterile and others completely fertile
385 (Table S2).
386

386

387 At intermediate or low divergence, no significant reduction of fertility was found for the
388 parapatric pairs investigated, neither for *H. heurippa* X *H. c. cordula* [29] nor *H. t. florenciac* X
389 *H. t. linaresei* hybrids [24]. The exception is the allopatric pair *H. m. rosina* (Panama)/*H. m.*
390 *melpomene* (French Guiana) since the F₁ female (and possibly males) hybrids showed a lower
391 fertility [57], resulting in an additional barrier of 0.43 only for one direction ($MPxMG$).
392

392

393

394 Discussion

395 Quantifying reproductive isolation throughout a speciose clade of *Heliconius* butterflies shows
396 that different levels of genetic divergence correspond to marked quantitative and qualitative
397 differences in reproductive isolation. Higher divergence is associated with both the
398 accumulation of additional barriers and the strengthening of a common set of barriers, although
399 some axes of differentiation are quite labile depending on the ecological context.

400
401 The diversity of taxa at different levels of divergence and strengths of RI has been characterised
402 as a ‘speciation continuum’. This does not necessarily imply that these actually represent
403 sequential stages in speciation, nor that any particular example is on an inevitable path towards
404 complete speciation. For example, different stages might be at equilibrium between divergence
405 and gene flow or correspond to qualitatively different pathways to differentiation. Nevertheless,
406 the ‘speciation continuum’ is useful and perhaps analogous to the manner in which those
407 studying the evolution of complex structures, such as the eye or the flagellum, infer past
408 evolutionary trajectories from the comparative study of apparently intermediate structures in
409 extant animals. Such examples provide support for the plausibility of a particular route towards
410 a complex structure, or in the present case a route towards complete speciation, but do not prove
411 that any particular evolutionary route has been taken in nature. Our analysis therefore allows
412 assessment of the roles that different factors might take in shaping divergence, while accepting
413 that the current array of divergence states does not necessarily represent successive stages along
414 a unique path to speciation.

415

416 *Is reproductive isolation driven by a single trait or multidimensional factors?*

417 Isolation in the face of gene flow requires that certain factors counter the effects of
418 recombination between alleles that characterise diverging taxa [10, 58-60]. This might include
419 strong disruptive selection on a single (large-effect) trait [61], an association between ecological
420 divergence and reproductive isolation (via a ‘magic’ trait for instance [39]), or the coupling of
421 several isolating barriers [58]. Diverging *Heliconius* taxa showing a shift in colour pattern meet
422 all those criteria, making colour pattern divergence a major initiator and driver of reproductive
423 isolation in this group [38, 62].

424

425 Given that colour-pattern differentiation underlies the main isolating barriers (predation, mate
426 choice, habitat partitioning) and that all those barriers operate at low, intermediate and high
427 divergence, one may wonder whether increased isolation results from the “stronger selection”
428 scenario [61], under which barriers associated with colour pattern differences are strengthened
429 along the continuum of divergence. This is the case, for instance, in *Pundamilia* cichlid fish, in
430 which increased isolation is associated with increased divergence on one main axis of
431 differentiation, male coloration in relation with habitat transparency [63]. The alternative
432 hypothesis would be that increased isolation is the product of “multifarious selection” [61], with
433 the addition of independent traits and more isolating barriers at higher divergence [64, 65]. For
434 instance, between colour-pattern races of poison frog, isolation is much higher for a pair which
435 also exhibit size differences associated with habitat specialization [66].

436

437 Those predictions can be tested by comparing the strength of the barriers potentially associated
438 with colour pattern divergence along the *Heliconius* continuum. The lower stages of divergence
439 reported in *Heliconius* correspond to wing-pattern races, for which selection causes genetic
440 differentiation only around wing-patterning loci [48] and maintain weak isolation. At this stage,
441 selection on different mimicry associations maintains spatial segregation through predation
442 against migrants [42], and is likely to cause post-mating isolation through predation against
443 non-mimetic hybrids. The third barrier, male preference based on colour, is already acting at
444 low-divergence but its contribution is variable and asymmetric. What is the fate of those barriers
445 at higher divergence? Isolation due to **predation** against hybrids has not been quantified in
446 many pairs of taxa. It does appear stronger for the *H. c. chioneus* x *H. m. rosina* hybrids (high
447 divergence), than for *H. c. alithea* F₁ (low divergence) for instance. It is worth noting that
448 predation itself is of the same magnitude in both cases, reducing the survival of any deviant
449 form by about 30%. RI due to predation is thus lower in *C. alithea* hybrids because they are
450 similar to one parent (white) while *H. c. chioneus* x *H. m. rosina* hybrids differ from both
451 parents and suffer from predation in all habitats. Therefore, isolation against hybrids depends
452 on dominance and segregation of colour patterns in hybrids, with the hybrid being generally
453 more different at higher level of divergence (except for the mimetic pairs). **Habitat isolation**
454 gets stronger at high divergence. Just like for pairs of taxa at low divergence, fine-scale
455 partitioning between taxa at high divergence may follow the distribution of their co-mimics, as
456 observed for instance between *H. c. chioneus* and *H. m. rosina* across the transition from closed
457 forest to disturbed forest and edge habitat [37]. However, habitat specialization for closed forest
458 habitat is also exhibited by other members of the cydno clade such as *H. timareta* (co-mimic
459 with *H. melpomene*) or *H. heurippa* (which has no co-mimic), suggesting that spatial
460 partitioning at high divergence is not only conditioned by mimicry, but also by other ecological
461 preferences which remain unknown but may involve abiotic conditions, adaptation to altitude
462 or host-plants. The component of **mate choice** clearly attributable to visual cues, deduced from
463 experiments with models, is generally strengthened at high and intermediate divergence, though
464 not consistently between species. In addition, assortative mating is likely to involve a chemical
465 component for most pairs of taxa at high divergence. Again, as hybrids tend to be quite different
466 from parental species at higher divergence, sexual selection against hybrids is also stronger at
467 high divergence. Overall, increased isolation does involve a strengthening of isolating barriers
468 directly linked to colour pattern differences, but higher RI also rests largely on the addition of
469 other isolating dimensions.

470
471 To assess the relative importance of colour pattern shift at later stages of speciation, it is also
472 useful to consider species pairs that do not exhibit colour pattern divergence, such as the co-
473 mimics *H. timareta* and *H. melpomene*. Genomic evidence suggests that these species were
474 initially divergent in colour pattern and became co-mimics after secondary introgression of
475 wing pattern alleles from *H. melpomene* into *H. timareta* [67, 68]. Under this scenario, if colour
476 pattern divergence plays an important role in the isolation of species at higher divergence,
477 reproductive isolation is expected to be weakened secondarily by mimicry and gene flow. Such
478 collapse of differentiation has sometimes been observed, notably between pairs of taxa that rely
479 on one main axis of differentiation, habitat-related for instance [69]. Compared with *H. c.*
480 *chioneus* and *H. m. rosina*, the co-mimics *H. t. thelxinoe* and *H. m. amaryllis* indeed display a

481 ~2% reduction of total estimated RI and a slightly lowered genomic divergence [21]. Both in
482 the Colombian and Peruvian mimetic pairs, natural hybrids are also marginally more frequent
483 (1-3%) [20, 31, 70]. This reduction of RI between co-mimics follows the prediction but shows
484 that lifting the wing-pattern barrier has a rather limited effect on species differentiation because
485 RI relies on multiple other isolating mechanisms (habitat specialisation, assortative mating
486 based on chemical communication [34], partial hybrid sterility and likely host-plant
487 divergence). This implies that reproductive isolation between pairs at high level of divergence
488 is strong enough to allow the secondary loss of certain barriers to gene flow, in this case through
489 introgression of wing-pattern alleles, without compromising genome-wide differentiation. It
490 supports the hypothesis that multiple diverging dimensions add cumulatively to reproductive
491 isolation and favour the completion of speciation in the face of gene flow [61].
492

493 *How do isolating mechanisms evolve?*

494 The continuum of reproductive isolation spanned in this study also corresponds to a continuum
495 of time since divergence, raising the questions of how the multiple barriers accumulate through
496 time, which result from selection, which are a by-product of isolation through drift, and what is
497 the relative importance of ecological and non-ecological processes.
498

499 Pre-mating sexual isolation stands out as one of the strongest barriers at all levels of divergence
500 and gets stronger along the continuum of divergence. This observation is consistent with the
501 rapid evolution of pre-mating isolation generally reported for speciation with gene flow [2], in
502 fish [9, 71], drosophila [51] or plants [64]. As for darter fish [72], the rapid evolution of strong
503 assortative mating in *Heliconius* appears to be associated with sexual selection, notably for
504 chemosensory traits [73] which, as indicators of mate quality, are common targets of sexual
505 selection [74].
506

507 An increase in pre-zygotic isolation between hybridizing populations may also reflect
508 reinforcement, under selection against interspecific mating [75]. In *Drosophila* for instance, the
509 fast evolution of mate choice has been linked to reinforcement processes, with pre-mating
510 isolation being stronger for pairs with geographic overlap [51] and pairs with higher
511 hybridization costs [76]. Here, higher stages of divergence are characterized by a decrease in
512 hybrid fitness, such that stronger pre-mating isolation may reflect stronger selection against
513 hybridization. In addition, the higher geographic overlap seen in pairs at high divergence also
514 provides more opportunities for selection against hybridisation to operate. Evidence for
515 reinforcement comes from higher pre-mating isolation observed in the sympatric *H. c.*
516 *chioneus/H. m. rosina* than in the allopatric *H. c. chioneus/H. m. melpomene* (French Guiana)
517 as well as an increased mate choice between *H. c. galanthus* and *H. pachinus* in populations
518 close to the contact zone [27].
519

520 Under a hypothesis of reinforcement, pre-mating isolation comes as a response to hybrid
521 unfitness, so it may seem paradoxical to observe rather weak or moderate post-mating barriers.
522 It could be that their current contributions do not reflect their past importance or that the
523 accumulation of several weak barriers is sufficient to select for assortative mating. Our analysis
524 may also underestimate the strength of extrinsic post-mating barriers, which are experimentally

525 more difficult to assess. Notably, little is known about the ecology of hybrids, and poor hybrid
526 performance may represent a significant barrier when parental species have markedly different
527 habitat preferences (e.g. altitude between *H. timareta* and *H. melpomene*).

528
529 Habitat specialisation associated with fine-scale spatial segregation and host-plant divergence
530 is observed for all pairs at high divergence but for none at low divergence. Interestingly, species
531 at intermediate divergence do not show clear habitat or host-plant differences either, suggesting
532 that habitat specialisation might be one of the key barriers allowing geographic overlap and
533 leading to high divergence. Such a transition from parapatric, ecologically-similar morphs to
534 overlapping habitat-specialized taxa is also reported along the stickleback speciation continuum
535 [9] and perhaps constitutes a tipping point in the evolution of isolation[13].

536
537 The last post-mating barrier widely observed at high divergence but generally absent at lower
538 levels of divergence is hybrid female sterility (with the exception of allopatric races of *H.*
539 *melpomene* [57], at intermediate divergence). This result is quite general in the literature: when
540 speciation occurs with gene flow, post-mating incompatibilities tend to accumulate more slowly
541 than ecological and pre-mating isolation [51, 71, 77], and follow Haldane's rule by first
542 affecting the heterogametic sex [78]. Generally, the strongest isolation was found between
543 allopatric pairs coming from distant areas (Panama VS French Guiana) whereas in sympatry,
544 F₁ female sterility can be variable, from fully sterile to fully fertile, suggesting that sterility is
545 variably affected by local gene flow. *Heliconius* female sterility is typically caused by
546 interactions between the Z chromosome and autosomal loci [29, 32, 57]. Among sympatric
547 pairs of taxa at high divergence such as *H. timareta* and *H. melpomene* or *H. cydno* and *H.*
548 *melpomene*, Z chromosomes are very divergent while autosomes show a strong signal of
549 admixture [21]. Admixture might prevent the accumulation of incompatibilities on autosomes
550 (or may allow its purge following secondary contact), therefore limiting the evolution of female
551 sterility. Such hypothesis would question the stability of this intrinsic barrier, traditionally
552 assumed to be irreversible.

553 Conclusion

554 In summary, we have quantified most of the known components of reproductive isolation across
555 a recent adaptive radiation. Contrasting pairs of hybridizing taxa showing different levels of
556 divergence suggests that speciation involves the strengthening of some isolating barriers but,
557 importantly, seems to require the accumulation of additional barriers. Indeed, the synergistic
558 action of wing pattern shifts and other isolating mechanisms appears to be important for
559 reproductive isolation in *Heliconius*, especially at early stages of divergence. Nevertheless, the
560 case of co-mimetic hybridizing species reveals that certain isolating barriers, and especially
561 wing pattern differences, may in fact be quite labile or partially reversible. This shows that a
562 seemingly key factor in the early stages of differentiation may have its role taken over by other
563 barriers at later stages of divergence. A key promoter of the stability and completion of species
564 divergence thus appears to be the multidimensionality of reproductive isolation.

565

566 **Author's contributions**

567 CM and MJ designed the analysis, and the study of the Peruvian taxa. CM performed the meta-
568 data analysis and the new data acquisition on the Peruvian species. CS performed the new data
569 acquisition on the Colombian species. RM and CJ studied the Panamanian species. CM and MJ
570 wrote the manuscript with contributions from all authors. All authors gave final approval for
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