

An introgressed wing pattern acts as a mating cue

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1 **An introgressed wing pattern acts as a mating cue**

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27 **Key words:** *Heliconius*, reproductive isolation, homoploid hybrid speciation, magic trait

28 **Running title:** An adaptive trait elicits assortative mating

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30

31 **Abstract**

32

33 *Heliconius* butterflies provide good examples of both homoploid hybrid speciation and
34 ecological speciation. In particular, examples of adaptive introgression have been detected
35 among the subspecies of *Heliconius timareta*, which acquired red color pattern elements
36 from *H. melpomene*. We tested whether the introgression of red wing pattern elements into *H.*
37 *timareta florenci*a might also be associated with incipient reproductive isolation from its close
38 relative, *H. timareta* subsp. nov., found in the eastern Andes. No choice experiments show a
39 50% reduction in mating between females of *H. t.* subsp. nov. and males of *H. t. florenci*a, but
40 not in the reciprocal direction. In choice experiments using wing models, males of *H.*
41 *timareta* subsp. nov. approach and court red phenotypes less than their own, while males of *H.*
42 *t. florenci*a prefer models with a red phenotype. Intrinsic post-zygotic isolation was not
43 detected in crosses between these *H. timareta* races. These results suggest that a color pattern
44 trait gained by introgression is triggering reproductive isolation between *H. timareta* subsp.
45 nov. and *H. t. florenci*a.

46 **Introduction**

47

48 The mode and tempo of speciation remains controversial among evolutionary biologists
49 (Mayr 1942; Turelli et al. 2001; Coyne and Orr 2004; Scopece et al. 2007). One highly
50 debated area is the possible role of hybridization in species formation, especially among
51 zoologists, who often see hybridization as a process that retards speciation (Dowling et al.
52 1997; Mallet 2007). While it is true that gene flow can impede divergence in sympatry, it is
53 becoming recognized that hybridization might also contribute useful genetic variation, i.e.
54 adaptive introgression (Arnold 1997; Dowling et al. 1997; Seehausen 2004; Mallet 2007;
55 Abbott et al. 2013). Currently several examples of adaptive introgression in animal species
56 are known and include warfarin resistance in mice, coat color in wolves, insecticide resistance
57 in *Anopheles*, wing color pattern in *Heliconius* and several traits gained by modern humans
58 from Neanderthals and Denisovans (Anderson et al. 2009; Green et al. 2010; Coulson et al.
59 2011; Song et al. 2011; Consortium 2012; Mendez et al. 2012b; Mendez et al. 2012a; Pardo-
60 Diaz et al. 2012; Hedrick 2013; Mendez et al. 2013; Clarkson et al. 2014; Norris et al. 2015).
61 Adaptive introgression and hybridization are processes with potential to facilitate hybrid
62 speciation when the novel traits or parental genome reorganization promote reproductive
63 isolation (RI) and/or adaptive divergence (Abbott et al. 2013; Seehausen et al. 2014). Hybrid
64 swarms, for example, might contribute to speciation by founding populations with novel
65 characteristics not seen in parents, and a number of examples are known where recently
66 derived species show evidence for admixed genomes derived from different parental taxa
67 (Edelist et al. 2009; Whitney et al. 2010; Cypionka et al. 2012). Nonetheless, the evidence
68 for hybrid speciation remains controversial, and in particular, strong evidence for traits of
69 hybrid origin contributing to RI remains elusive in most systems (Schumer et al. 2014). A
70 simple way to test whether adaptive introgression leads to speciation in animals is by
71 assessing its potential to generate RI in early stages of divergence. This however has been

72 tested only a handful of times (Schumer et al. 2014; Selz et al. 2014) and, in particular, the
73 contribution of adaptive introgression to develop novel mating preferences has only been
74 investigated in artificial hybrids that do not occur in nature (Doherty and Gerhardt 1983;
75 Segura et al. 2011; Selz et al. 2014).

76

77 Butterflies of the genus *Heliconius* are famous for their adaptive wing color patterns (Mallet
78 and Jackson 1980; Jiggins 2008; Merrill et al. 2011) and provide one of the best animal
79 examples in which hybridization is known to play a role in speciation (Mavarez et al. 2006;
80 Melo et al. 2009). For instance, *Heliconius heurippa* is a novel non-mimetic species
81 established as a result of hybridization, leading to both a novel wing pattern and a novel
82 derived mating preference, constituting a case of ecological speciation where an adaptive
83 character, acquired by hybridization, drives RI (Mavarez et al. 2006; Melo et al. 2009; Salazar
84 et al. 2010). Additional cases of adaptive introgression in *Heliconius* include the species *H.*
85 *timareta* (Consortium 2012; Pardo-Diaz et al. 2012). Phylogenetic analysis shows this taxon
86 as sister species to *H. cydno*, a species usually displaying yellow and white wing coloration
87 (Beltran et al. 2007; Giraldo et al. 2008). However, recent studies have uncovered several
88 previously undescribed populations of *H. timareta* with red pattern elements (Giraldo et al.
89 2008; Merot et al. 2013; Nadeau et al. 2014). In the eastern Colombian Andes the endemic
90 race *H. t. florencia*, displays an orange ‘dennis-ray’ wing color pattern (Giraldo et al. 2008),
91 the most common *Heliconius* mimicry Müllerian ring in the Amazon basin (Mallet and
92 Jackson 1980) whereas in Peru, the race *H. t. thelxinoe* shows a forewing red-banded
93 phenotype (Merot et al. 2013). Such discoveries were unexpected because mimicry between
94 closely related sympatric species such as *H. timareta* and *H. melpomene* had been considered
95 unlikely (Giraldo et al. 2008; Merot et al. 2013). However, recent analysis of genomic data
96 and genetic markers across the red color interval, have shown that red color patterns of these
97 *H. timareta* races have been acquired through multiple adaptive introgression events from *H.*

98 *melpomene*, permitting these two species to become mimetic in the Florencia region of
99 Colombia and in the San Martin region of Peru (Consortium 2012; Pardo-Diaz et al. 2012;
100 Merot et al. 2013).

101 In the light of evidence that the red coloration of *H. t. florencia* has been acquired via
102 hybridization, we here aim to determine whether the introgression of the rayed wing pattern
103 from *H. melpomene* into *H. t. florencia* is associated with RI from its close relatives. We
104 study closely related taxa in the melpomene/cydno/timareta clade found in the eastern Andes.
105 These include the ‘dennis-ray’ *H. m. malleti* and an undescribed endemic taxon found near to
106 San Vicente del Caguán (Colombia), *H. timareta* subsp. nov., for which morphological and
107 molecular data support its identity as another subspecies of *H. timareta*. This taxon has a
108 black background with a yellow band in the forewing, similar to nearby forms of *H. cydno*
109 (Figure 1; Giraldo et al. *in prep*). Although this *H. t.* subsp. nov. occurs geographically close
110 to *H. t. florencia*, a contact zone is unknown for these subspecies (Figure 1, Linares pers.
111 obs.), mostly due to security issues in the region.

112 Here, we evaluated whether pre-zygotic isolation barriers have evolved between *H. t.*
113 *florencia* and *H. t.* subsp. nov.. We also included *H. m. malleti* and *H. c. cordula* in these
114 experiments, in order to determine the role of the novel *H. t. florencia* wing color pattern in
115 RI from other parapatric and sympatric taxa from the same geographic region. In addition,
116 these comparisons across multiple stages of divergence (from races to ‘good’ species) shed
117 lights on how RI develops along the speciation continuum. This will help to understand the
118 importance of wing color pattern acquired through adaptive introgression as a cause of
119 speciation in the *Heliconius* butterflies.

120 **Methods**

121

122 SAMPLING AND EXPERIMENTAL POPULATIONS

123

124 During 2009 and 2010 we collected a minimum of 25 wild individuals of each *H. t. florencía*
125 (*Tf*) and *H. m. malleti* (*Mm*) from Las Doraditas (2°41'04''N-74°53'17''W, Caquetá,
126 Colombia), *H. c. cordula* (*Cc*) from San Cristobal (7°47'566''N-72°11'566''W, Venezuela),
127 and *H. timareta* subsp. nov. (*Tn*) from Las Morras (01°45'02'' N-75°37'55''W, Caquetá,
128 Colombia) and Guayabal (2°41'04''N-74°53'17''W, Caquetá, Colombia) (Figure 1). We used
129 these wild individuals to establish experimental populations in outdoor insectaries of 2x3x2m³
130 in La Vega (Colombia), that were provided with the host plants *Passiflora oerstedii*, *P. edulis*,
131 *P. maliformis* and *P. ligularis* for oviposition and larvae feeding. For the adults, we provided
132 the nectar and pollen source plants *Lantana sp.*, *Gurania sp.* and *Psiguria sp.*, and artificial
133 nectar solution (Merrill et al. 2011).

134

135 MATING EXPERIMENTS

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137 To determine the presence and strength of pre-zygotic barriers to gene flow between *H. t.*
138 *florencia* and *H. t.* subsp. nov., *H. c. cordula* and *H. m. malleti*, we used two types of
139 experiments, no-choice mating experiments and color pattern models. We expect that as
140 species divergence increases the strength of RI does. Thus, given the recent introgression of
141 the 'dennis-ray' in *H. t. florencía* we expect that both *H. timareta* races show some
142 indications of RI based in coloration pattern. This isolation should accentuate between the
143 species *H. timareta* and *H. cydno*, whilst between the more divergent *H. timareta*-*H. cydno*
144 and *H. melpomene*, isolation should be strong despite some of them display similar wing
145 color pattern.

146

147 *No-choice mating experiments*

148

149 We classified the no-choice matings, including direct and reciprocal, into three categories: (i)
150 control (same race), (ii) conspecific (same species, different race) and (iii) heterospecific
151 (Supplementary table 1). For each combination, a virgin female was placed with a male of at
152 least 8 days old inside an insectary for a maximum period of 8 days. The success or failure of
153 mating was recorded either by direct observation of mating or by the presence of the
154 spermatophore inside the female abdomen. After mating, the female was isolated in a
155 different insectary while the male was returned to the stock. Mated males were used only
156 once whereas unmated males were reused (Mavarez et al. 2006; Muñoz et al. 2010).

157 A Bayesian hierarchical model was used to estimate the probability of success for each
158 mating type. For the full model (Supplementary table 1, Supplementary Figure 1A), we
159 assume there is an overall rate of mating success μ for any mating class and each one of these
160 is assumed to have a rate of mating success θ_i which comes from a distribution centered
161 around μ . The number of times a mating success was registered y_i follows a binomial
162 distribution with rate of success θ_i and n_i trials. For the population specific model, we assume
163 different groups of crosses (Supplementary table 1, Supplementary Figure 1B) to have a
164 preference p_k , drawn from the overall distribution centered around μ . In this case θ_{lk} is the rate
165 of mating success for each type of cross l in each group of crosses k . As above, y_{lk} is the
166 number of successes in each experiment and follows a binomial distribution with rate of
167 success θ_{lk} and a total number of trials n_{lk} . These graphical models were implemented in the
168 JAGS software (Plummer 2003) using the R package R2jags (Su and Yajima 2009). We used
169 six independent Monte Carlo Markov Chains each with 20,000 collected samples and 5,000
170 burn-in samples. The \hat{R} statistic was used to verify convergence and autocorrelation as well as
171 to check that samples are good approximations to posterior distributions (Gelman et al. 1996).
172 Further, we used Bayes factors (BF henceforth) (Kass and Raftery 1995) to determine

173 whether rates of mating success are the same or different between mating types. In each case,
174 the Savage-Dickey approximation method (Lee and Wagenmakers 2013) was used to
175 estimate the value of the BF by comparing prior and posterior densities of the parameters (i.e.
176 calculating the ratio between of evidence supporting the null hypothesis and that of the
177 alternative hypothesis (H_0/H_1)). In our case, the null hypothesis is that success rate is no
178 different between mating types (i.e. $\delta = 0.5$, where δ is the difference between the success
179 rates of any pair of mating types) and thus, BF values below 1 support the alternative
180 hypothesis. Finally, for comparison purposes and following previous studies in *Heliconius*
181 (Jiggins et al. 2001b; Muñoz et al. 2010), we estimated mating preference using likelihood
182 (supplementary likelihood analysis).

183

184 *Color pattern models*

185

186 Color pattern models consisted of dissected wings of dead females and were used to test the
187 role of the color pattern preference of the males of *H. t. florencía* and *H. t.* subsp. nov.. A
188 single male of at least 8 days old of either *H. t. florencía* or *H. t.* subsp. nov. was presented
189 simultaneously with two female models, one being a control model (same race) and the
190 second, the experimental model, which could be either *H. m. malleti*, *H. c. cordula*, *H. t.*
191 *florencia* or *H. t.* subsp. nov. Both, the control and the experimental models were hanging
192 from a nylon string in the center of a spherical area (60 cm diameter) and gently shaken in
193 order to simulate real flying. We recorded the male response as approach (entered the sphere)
194 or courtship (fluttered towards the model) (Melo et al. 2009). In total, we tested 60 males of
195 *H. t. florencía* and 90 males of *H. t.* subsp. nov. and for each of them, we recorded a total of
196 20 approaches and courtships (Supplementary table 2).

197 In order to test the male response to the models we analyzed mate preference data using a
198 hierarchical random effects Bayesian model for count data, which accounts for variation at

199 both individual and population levels and has been recently implemented in ecology and
200 evolution studies that analyze count data (Merrill et al. 2011; Lee and Wagenmakers 2013;
201 Finkbeiner et al. 2014). In our model we estimated the rate π_j with which males of type j
202 approached or courted experimental over control wing models, thus being the key parameter
203 of interest. We assumed there is an overall preference μ of choosing the control wing model
204 over the experimental in any case, and also, that each male of *H. t. florencica* and *H. t. subsp.*
205 *nov.* has a preference for their control wing model type that comes from the distribution
206 centered around μ (supplementary figure 2). It was also assumed that there is between-
207 butterfly individual differences drawn from a distribution with mean π_j so that the i th butterfly
208 on the j th condition has a latent preference q_{ij} . Finally, we assumed that the number of times
209 the control type was chosen (y_{ij}) follows a binomial distribution out of a total of n_{ij} events.
210 Beta distributions were used to model the preferences π_j and q_{ij} . The graphical model
211 illustrating our Bayesian approach (supplementary figure 2) was implemented in JAGS
212 (Plummer 2003) as described above. We also calculated BFs (Kass and Raftery 1995) using
213 the Savage-Dickey approximation method (Lee and Wagenmakers 2013) to: (i) address
214 whether males of *H. t. florencica* and *H. t. subsp. nov.* have an actual preference for their
215 control wing pattern or if they choose a different wing pattern as frequently as their own and,
216 (ii) address whether pairs of group mean preferences were the same or different (see BF
217 interpretation above). Once more, for comparison purposes we estimated color pattern
218 preference using likelihood (supplementary likelihood analysis).

219

220 ANALYSIS OF POST-MATING ISOLATION

221

222 Using the successful matings obtained in the no-choice mating experiments, we calculated
223 hatching proportion as a measure of egg viability relative to control crosses. For this, once the
224 female was mated we isolated her in an individual insectary with food resources and host

225 plant for oviposition. Eggs were collected daily and larvae were reared individually. We
226 recorded the number of eggs laid and their hatching success. The data were analyzed with the
227 likelihood approximation implemented in BETABINO 1.1 (Jiggins et al. 2001a). Basically, a
228 betabinomial distribution is used with count data (number of eggs) to obtain the maximum
229 probability of observing an event (success of hatching) through different classes (category of
230 no-choice mating). This likelihood function considers the variability within replicates of the
231 same category and between different categories of no-choice matings. The program calculates
232 the maximum log-likelihood under four models considering variation in the hatching
233 proportion among classes. Likelihood ratio tests were used to differentiate among alternative
234 models with dissimilar number of parameters (Jiggins et al. 2001a; Naisbit 2002; Salazar et
235 al. 2005). With these experiments, we expect to observe intrinsic isolation barriers only
236 between the more divergent taxa (*H. timareta*-*H. cydno* and *H. melpomene*).

237

238 **Results**

239

240 **MATING EXPERIMENTS**

241

242 The Bayesian approach with hierarchical models used here allowed us to quantify uncertainty
243 of individual and population preferences that we had not been able to estimate using
244 likelihood methods alone that assume a single parameter to describe the preference of all
245 individuals (supplementary likelihood analysis). However, the results obtained by both
246 approaches were largely consistent.

247

248 *Conspecific comparisons*

249

250 In no-choice mating experiments we performed a total of 23 conspecific comparisons that
251 involved both *H. timareta* races and contrasted them against 105 control comparisons (Figure
252 2, supplementary Table 3). In trials involving ♀ *H. t. subsp. nov.* x ♂ *H. t. florencia*, the
253 frequency of successful mating was about half that of control matings (Figure 2, BF =
254 0.01384), while in trials with ♀ *H. t. florencia* x ♂ *H. t. subsp. nov.*, matings occurred in the
255 same proportion as controls (Figure 2, BF = 5.54426). Although it is clear that there is a
256 reduction of inter-population mating, these experiments had a small sample size due to
257 availability of specimens so it is not clear whether the asymmetric mating probability reflects
258 a biological reality. In wing model experiments, males of *H. t. florencia* and *H. t. subsp. nov.*
259 showed a similar preference strength in approaches and courtships (Figure 3). The males of
260 *H. t. florencia* discriminated against the wing models of *H. t. subsp. nov.* in approaches and in
261 courtships (Figure 3A). Similarly, when *H. t. subsp. nov.* males were exposed to wing
262 models, they preferred their own color pattern over that of *H. t. florencia* in approaches and in
263 courtships (Figure 3B). The model that best fits the no-choice conspecific mating experiments
264 consisted of three parameters (Pop. Sp. 1 in supplementary table 1; pD=5.7, DIC=26.3), being
265 better than the initial full model with a single mating probability (full in supplementary Table
266 1; pD=14.3, DIC=57.8). The first parameter grouped control crosses ($p_1=0.863$), the second
267 parameter only included the cross ♀ *H. t. florencia* x ♂ *H. t. subsp. nov.* ($p_3=0.810$) and the
268 last parameter consisted only of the cross ♀ *H. t. subsp. nov.* x ♂ *H. t. florencia* ($p_2=0.644$).
269 Overall, our results seem to support that incipient mating preferences are triggering RI
270 between *H. timareta* races.

271

272 *Heterospecific comparisons*

273

274 A total of 163 heterospecific comparisons were made and further compared with those of
 275 controls (supplementary Table 3). Both *H. t. florencica* and *H. t.* subsp. nov. preferred to mate
 276 with their own when tested against *H. m. malleti* ($BF=4.41 \times 10^{-13}$ - 4.35×10^{-6} ; Figure 2). The
 277 extent of such pre-zygotic isolation is similar to that observed between *H. m. malleti* and *H. c.*
 278 *cordula* ($BF= 1.27 \times 10^{-5}$ - 4.09×10^{-3} ; Figure 2) and between other *H. cydno* and *H. melpomene*
 279 races studied previously (Naisbit et al. 2001). Similarly, matings between the more closely
 280 related *H. timareta* races and *H. c. cordula* were as frequent as those of their controls (BF
 281 $=2.29 - 7.31$, Figure 2), except for ♀ *H. t.* subsp. nov. x ♂ *H. c. cordula* whose mating was
 282 only 35.5% as likely ($BF= 4 \times 10^{-3} - 0.18$, Figure 2). The males of *H. t. florencica* discriminated
 283 against the wing models of *H. c. cordula* in approaches and courtships but failed to
 284 differentiate models of *H. m. malleti*, that display their same wing phenotype (Figure 3A).
 285 This suggests that the presence of red wing elements, and in general the color pattern, plays a
 286 major role in mate discrimination in *H. t. florencica*. Similarly, when *H. t.* subsp. nov. males
 287 were exposed to wing models, they preferred their own color pattern over that of *H. m.*
 288 *malleti* and *H. c. cordula* when approaching and courting (Figure 3B) indicating that initial
 289 recognition of color pattern helps identifying possible mates and other factors likely of
 290 chemical nature, determine the success of a mating in *H. t.* subsp. nov.. For heterospecific no-
 291 choice experiments, an initial full model with a single mating probability (supplementary
 292 Table 1) was established across all trials ($pD=14.3$, $DIC = 57.8$). To test different hypotheses,
 293 the Bayesian hierarchical model was fitted in a stepwise manner by adding parameters to the
 294 initial model. When mating probabilities were estimated in a model of four parameters
 295 separating (i) control crosses, (ii) crosses involving *H. m. malleti* females, (iii) crosses with *H.*
 296 *timareta* females and (iv) crosses involving *H. c. cordula* females, this led to a significant
 297 improvement in the fitting of the model ($pD=13.6$, $DIC=56.3$, Pop. Sp. 2 in supplementary
 298 Table 1). This possibly reflects different mating preferences of females from different species,
 299 mostly females of *H. m. malleti* which are highly selective ($p_2=0.091$).

300

301 **ANALYSIS OF POST-MATING ISOLATION**

302

303 The likelihood model that better explained the observed hatching proportions was that of a
304 common mean with different variances (mvvv; $G_{17}=33.19$; $p=0.0106$). None of the
305 heterospecific crosses involving either race of *H. timareta* and/or *H. c. cordula* showed
306 significant differences in egg hatching as compared to the control crosses (Table 2; $G_9=13.6$;
307 $p=0.13$). F_1 hybrid males were always fertile ($G_7=9.22$; $p=0.23$; Table 2) while F_1 hybrid
308 females showed significant reduction in their hatching proportions when compared to those of
309 control, conspecific or heterospecific crosses ($G_7=15.87$; $p=0.026$ and $G_9=18.89$; $p=0.0261$,
310 respectively). Interestingly, this significant reduction seems to be due only to F_1 hybrid
311 females from the cross ♀ *H. m. malleti* × ♂ *H. t. florenci*, as when they were removed from
312 the comparisons, the remaining F_1 females (that is, the ones resulting from the crosses ♀ *H. t.*
313 *florenci* × ♂ *H. t.* subsp. nov., ♀ *H. t.* subsp. nov. × ♂ *H. t. florenci* and ♀ *H. c. cordula* × ♂
314 *H. t.* subsp. nov.) did not show any signal of egg inviability ($G_6=8.828$; $P=0.1835$ in the
315 comparison with control crosses and $G_8=12.509$; $P=0.1299$ in the comparison with
316 heterospecific crosses). Consistently, when females from the reciprocal cross (♀ *H. t.*
317 *florenci* × ♂ *H. m. melpomene*) were tested, none of the eggs they laid hatched (Table 1).
318 These results indicate that there is no post-zygotic isolation between *H. cydno* and *H.*
319 *timareta*, but there is between *H. melpomene* and *H. timareta*, consistent with previously
320 observed crosses between other races of *H. melpomene* and *H. cydno* (Jiggins et al. 2001a;
321 Naisbit 2002; Salazar et al. 2005).

322 **Discussion**

323

324 Recently there have been studies documenting adaptive introgression and hybrid speciation in
325 animals (Mavarez et al. 2006; Schwarz et al. 2007; Anderson et al. 2009; Green et al. 2010;

326 Hermansen et al. 2011; Song et al. 2011; Consortium 2012; Pardo-Diaz et al. 2012; Hedrick
327 2013; Mendez et al. 2013; Clarkson et al. 2014; Lucek et al. 2014; Norris et al. 2015)
328 however, few have experimentally shown whether introgression directly affects adaptation
329 and/or leads to speciation when the hybrid and parents are not temporarily and/or spatially
330 separated (Schwander et al. 2008; Melo et al. 2009; Schumer et al. 2014; Selz et al. 2014). In
331 particular, the potential of adaptive introgression to promote RI in animal systems remains a
332 largely unexplored question (Schumer et al. 2014).

333

334 An important requirement to address this question is having an animal system where adaptive
335 introgression occurred recently to assess whether it is triggering RI between the forms of the
336 newly formed polymorphic population. To our knowledge only few cases have investigated
337 the contribution of recent hybridization to RI. Four studies showed that hybrids prefer to mate
338 with themselves rather than with the parental species (Doherty and Gerhardt 1983; Melo et al.
339 2009; Segura et al. 2011; Selz et al. 2014) however, three out of those four cases namely
340 *Anastrepha* flies, cichlid fishes and *Hyla* frogs, tested preference in F₁ artificial hybrids that
341 do not occur in nature.

342

343 In *Heliconius*, *H. timareta* has recently acquired wing pattern elements by hybridizing with *H.*
344 *melpomene* (Consortium 2012; Pardo-Diaz et al. 2012). This gene sharing allowed the
345 diversification of *H. timareta* across the east of the Andes by allowing it to enter mimetic
346 rings already established between *H. melpomene* and *H. erato*. The present study shows that
347 besides the intrinsic adaptive value of the novel mimetic and aposematic wing coloration in
348 *H. timareta*, the introgression of this trait into this species contributes to some degree of
349 incipient RI.

350

351 In the south east of the Colombian Andes the introgression of the ‘dennis-ray’ pattern from *H.*
352 *m. malleti* into the ancestor of *H. timareta* led to the diversification of this species, resulting

353 in the co-existence of the races *H. t. florencía* ('dennis-ray') and *H. t.* subsp. nov. that,
354 according to our data, are developing incipient assortative mating based on presence/absence
355 of the 'dennis-ray'. Specifically, we observed a reduction in mating frequency in no-choice
356 experiments between *H. timareta* races. This reduction can be due to several behavioral and
357 ecological factors, but is likely mostly explained by the fact that females of *H. t.* subsp. nov.
358 and males of *H. t. florencía* are less prone to mate with each other. Furthermore, males of
359 both *H. t.* subsp. nov. and *H. t. florencía* approached and courted wing models of the other
360 subspecies substantially less than those of their own. Thus, it seems that mating success is
361 largely due to the males' color pattern preference. However, females of *H. t. florencía* and
362 males of *H. t.* subsp. nov. mated with each other despite the choosiness showed by these
363 males in wing model experiments. This may be explained by the nature of no-choice
364 experiments, which simulate natural situations of one to one encounters in the field, and
365 measure reluctance but not choice. Thus, males of *H. t.* subsp. nov. prefer females of their
366 own if they are given the choice but, when that is not the case, they are opportunistic and
367 mate with *H. t. florencía*.

368

369 In agreement with the incipient RI detected in our experiments, we have collected hybrids
370 between *H. t. florencía* and *H. t.* subsp. nov. in the wild (~3%; 5 out of 150 individuals
371 sampled, that have a broader forewing band typical from *H. t.* subsp. nov. and 'dennis-ray'
372 from *H. t. florencía*). However, this value is likely an underestimation. Given the dominant
373 inheritance of the 'dennis-ray' phenotype, F₁ hybrids between these races will look
374 phenotypically identical to *H. t. florencía* and can be mistakenly classified as 'pure'. This is
375 indeed likely, as some wild-caught *H. t. florencía* females have produced offspring with both
376 rayed and non-rayed phenotypes (Linares pers. comm.). Finally, a comprehensive sampling
377 across the zone of contact has not been possible due to political instability. In the light of the
378 lack of evidence for the extent of hybridization in the wild and that the degree of RI between
379 these morphs is likely insufficient to merit species status (and they may not necessarily

380 diverge into good species), we prefer to think of this study as an example of what may happen
381 during early stages of hybrid trait speciation *sensu* Jiggins et al. (2008).

382

383 On the other hand, when we examined mating behavior involving comparisons between *H.*
384 *timareta* with other species, interesting observations emerged. First, matings between females
385 of *H. t.* subsp. nov. and males of the closely related taxon *H. c. cordula* were infrequent
386 (Figure 2), despite these two species have a very similar wing coloration (mainly
387 differentiated by the presence of iridescence and brown hind wing forceps in *H. c. cordula*;
388 Figure 1). This RI may be the result of both female and male choice. Males may be using
389 iridescence as a mating cue. We observed that although males of *H. t.* subsp. nov. (non-
390 iridescent) approach wing models of *H. c. cordula* (iridescent), they avoid courting them
391 (Figure 3). In addition, as *Heliconius* females have odor receptors (Briscoe et al. 2013) and
392 the males produce sex pheromones (Vanjari pers. comm.), females of *H. t.* subsp. nov. may be
393 recognizing their conspecifics males from those of *H. c. cordula* using chemical cues,
394 although this remains untested. However, this isolation is asymmetrical as *H. c. cordula*
395 females mate freely with *H. t.* subsp. nov. males (Figure 2). Second, the pre-mating isolation
396 between *H. timareta* and *H. melpomene* is strong and mediated by color and, perhaps,
397 chemical cues. Females of *H. t.* subsp. nov. almost never mated males of *H. m. malleti* (only 1
398 successful cross in 30 attempts) and the reciprocal cross never occurred in our experiments
399 (Figure 2), perhaps explained by the differences in color pattern between these species. In
400 consequence, males of *H. t.* subsp. nov. approached and courted wing models of *H. m. malleti*
401 in less than 30% of the trials (Figure 3). In contrast, phenotypically identical co-mimics *H. t.*
402 *florencia* and *H. m. malleti* were strongly assortative, but did mate more frequently than the
403 non-mimetic pair (less than 20%; Figure 2 and (Giraldo et al. 2008)). Furthermore, males of
404 *H. t. florencia* approached and courted wing models of *H. m. malleti* as much as theirs (Figure
405 3). This suggests that recognition is likely primarily based on pheromones. There is evidence
406 supporting this, as males of *H. t. florencia* and *H. m. malleti* are known to produce different

407 pheromone blends (Vanjari pers. comm.). The isolation we found between *H. timareta* and *H.*
408 *melpomene* is also consistent with previous studies that found that interspecific crosses
409 between *H. cydno* (closely related to *H. timareta*) and *H. melpomene*, are highly infrequent
410 (Jiggins et al. 2001b; Mavarez et al. 2006).

411

412 There was no egg inviability in F₁ individuals from crosses between *H. cydno* and *H. timareta*
413 or between *H. timareta* races, while eggs laid by F₁ female hybrids between any race of *H.*
414 *timareta* and *H. melpomene* always failed to hatch (Table 1), a result also observed between
415 *H. cydno* and *H. melpomene* (Naisbit 2002). These results are consistent with the idea that the
416 early stages of speciation are driven by divergent ecological or sexual selection, with intrinsic
417 postzygotic isolation arising later in the speciation continuum (Seehausen et al. 2014). Here,
418 races of the same species (*H. t.* subsp. nov. and *H. t. florencica*) show incipient mating
419 preference, closely related species (*H. timareta* – *H. cydno*) have stronger assortative mating
420 without intrinsic genetic incompatibilities and, finally, more distant species (*H. timareta/H.*
421 *cydno* – *H. melpomene*) have developed both prezygotic and postzygotic isolation.

422

423 The presence of prezygotic isolation barriers in early stages of speciation has also has been
424 documented between sister taxa of recent origin such as *Pundamilia* cichlids (Seehausen
425 2009), *Littorina* ecotypes (Conde-Padín et al. 2008; Saura et al. 2011), races of *Rhagoletis*
426 (Powell et al. 2014), *Ophrys* spp. orchids (Scopece et al. 2007), *Haplochromine* cichlids
427 (Stelkens et al. 2010) and other *Heliconius* butterflies (Merrill et al. 2011). However, in none
428 of those cases RI resulted as consequence of an introgressed trait. Additionally, our study is
429 one of the few documenting the subsequent evolution of intrinsic postzygotic barriers in later
430 stages of speciation (although see (Naisbit 2002; Stelkens et al. 2010; Merrill et al. 2011)).

431

432 In summary, we have confirmed that *H. timareta* is a taxon more closely related to *H. cydno*,
433 but that the introgression of red color wing elements from *H. melpomene* has contributed to

434 the divergence between *H. t. florencía* and *H. t.* subsp. nov. through the development of
 435 incipient assortative mating. We do not know whether this incipient mate recognition will
 436 lead to the formation of two different species but, at present, this case reflects the potential of
 437 adaptive introgression to promote and facilitate hybrid trait speciation.

438

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440

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448

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653

654 **Figure legends**

655

656 **Figure 1.** Geographic distribution and phenotypes of *H. timareta* across South America. The
657 species *H. m. malleti* and *H. c. cordula* are also depicted. The distributions of taxa are
658 estimated from locality data compiled by Neil Rosser and Claire Merot (Rosser et al. 2012;
659 Merot et al. 2013). Circles represent possible (but not confirmed) contact zones. Background
660 map image was downloaded from ETOPO (Amante and Eakins 2009).

661

662 **Figure 2.** Mating frequency in no-choice mating experiments with virgin adult females. *Tn*:
663 *H. timareta* subsp. nov.; *Tf*: *H. timareta florenciana*; *Cc*: *H. cydno cordula*; *Mm*: *H. m. malleti*.
664 Cross type is specified as *female x male*. Error bars represent 95% credible interval of the
665 posterior distribution.

666

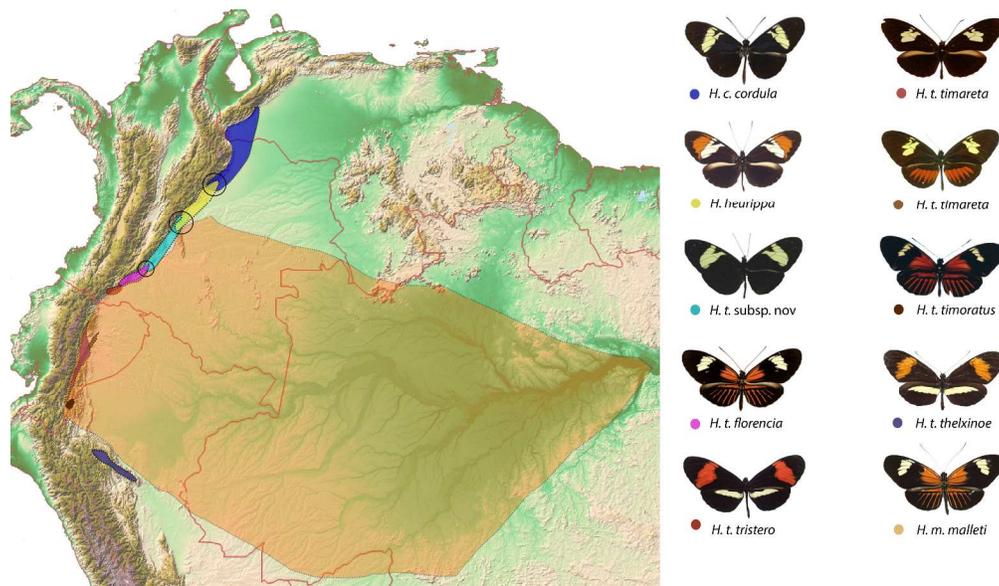
667 **Figure 3.** Violin plots showing the entire posterior distribution of the approaches and
668 courtships of males of (A) *H. t. florenciana* and (B) *H. timareta* subsp. nov. (depicted at the top
669 of each panel) to female wing models of *H. m. malleti*, *H. c. cordula*, *H. t.* subsp. nov. and/or
670 *H. t. florenciana* (bottom of each panel). The y-axis corresponds to the preference towards the
671 experimental model π_j . Values above 0.5 suggest preference for the own pattern while those
672 below 0.5 suggest preference for the experimental model.

673 **Table 1.** Proportion of viable eggs in control (same races), conspecific (same species, different race), heterospecific and F₁ crosses

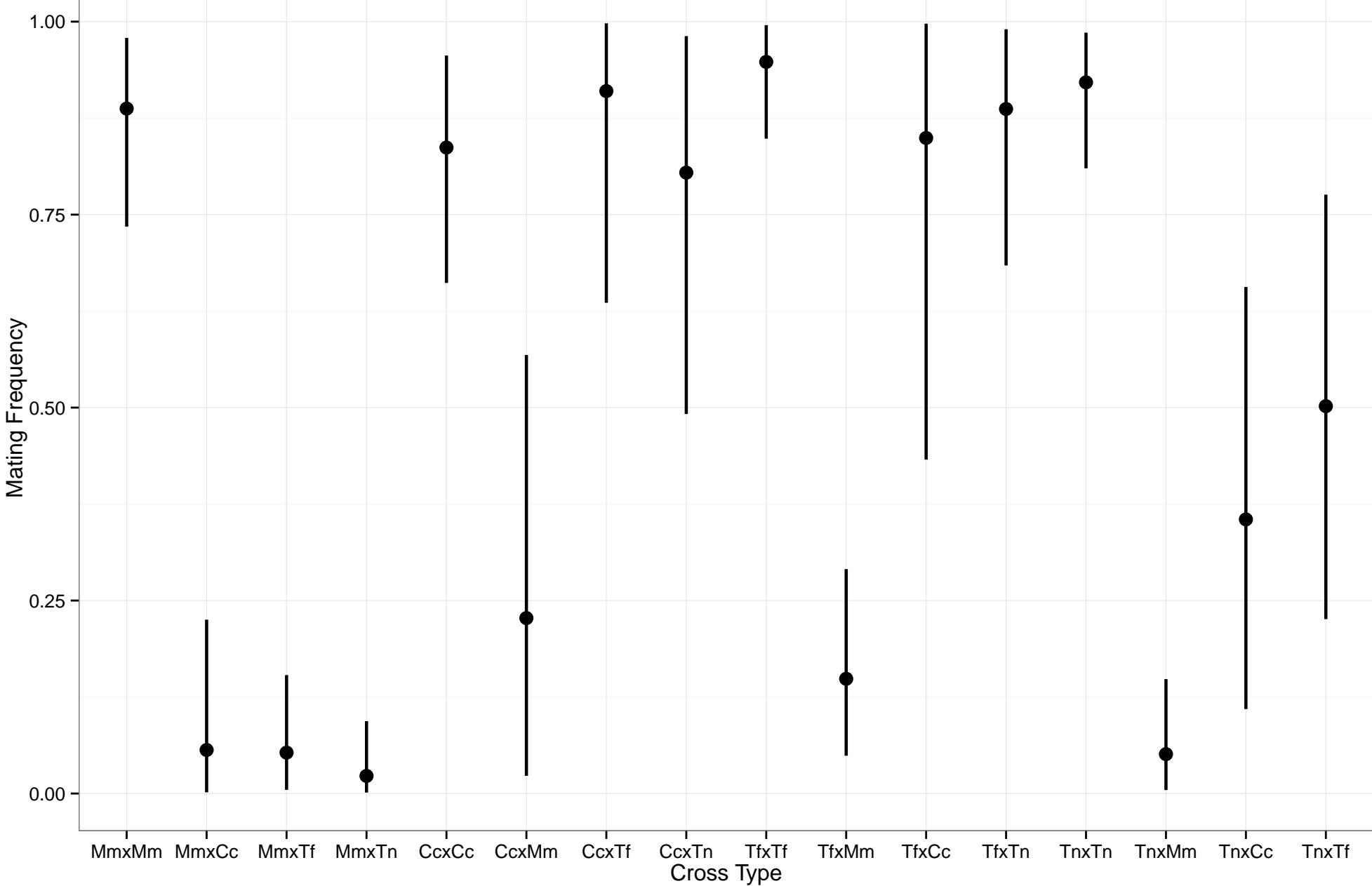
Cross type (♀ genotype x ♂ genotype)				No. of broods	No. of eggs	Proportion of viable eggs	SE	Variance	SE
Control	<i>Tn</i>	<i>x</i>	<i>Tn</i>	4	288	0.63	0.07	0.01	0.01
	<i>Tf</i>	<i>x</i>	<i>Tf</i>	4	111	0.76	0.07	0.15	0.01
	<i>Cc</i>	<i>x</i>	<i>Cc</i>	29	1377	0.64	0.05	0.06	0.01
	<i>Mm</i>	<i>x</i>	<i>Mm</i>	4	103	0.60	0.13	0.05	0.04
Conspecific	<i>Tf</i>	<i>x</i>	<i>Tn</i>	3	210	0.70	0.03	0.0001	--
	<i>Tn</i>	<i>x</i>	<i>Tf</i>	4	192	0.55	0.05	0.003	0.006
Heterospecific	<i>Cc</i>	<i>x</i>	<i>Tn</i>	3	236	0.53	0.04	0.003	0.005
	<i>Tn</i>	<i>x</i>	<i>Cc</i>	3	138	0.61	0.04	0.0001	--
	<i>Tn</i>	<i>x</i>	<i>Mm</i>	5	485	0.51	0.06	0.01	0.01
	<i>Mm</i>	<i>x</i>	<i>Tf</i>	2	83	0.43	0.18	0.06	0.04
	<i>Mm</i>	<i>x</i>	<i>Tn</i>	0	--	--	--	--	--
F ₁	<i>[Tf x Tn]</i>	<i>x</i>	<i>Tn/Tf</i>	5	390	0.53	0.02	0.0001	--
	<i>[Tn x Tf]</i>	<i>x</i>	<i>Tn/Tf</i>	6	375	0.60	0.06	0.01	0.01
	<i>Tf</i>	<i>x</i>	<i>[Tf x Tn]</i>	1	43	0.64	0.07	0.0001	--
	<i>Tn</i>	<i>x</i>	<i>[Tn x Tf]</i>	4	261	0.55	0.03	0.0001	0.003

<i>[Cc x Tn]</i>	x	<i>Cc/Tn</i>	4	331	0.59	0.02	0.0001	0.002
<i>[Mm x Tf]</i>	x	<i>Mm</i>	2	70	0.28	0.05	0.0001	--
<i>[Tf x Mm]</i>	x	<i>Tf</i>	2	80	0	--	--	--
<i>[Tn x Mm]</i>	x	<i>Mm/Tn</i>	12	816	0	--	--	--
<i>Mm</i>	x	<i>[Mm x Tf]</i>	2	106	0.64	0.20	0.10	0.06
<i>Tn/Mm</i>	x	<i>[Tn x Mm]</i>	8	970	0.41	0.05	0.02	0.01

674 Crosses are specified as female genotype x male genotype. The symbol (/) means or.



Geographic distribution and phenotypes of *H. timareta* across South America. The species *H. m. malleti* and *H. c. cordula* are also depicted. The distributions of taxa are estimated from locality data compiled by Neil Rosser and Claire Merot (Rosser et al. 2012; Merot et al. 2013). Circles represent possible (but not confirmed) contact zones. Background map image was downloaded from ETOPO (Amante and Eakins 2009).
301x175mm (300 x 300 DPI)





*H. timareta
florencia*



*H. timareta
subsp. nov.*

