Genetic dissection of assortative mating behavior

Richard M. Merrill [1,2,3,*], Pasi Rastas[2], Simon H. Martin[2], Maria C. Melo[3,4], Sarah Barker[2], John Davey[2,5], W. Owen McMillan[3] & Chris D. Jiggins[2]

1 Division of Evolutionary Biology, Ludwig-Maximilians-Universität, München, Germany **2** Department of Zoology, University of Cambridge, Cambridge, UK **3** Smithsonian Tropical Research Institute, Panama City, Panama **4** IST Austria, Klosterburg, Austria **5** Department of Biology, University of York, York, UK

*merrill@bio.lmu.de

Abstract

The evolution of new species is made easier when traits under divergent ecological selection are also mating cues. Such ecological mating cues are now considered more common than previously thought, but we still know little about the genetic changes underlying their evolution, or more generally about the genetic basis for assortative mating behaviors. Both tight physical linkage and the existence of large effect preference loci will strengthen genetic associations between behavioral and ecological barriers, promoting the evolution of assortative mating. The warning patterns of *Heliconius melpomene* and *H. cydno* are under disruptive selection due to increased predation of non-mimetic hybrids, and are used during mate recognition. We carried out a genome-wide quantitative trait locus (QTL) analysis of preference behaviors between these species and showed that divergent male preference has a simple genetic basis. We identify three QTLs that together explain a large proportion (~60%) of the differences in preference behavior observed between the parental species. One of these QTLs is just 1.2 (0-4.8) cM from the major color pattern gene *optix*, and, individually, all three have a large effect on the preference phenotype. Genomic divergence between *H. cydno* and *H. melpomene* is high but broadly heterogenous, and admixture is reduced at the preference-*optix* color pattern locus, but not the other preference QTL. The simple genetic architecture we reveal will facilitate the evolution and maintenance of new species despite on-going gene flow by coupling behavioral and ecological aspects of reproductive isolation.

Introduction

During ecological speciation, reproductive isolation evolves as a result of divergent natural selection [1]. Although ecological barriers can reduce gene flow between divergent populations, speciation normally requires the evolution of assortative mating [1,2]. This is made easier if traits under divergent ecological selection also contribute to assortative mating, as this couples ecological and behavioral barriers [3–6]. Ecologically relevant mating cues (sometimes known as 'magic traits' [2,6]) are now predicted to be widespread in nature [6,7], and the last few years have seen considerable progress in our understanding of their genetic basis. For example, studies have explored the genetic basis of beak shape in Darwin's finches [8], body shape in sticklebacks [9,10], cuticular hydrocarbons in *Drosophila* [11], and wing patterns in *Heliconius* butterflies [12–14]. However, the extent to which these traits contribute to assortative mating depends on the evolution of corresponding preference behaviors, and the underlying genetic architecture.

We still know little about the process by which ecological traits are co-opted as mating cues, and in particular, how matching cues *and* preference behaviors are controlled genetically (but see [15]). Both the substitution of large effect preference alleles, and physical linkage will strengthen linkage disequilibrium ('LD', *i.e.* the non-random association of alleles at different loci [16]) between cue and preference. Strong LD between barrier loci is expected to both maintain and facilitate the evolution of new species in the face of gene flow. This is the result of two key, but related processes. First, LD between barrier loci will result in the coupling of barrier effects, and where these effects coincide the overall barrier to gene flow is increased [4,16]. Second, LD between pre- and post-mating barrier loci will facilitate an increase in premating isolation in response to selection against hybridization (*i.e.*

reinforcement, *sensu* [18]), by transferring the effects of selection from the latter to the former [19].

In central Panama, the butterfly *Heliconius melpomene rosina* is a precise Müllerian mimic of *H. erato* and normally occurs in forest-edge habitats, whereas the closely related species *H. cydno chioneus* mimics *H. sapho* and is more common in closed-forest habitats, although *H. melpomene* and *H. cydno* are often seen flying together (Fig. 1a & b) [20]. Hybrids are viable but occur at very low frequency in the wild (estimated at ~0.1%), consistent with strong assortative mating shown in insectary experiments. Specifically, heterospecific mating was not observed in 50 choice and no-choice trials between Panamanian *H. melpomene* and *H. cydno* ([21,22] ; see also [23]).

The amenability of *Heliconius* color patterns to experimental manipulation has led to the demonstration that color pattern is both under strong disruptive selection due to predation [24], and also that males prefer live females and paper models with the same color pattern as themselves [24]. These results led Servedio and colleagues [6] to conclude that, unlike other putative examples, both criteria for a magic trait have been confirmed with manipulative experiments in *H. melpomene rosina* and *H. cydno chioneus*. Although female preferences undoubtedly contribute to assortative mating [25–27], male preferences act first in these species such that strong observed male discrimination against heterospecific females will have a disproportionate contribution to overall reproductive isolation [28]. As highlighted by Coyne and Orr [29], the order in which reproductive isolation acts influences their relative contribution to overall isolation. In this case, the ordering of behavioral decisions is likely predetermined by their sensory systems: *Heliconius* lack specialized olfactory structures to support long range detection of chemical signals,

103 so are only likely to use these in close proximity, whereas they have very good long-
104 range vision [30]. As such, not only is male preference in *Heliconius* butterflies
105 experimentally more tractable than other components of behavioral isolation, it is also
106 an important barrier to gene flow.

107 Crossing experiments have shown that the shift in mimetic warning pattern
108 between *H. melpomene rosina* and *H. cydno chioneus* is largely controlled by just
109 three major effect loci [31]. Genes underlying these loci have now been identified: the
110 transcription factor *optix* controls red patterns [12], the *WntA* gene controls forewing
111 band shape [13] and yellow patterns map to the gene *cortex* [14]. In addition, a
112 further locus, *K*, segregates in crosses between *H. melpomene rosina* and *H. cydno*
113 *chioneus* with more minor effect [31]. Further modularity occurs within these loci. For
114 example, different regulatory elements of *optix* each result in distinct red pattern
115 elements [32]. The modular nature of individual color pattern loci and their
116 functionally sufficient enhancers means that they can be combined to produce
117 considerable phenotypic diversity [32,33]. These loci are large-effect 'speciation
118 genes', in that they control traits that generate strong reproductive isolation [34].

119 Two of these color pattern loci, *optix* and *K,* have previously been associated
120 with *Heliconius* courtship behaviors [25,35,36]; however, these studies do not provide
121 evidence for tight physical linkage (<20cM) between warning pattern and preference
122 loci. Our own previous study tested for an association between Mendelian color
123 pattern loci and preference behaviors [25], but did not correct for the segregation of
124 alleles across the genome, so that reported levels of support are likely inflated [37];
125 and an earlier study of the parapatric *taxa H.* cydno *and H. pachinus* [35] is limited by
126 small sample size [37]. Regardless of the level of statistical support for preference
127 QTL, these studies both lack the resolution to demonstrate the degree of tight

128 physical linkage between loci contributing to reproductive isolation that would be
129 expected to aid speciation. Perhaps the best evidence comes a study of wild *H.*
130 *cydno alithea* [36]. This population is polymorphic for a yellow or white forewing (due
131 to the segregation of alleles at the *K* locus), and males with a yellow forewing prefer
132 yellow females. These results are important because they suggest a key component
133 of speciation: Specifically, coupling between potential behavioral and ecological
134 barriers. However, because they rely on segregation within a wild population, rather
135 than laboratory crosses, it is not possible to distinguish physical linkage from genetic
136 associations between cue and preference alleles due to non-random mating. The
137 extent to which warning pattern and behavioral loci are physically linked in
138 *Heliconius*, as well as the existence of major preference loci elsewhere in the
139 genome remains unknown. To address this, and to complement our extensive
140 knowledge of the genetics of their color pattern cues, here we use a genome-wide
141 quantitative trait locus (QTL) approach to explore the genetics of male preference
142 behaviors between the sympatric species *H. melpomene rosina* and *H. cydno*
143 *chioneus*.
144
145
146 **Results**
147 We studied male mating preference among F1 and backcross hybrid families
148 between *H. melpomene rosina* and *H. cydno chioneus*, in standardized choice trials
149 [25,38] (Figs. 1 and S1). We introduced individual males into an experimental cage
150 and recorded courtship directed towards two virgin females, one of each species. In
151 total, we collected data from 1347 behavioral trials, across 292 individuals. Multiple

152 trials were performed for each backcross male, from which we determined the
153 relative courtship time directed towards *H. melpomene* and *H. cydno* females.
154
155 **Three loci contribute to species differences in preference behavior.** As reported
156 previously [25], F1 males have a strong preference for the red *H. melpomene*
157 females, and little segregation in mate preference is observed among the backcross
158 to *melpomene* (and whose mean preference does not differ significantly from that of
159 pure *H. melpomene* males: $2\Delta \ln L = 1.33$, $d.f. = 1$, $P > 0.2$), implying that *melpomene*
160 mate preference alleles are dominant. In contrast, courtship behavior segregates
161 among *H. cydno* backcross males, permitting analysis of the genetic basis for this
162 mating behavior (Fig. 1C). Consequently, all subsequent analyses were performed
163 on backcross to *cydno* males. We used a genome-wide quantitative trait locus (QTL)
164 mapping approach to identify the genomic regions underlying divergence in mate
165 attraction. Linkage maps were constructed from genotype data of 331 backcross-to-
166 cydno individuals and their associated parents [39], including 146 individual males for
167 which we had recorded attraction behaviors.
168 We identified three unlinked QTLs on chromosomes 1, 17 and 18 associated
169 with variation in the relative amount of time males spent courting red *H. melpomene*
170 and white *H. cydno* females (Fig. 2A). Of these, one is tightly linked to the *optix* locus
171 on chromosome 18, which controls the presence/absence of a red forewing band.
172 Specifically, the QTL peak for the behavioral QTL on chromosome 18 (at 0cM) is just
173 1.2cM from *optix.* The associated 1.5-LOD support interval is between 0 and 6.0cM,
174 suggesting that the true location of the QTL is no more than 4.8cM from the *optix*
175 coding region (whose genetic position is at 1.2cM) (Fig. 3); however, given that the
176 peak support (i.*e.* highest LOD score) for our behavioral QTL is at 0cm and that this

177 rapidly drops off, physical linkage between wing patterning cue and preference loci is
178 likely much tighter than a strict 1.5-LOD interval might suggest. In contrast, the QTL
179 on chromosome 1 is at least 30cM from the gene *wingless*, which although unlikely to
180 be a color pattern gene itself has previously been associated with the *K* wing pattern
181 locus between taxa within the *cydno* clade [35]. No known wing pattern loci reside on
182 chromosome 17 and this chromosome does not explain any of the pattern variation
183 segregating in our BC pedigrees (Merrill, unpublished data).
184 Modeling supports additive effects of all three detected loci (Table 1), and in
185 our mapping population these three QTLs together explain ~60% of the difference in
186 male preference behavior between the parental species (Fig. 2B). Given the sample
187 sizes feasible in *Heliconius*, our analysis lacks the power to resolve smaller effect
188 QTLs. We also found no evidence of pairwise interactions between individual QTLs
189 in our model of relative courtship time, which again is unsurprising given achievable
190 sample sizes. However, genome scans considering individuals with alternative
191 genotypes at the QTL on chromosome 18 separately revealed a significant QTL on
192 chromosome 17 (LOD = 3.52, P=0.016) for heterozygous (i.e. LG18@0cM =
193 CYD:MEL), but not for homozygous (i.e. LG18@0cM = CYD:CYD) males (Fig. S2),
194 though this result is not supported by non-parametric interval mapping (LOD = 2.4,
195 P=0.132). Nevertheless, these results perhaps suggest that alleles on chromosomes
196 17 and 18, or the specific behaviors they influence, may interact.
197
198 **Preference QTL are of large effect.** Individually, the measured effect of each of the
199 three QTLs we identified was large, explaining between 23 and 31% of the difference
200 between males of two parental species (Fig. 2B). However, in studies with relatively
201 small sample sizes such as ours (n = 139), estimated effects of QTL are routinely

202 over-estimated (a phenomenon known as the “Beavis effect”, after [40]). This is
203 because effect sizes are determined only after significance has been determined,
204 and QTL with artificially high effect sizes – due to variation in sampling – are more
205 likely to achieve ‘significance’.

206 To determine the extent to which the effects of our QTL may be over-
207 estimated, we simulated QTL across a range of effect sizes, and compared the
208 distribution of measured effects for all simulations to those which would be significant
209 in our analysis (Fig. S3). Our simulations suggest that the reported effects of our QTL
210 are not greatly over-estimated. We first considered what proportion of ‘significant’
211 simulations would be smaller than our empirically measured effects (Fig. 4A). A
212 highly conservative threshold of 95% would suggest that the QTL on chromosome 1
213 and 18 explain at least 10% and 20% of the difference in behavior between the
214 parental species, respectively. Adopting the median values, our simulations would
215 suggest true effects of 25%, 15% and 30%, or greater, for the QTL on chromosomes
216 1, 17 and 18, respectively. Given simulated effect sizes similar to those measured
217 empirically, there was little bias among simulation runs that achieved the genome-
218 wide significance threshold (Fig. S3). This suggests that the true effect sizes of our
219 QTL are likely to be large, with somewhat less support for the QTL on chromosome
220 17.

221 Although our simulations suggest the effects we have measured are
222 reasonable, ideally we would estimate effect sizes from a population of individuals
223 that were not used to determine significance. In evolutionary biology, follow-up
224 experiments such as this are uncommon; collecting phenotypic data across a large
225 number of hybrid individuals is often a considerable undertaking, and this is similarly
226 true for *Heliconius* behaviors. Nevertheless, we were able to follow-up our results for

227 the QTL on chromosome 18, using a sample of a further 35 backcross males for
228 which preference behavior was measured, but for which we were unable to generate
229 genotype data (and so were not included in our initial QTL analysis). As reported
230 above, the QTL peak (at 0cM) on chromosome 18 is in very tight linkage with the
231 *optix* color pattern locus (at 1.2cM), which controls the presence and absence of the
232 red forewing band. Presence of the red forewing band is dominant over its absence,
233 so that segregation of the red forewing band can be used to perfectly infer genotype
234 at the *optix* locus, even without sequence data. This analysis supports our previous
235 result that the QTL on linkage group 18 is of large effect (Fig. 4B): among these 35
236 hybrid males, the *optix* locus explains 27% of the difference in behavior between the
237 parental species (*c.f.* 31% for the larger mapping population).
238
239 **Admixture is reduced at the preference-color pattern locus on chromosome 18.**
240 To consider the effects of major color pattern cue and preference loci on localized
241 gene flow across the genome we used the summary statistic f_d to quantify admixture
242 between *H. cydno chioneus* and *H. melpomene rosina* (Fig. 3 and S4). f_d is based on
243 the so-called ABBA-BABA test and provides a normalized measure that
244 approximates the proportional effective migration rate (*i.e.* $f_d = 0$, implies no localized
245 migration of alleles, whereas $f_d = 1$, implies complete localized migration of alleles)
246 [41,42]. At the physical location of our behavioral QTL on chromosome 18, which is
247 in tight linkage with the *optix* color pattern locus, there is a substantial reduction in
248 admixture across a ~1 megabase region. At our other two QTLs, reduced f_d values
249 (<0.1) are observed for individual 100kb windows associated with all behavioral QTL
250 (specifically, within the 1.5-LOD intervals); but, this is true for many sites across the
251 genome. In addition to mating behavior these two species differ among a number of

252 other behavioral and ecological axes and genomic divergence is highly
253 heterogenous.
254
255 **Different preference QTL affect different aspects of behavior.** The male
256 preference QTLs we have identified may influence differences in male attraction
257 towards red *H. melpomene* females, or white *H. cydno* females, or towards both
258 female types. To further explore the influence of segregating alleles at these loci we
259 considered the influence of all three QTLs on courtships directed towards each
260 female type separately (Fig. 5). We have already robustly established a significant
261 effect of these loci on variation in the relative amount of time males spent courting
262 each female type (see Fig. 2A). Consequently, although we corrected for multiple
263 testing arising from considering three QTL across the two data sets [37], in these
264 *post-hoc* analyses we did not account for multiple segregating loci across the entire
265 genome (in contrast to the results reported above). This greatly increases our power
266 to detect any influence of the QTLs on attraction towards the two species individually,
267 but also increases the likelihood of false positives. The QTL on chromosome 1
268 influenced the number of courtships directed towards *H. cydno* females ($F_{1,145}$ =
269 10.85, $P < 0.01$), but had no significant effect on how males behaved towards *H.*
270 *melpomene* females ($F_{1,145}$ = 1.35, $P > 0.2$). In contrast, the QTL on chromosome 17
271 influenced the degree of courtship directed towards *H. melpomene* ($F_{1,145}$ = 10.08, P
272 = 0.011), but not *H. cydno* females ($F_{1,145}$ = 0.41, $P > 0.2$). Similarly, the QTL on
273 chromosome 18 had a significant effect on courtships directed towards *H.*
274 *melpomene* ($F_{1,145}$ = 9.93, P = 0.012) females (though we note that prior to Bonferroni
275 correction there is also some support for an effect on courtships directed towards *H.*
276 *cydno* females: $F_{1,145}$ = 6.56, P = 0.01).

Discussion

Here, we reveal a genetic architecture that will strengthen genetic associations (*i.e.* LD) between key components of reproductive isolation, and so facilitate ecological speciation in the face of gene flow. Specifically, we demonstrate that just three QTLs are largely responsible for an important component of behavioral isolation between two sympatric species of *Heliconius* butterfly. One of these resides only 1.2 (0-4.8) cM from a major color pattern gene. Our results also suggest that all three preference loci are of large phenotypic effect. Because LD between cue and preference loci will arise as a natural consequence of mating preferences [43], these large effect preference loci will further increase LD between ecological and behavioral components of reproductive isolation. Additional smaller effect loci undoubtedly also contribute to variation in male preference, which we would be unlikely to detect without very large sample sizes (a caveat shared with many QTL studies of ecologically relevant behaviors e.g. [15,44,45]). Regardless, our results suggest that during speciation, divergence between populations in both mating cue and the corresponding preference behaviors can have a surprisingly simple genetic architecture.

By ensuring robust genetic associations between components of reproductive isolation, physical linkage between loci for traits influencing pre- and post-mating isolation is expected to facilitate speciation with gene flow [19]. Two of the behavioral QTL we have identified are situated on chromosomes with major color pattern loci (chromosome 1 includes the *K* locus, and chromosome 18 includes the *optix* locus). Both *optix* and the *K* locus have previously been associated with variation in *Heliconius* courtship behaviors [25,35,36]. Nevertheless, we have not previously been able to robustly estimate the position of QTLs along the chromosome. The QTL

302 we identify on chromosome 1 is not tightly linked to the *K* locus. It remains to be seen
303 whether this QTL underlies the association between male preference behavior and
304 the *K* locus phenotype (a shift between white and yellow color pattern elements)
305 previously observed in crosses between *H. cydno* and *H. pachinus* [35], and within a
306 polymorphic population of *H. cydno* [36]. (Although the *K* locus phenotype
307 segregating in crosses between *H. cydno* and *H. melpomene* [39] has not been
308 mapped, it is very likely that it is the same locus as that observed in *H. cydno* and *H.*
309 *pachinus*). In contrast, our results reveal that the QTL for male attraction on
310 chromosome 18 is tightly linked to the *optix* locus, which controls presence/absence
311 of a red forewing. The mechanistic basis for linkage of trait and preference loci
312 remains unclear. There is no evidence for an inversion at this locus [39]; it also
313 seems unlikely that the same mutations control both wing pattern and the
314 corresponding attraction behavior. However, *optix* is known to function during eye
315 and neural development in *Drosophila* [46], and is expressed in the optic lobe and
316 medulla of pupal *Heliconius* [47], so it is plausible – if unlikely [48] – that the two traits
317 could be controlled by different regulatory elements of the same gene.
318 Our work joins a small collection of studies in animals where physical linkage
319 is reported to couple loci contributing to preference behaviors and ecological barriers
320 [15,25,35,36,49], as predicted by Felsenstein [19]; and more broadly between loci for
321 cue and preference between incipient species [50–55]. In a seminal study,
322 published almost 20 years ago, Hawthorn and Via [49], showed that QTL for
323 preference and performance for different host plants co-segregate in pea aphids.
324 These insects mate on their host providing a rapid path to speciation. The resolution
325 of molecular markers available at the time allowed linkage to be confirmed to no
326 more than ~10cM, but even this could substantially impede the break-down of LD:

327 whereas LD between unlinked loci declines by 50% in one generation of random
328 mating, LD between two loci that are 10cM apart would decline by only ~9% per
329 generation (cyclical parthenogenesis would further reduce recombination in these
330 aphid species). Extending the same logic to our results, LD between the preference
331 locus and *optix* on chromosome 18 would be expected to decline by 1.2 (0-4.6) %
332 per generation (Fig S5), assuming random mating. However, alleles at the behavioral
333 locus result in a preference for the trait controlled by *optix:* LD will be further
334 maintained by non-random mating because warning pattern is a magic trait. As such
335 LD is likely to decline much more slowly than this simple model would suggest.
336 More recently, Bay and colleagues [15] have reported widespread physical
337 linkage between loci for divergent mate choice and ecological phenotypes in benthic
338 and limnetic populations of three-spine sticklebacks. Two lines evidence support this.
339 First, individual QTLs for mate choice and morphology map to chromosome 14.
340 Second, a polygenic QTL model predicting hybrid position along the benthic-limnetic
341 morphological axis, generated by a previous study [10], explains a significant
342 proportion of variance in mate choice, consistent with physical linkage of ecological
343 and mate choice loci. Our results complement this previous work by explicitly
344 demonstrating tight linkage between assortative mating and ecological traits. In
345 addition, our study shows a much simpler genetic architecture, which should further
346 facilitate the maintenance of LD between traits and which is predicted to facilitate
347 speciation [2].
348 When mate choice is based on a preference for divergent ecological traits, this
349 will inevitably couple ecological and behavioral components of reproductive isolation.
350 Furthermore, the strength of LD generated will be proportional to the strength of the
351 mating preference, so a genetic architecture with large-effect loci controlling

352 assortative mating will generate stronger LD than a more polygenic architecture. Both
353 our simulations and replication analysis support the existence of large effect QTLs
354 controlling an important interspecific difference in preference behavior. Even if we
355 adopt an especially cautious approach, the QTLs on chromosomes 1 and 18 would
356 explain *at least* 10% and 20% of the difference in male preference behavior,
357 respectively. However, our follow-up analysis, exploiting individuals that were not
358 used to determine significance (thereby evading the Beavis effect), suggests that
359 these estimates are overly conservative; these data explicitly reinforce our initial
360 estimate for the QTL on chromosome 18, which explains ~30% of the difference
361 between parents. One potential caveat is that the position of the putative QTL and
362 that of *optix* are not the same, but 1.2cM apart; however, any recombination between
363 these loci in the individuals tested will be rare (we expect just 0.42 recombination
364 events between these two loci across 35 individuals), and likely has very limited
365 impact on our estimates of effect size.

366 We observed a dramatic reduction in admixture (estimated using f_d) at the
367 proximal end of chromosome 18, and specifically on the distal side of *optix* coincident
368 with our QTL. It is tempting to ascribe this to the combined effects of the major
369 preference locus we have identified and the color pattern gene *optix.* However, in the
370 populations studied here, the phenotypic effect of *optix* is more striking than the other
371 color pattern loci, and selection against introgression is likely be stronger at this
372 locus. As a result, tight linkage with *optix* makes it impossible to determine any
373 effects of the preference locus alone. Similarly, it is difficult to infer a signal of
374 reduced admixture due to the behavioral QTLs on chromosomes 1 and 17. Levels of
375 F_{st} are high across the genome between *H. cydno* and *H. melpomene* and patterns of
376 admixture across the genome suggest widespread selection against introgression

377 [42]. At this point, the patterns of divergence between *H. cydno* and *H. melpomene*
378 are so heterogenous, it is difficult to disentangle the many processes that could be
379 driving reduced admixture.

380 A general caveat of our results, alongside other studies of the genetics of
381 assortative mating in *Heliconius* [35,36] and elsewhere (e.g. [15,56]), is that it is hard
382 to distinguish between loci affecting preference behaviors *per se*, from other traits
383 that influence the behavior of the opposite sex. Here, we measured the time
384 *Heliconius* males spend courting a particular female, which may depend not only on
385 male attraction, but also on the female's response to male behavior (and in turn the
386 male's response to the female's behavior). Recent work suggests that *H. cydno*
387 females respond differently to *H. pachinus* than conspecifics males [27]. Although
388 there is currently no evidence that female *Heliconius* use color pattern as an
389 interspecific mating (or rejection) cue (but see [57]), it is not inconceivable, and this
390 could perhaps account for the apparent linkage between male interest and forewing
391 color observed in our study and elsewhere [35,36]. In addition, it is possible that
392 either of these QTLs we identified might influence male pheromones, which has been
393 shown to influence female acceptance behaviors within *H. melpomene* [58].
394 Nevertheless, using the same hybrids as studied here, we previously demonstrated
395 that individuals that have inherited the red band allele from *H. melpomene* are more
396 likely to court artificial females with the red *melpomene* pattern, implying that the QTL
397 on chromosome 18, at least, influences male response to a visual cue [25].
398 Regardless of the exact proximate mechanisms involved, the QTLs we identify here
399 influence an important component of male assortative mating behavior.

400 Overall, the scenario we describe reflects one that modeling broadly predicts
401 will generate a strong overall barrier to gene flow through reinforcement [4]:

402 Specifically, the effects of barrier loci on prezygotic isolation are strong,
403 recombination between pre- and post-mating isolation barrier loci is reduced, and
404 hybridization imposes high costs. Indeed, experimental evidence shows that non-
405 mimetic hybrids between *H. melpomene* and *H. cydno* suffer not only increased
406 predation [24], but also reduced mating success [22] and fertility [59]. In addition,
407 males make a considerable reproductive investment by donating a nutrient-rich
408 spermatophore during mating [60,61], so indirect selection against poorly adapted
409 hybrids could strengthen divergent male preferences. Consistent with a role of
410 reinforcement, *H. melpomene* males from French Guiana, outside the range of *H.*
411 *cydno*, are less choosy than males from Panama, where the species co-occur and
412 are known to occasionally hybridize [21]; and similar patterns of reproductive
413 character displacement have been observed elsewhere in the *melpomene-cydno*
414 clade [62].
415 Reinforcement is further promoted when indirect selection, resulting from
416 coupling of prezygotic and postzygotic barrier effects, is supplemented by direct
417 selection [4,63]. In *Heliconius,* divergence in male preferences is likely initiated by
418 divergence in wing pattern, and male preferences are observed between populations
419 with few opportunities for hybridization e.g. [64]. Female re-mating is a rare event
420 [65], and males must compete to find virgin females within a visually complex
421 environment [26]. Divergence in female (and male) wing patterns is driven primarily
422 by strong selection for mimicry, and is likely to impose divergent sexual selection on
423 male preferences to improve their ability to find receptive females. This is similar to
424 examples of assortative mating driven by sensory drive, such as in cichlid fishes [66],
425 but it is perhaps less well appreciated that morphological traits under ecological

426 selection (such as *Heliconius* wing patterns) might impose divergent sexual selection
427 on male preferences in a similar fashion.
428 In addition to a simple genetic architecture, different QTLs appear to control
429 different aspects of preference behavior. Our *post-hoc* analyses suggest that
430 differences associated with QTL1 and QTL17 in the relative amount of time spent
431 courting each female type are driven by differences in attraction to either *H. cydno* or
432 *H. melpomene*, respectively, rather than both species. QTL18 also seems to
433 influence attraction to *H. melpomene* much more strongly than to *H. cydno* females.
434 This genetic modularity, where discrete, independently segregating loci appear to
435 affect different aspects of behavior, may facilitate evolutionary change and innovation
436 by providing a route for rapid evolution of novel behavioral phenotypes [44,67]. In
437 *Heliconius*, this might allow different aspects of mating behavior to evolve
438 independently. It might also allow novel composite behavioral preferences to arise
439 through hybridization and recombination. There is some evidence that this has
440 occurred during hybrid speciation in *Heliconius*. The wing pattern of the hybrid
441 species *H. heurippa* includes both red and yellow pattern elements, which are
442 believed to have originated from the putative parental species *H. melpomene* and *H.*
443 *cydno,* respectively (local Colombian races of *H. cydno* have a yellow, as opposed to
444 white, forewing band) [23]. Not only do *H. heurippa* males prefer this combined
445 pattern over the 'pure' red or yellow patterns of *H. melpomene* and *H. cydno* [23], but
446 'recreated *H. heurippa*', obtained in first generation backcrosses between *H.*
447 *melpomene* and *H. cydno*, prefer the pattern of *H. heurippa* over that of the two
448 putative parents [68]. This is consistent with a hypothesis in which introgression and
449 subsequent recombination of preference alleles are responsible for novel behavioral
450 phenotypes, although further work would be needed to confirm this.

451 In conclusion, the genetic architecture we demonstrate here will promote the
452 evolution of behavioral isolation by strengthening genetic associations between cue
453 and preference. Disassociation of alleles at loci that are physically close on the
454 chromosome is slower compared to that between alleles at more distant loci (due to
455 reduced crossing over), or at loci on different chromosomes. Similarly, the
456 substitution of large effect alleles will also increase linkage disequilibrium between
457 cue and preference, even if they are not physically linked, because preference alleles
458 of larger effect will more often find themselves in the same genome as alleles for the
459 corresponding cue, compared to preference alleles with smaller effects. We cannot
460 currently distinguish whether preference QTL result from single adaptive mutations,
461 or represent multiple functional loci that have built up during the course of speciation.
462 Nevertheless, the genetic basis of *Heliconius* mate preferences is remarkably similar
463 to that for differences in the wing pattern cue. Differences in individual color pattern
464 elements probably do involve multiple, sequential mutations (which target the same
465 gene(s)), but 'ready-made' alleles of large phenotypic effect can be brought together
466 in new combinations through adaptive introgression. The existence of large effect
467 preference loci, potentially influencing different aspects of behavior, could similarly
468 facilitate the origin of novel phenotypes through introgression, and further facilitate
469 rapid speciation.
470

471 **Methods**

472 **Butterfly collection, rearing and crossing design**. All butterfly rearing, genetic
473 crosses and behavioral experiments were conducted at the Smithsonian Tropical
474 Research Institute in Panama between August 2007 and August 2009.

475 We collected wild *Heliconius cydno chioneus* and *Heliconius melpomene rosina* from
476 Gamboa (9°7.4'N, 79°42.2' W, elevation 60 m) and the nearby Soberania National
477 Park, Panama. These were used to establish stocks maintained in insectaries in
478 Gamboa, which were further supplemented with wild individuals throughout the
479 experimental period. We established interspecific crosses by mating wild caught *H.*
480 *melpomene* males to *H. cydno* females from our stock population. In interspecific
481 crosses between *Heliconius cydno* females and *Heliconius melpomene* males, F1
482 hybrid females are sterile, restricting us to a backcrossing design. We generated
483 backcross broods to *H. cydno* and *H. melpomene* by mating F1 males to virgin
484 females from our stock populations. Brood mothers were kept individually in cages
485 (approx. 1 or 2 x 2x2m), and provided with ~10% sugar solution, a source of pollen
486 and *Passiflora* shoots for oviposition. Eggs were collected daily and caterpillars
487 raised individually in small pots until 4th or 5th instar, and then either in groups or
488 individually until pupation. Caterpillars were provided with fresh *Passiflora* leaves and
489 shoots daily.
490
491 **Behavioral assays.** We measured male attraction to *H. melpomene* and *H. cydno*
492 females in standardized choice trials [25,38]. Males were allowed to mature for at
493 least 5 days after eclosion before testing. Males were introduced into outdoor
494 experimental cages (1x1x2m) with a virgin female of each species (0 – 10 days
495 matched for age). Fifteen-minute trials were divided into 1 min intervals, which were
496 scored for courtship (sustained hovering or chasing) directed towards each female as
497 having occurred or not occurred. Accordingly, if a male courted the same female
498 twice within a minute interval, it was recorded only once; if courtship continued into a
499 second minute, it was recorded twice. Where possible, trials were repeated for each

500 male (median = 5 trials). From these trials we generated a large dataset used in
501 subsequent analyses which includes the total number of 'courtship minutes' directed
502 towards *H. melpomene* and the number of 'courtship minutes' *H. cydno* females
503 (Table S2). The QTL analysis considered the proportion of total 'courtship minutes'
504 directed towards *H. melpomene, i.e.* 'courtship minutes' directed towards *H.*
505 *melpomene /* ('courtship minutes' directed towards *H. melpomene* + 'courtship
506 minutes' directed towards *H. cydno*) = "the relative amount of time males spent
507 courting red *H. melpomene* and white *H. cydno* females" = "relative courtship time".
508 In total we conducted 1347 behavioral trials, and collected data from 28 *H. cydno*, 16
509 *H. melpomene*, 23 F1 hybrid, 29 backcross-to-*melpomene* hybrid and 196
510 backcross-to-*cydno* hybrid males (of which 11 performed no courtship behaviors).
511
512 **Genotyping and linkage map construction.** Genotyping and construction of
513 linkage maps has been described elsewhere [39]. In brief, backcross hybrids and
514 associated parents were preserved in 20% DMSO and 0.25M EDTA (pH 8.0) and
515 stored at -20°C. DNA was extracted with Qiagen DNeasy Blood & Tissue Kits
516 following the manufacture's protocol for animal tissue. Individuals were genotyped
517 using a RAD-sequencing approach [69] and sequenced by BGI using the Illumina
518 HiSeq 2500. Sequences were then aligned to version 2 of the *H. melpomene*
519 genome [70] using Stampy v1.0.23 [71]. Duplicates were removed with Piccard tools
520 v1.135 (http://broadinstitute.github.io/picard/), and genotype posteriors called using
521 SAMtools v1.2. Interspecific linkage maps were constructed using Lep-MAP2 [72]
522 and modules from Lep-MAP3 as described in [39]. To obtain the genotypic data for
523 QTL mapping, the parental-phased data was obtained using Lep-MAP3
524 option outputPhasedData=1. This option imputes data based on input genotypes and

525 the map order. These data were then compared to the subset of markers in which
526 grandparents could be used to phase the data for each family and chromosome
527 using custom scripts. Family and chromosome was inverted when required to obtain
528 matching phases. Finally, the non-informative markers between inferred
529 recombinations were masked (i.e. set to missing) to account for the fact the exact
530 recombination position was not known for these regions.
531
532 **Data analysis.** All QTL analyses were performed on backcross-to-*cydno* hybrid
533 males in which the preference behaviors segregate. We were able to generate
534 genotype data for 146 of the 196 backcross-to-*cydno* hybrid males for which we
535 recorded behaviors in our choice trials. The remaining 50 individuals include males
536 from which we were unable to extract sufficient DNA, were poorly sequenced, or
537 were lost in the insectaries most often due to ants or other predators. For each
538 backcross individual, we calculated the probabilities of the two alternative genotypes
539 at every marker and centiMorgan (cM) position along the chromosomes, conditional
540 on the available marker data, using R/qtl package [73]. R/qtl uses a hidden Markov
541 model to calculate the probabilities of the true underlying genotypes given the
542 observed multipoint marker data. We then tested for an association between
543 phenotype and genotype at each position using generalized linear mixed models
544 (GLMMs) with binomial error structure and logit link function (implemented with the R
545 package lme4). We first considered the relative time males courted *H. melpomene* as
546 opposed to *H. cydno* females. For each position along the genome we modeled the
547 response vector of the number of 'courtship minutes' towards *H. melpomene* vs
548 'courtship minutes' towards *H. cydno* with the genotype probability as the
549 independent variable. LOD scores were obtained by comparing this to a null model in

550 which genotype probability was not included. An individual level random factor was
551 included in all models to account for over-dispersion. This approach is analogous to
552 the to the Haley-Knott regression implemented in R/qtl [54, 55], but more
553 appropriately accounts for the non-normal structure of our data and for differences in
554 total courtship data recorded for each individual [56]. Seven individuals were
555 excluded from these analyses for which, although tested in multiple trials, no
556 courtship towards either female type was recorded. Using permutation [57], we
557 determined the genome-wide significance threshold for the association between
558 marker genotype and phenotype (alpha = 0.05, n = 1000 permutations) as LOD =
559 2.99. By using our GLMM approach we had more power to detect QTL than would be
560 permitted by adopting non-parametric methods. Nevertheless, we repeated all QTL
561 analyses using non-parametric interval mapping in R/qtl, using the 'scanone' and
562 'model = "np" ' commands. Results of non-parametric analyses are reported in the
563 supplementary materials (Table S2).
564 To consider all three QTL identified in our initial genome scans together, we
565 again modeled the number of 'courtship minutes' towards *H. melpomene* vs
566 'courtship minutes' towards *H. cydno* but with the genotype at the max LOD score for
567 each QTL as explanatory variables. The fully saturated GLMM, including all three
568 pairwise interactions, was simplified in a stepwise manner using likelihood ratio tests,
569 but always retaining individual id as a random factor. To further test for effects of
570 each QTL we compared the penalized LOD scores of the full model (including all
571 three QTL as additive effects) to reduced models in which each QTL was eliminated
572 in turn. The penalized LOD score is calculated as: $pLODa(\gamma) = LOD(\gamma) - T|\gamma|$, where
573 γ denotes a model, $|\gamma|$ is the number of QTL in the model and T is a penalty

574 determined through permutation (i.e. the genome-wide significance threshold = 2.99)
575 [58] .
576 Finally, to determine the contribution of each QTL to variation in courtship time
577 towards *H. cydno* and *H. melpomene* females separately, we considered the total
578 number of 'courtship minutes' directed to each female type, correcting for the number
579 of trials. We included all 146 backcross males for which we had genotype data in this
580 analysis. We square-root transformed courtship minutes/trial and then used the
581 makeqtl() and fitqtl() functions in R/qtl [54] to determine significance. Model residuals
582 were inspected visually for an approximate normal distribution, and we tested for
583 homogeneity of variance with Levene's tests (*H. cydno* females: $F_{3,142} = 0.27$, P >
584 0.02; *H. melpomene* females: $F_{3,142} = 0.39$, P > 0.02). We corrected *P* values to
585 account for the 6 tests (i.e. 3 loci x 2 species) [37].
586
587 **Simulations**. We used simulations to estimate potential inflation of measured effect
588 sizes due to the Beavis effect. We generated 10000 simulated data-sets for each of a
589 range of 'true' effect sizes for each significant QTL (i.e. on chromosomes 1, 17 and
590 18), using the R package simr [74]. For each of these we determined the LOD score
591 and compared it to our genome-wide significance threshold (i.e. LOD = 2.99). This
592 allowed us to compare i) the entire range of simulated effects (where the mean is
593 expected to equal the 'true' effect size), with those that would be significant given our
594 sample size (n = 139) and linkage map (figure S1), and ii) the empirically measured
595 effects with simulated effects that would be significant (Fig. 3A).
596
597 **Admixture analysis.** We investigated heterogeneity in admixture across the genome
598 between *H. melpomene rosina* and *H. cydno chioneus* using f_d, which provides an

599 approximately unbiased estimate of the admixture proportion [41,42]. This analysis
600 made use of available whole genome sequence data for *H. melpomene rosina*
601 (N=10) and *H. cydno chioneus* (N=10) from Panama, with *H. melpomene*
602 *melpomene* from French Guiana (N=10) serving as the allopatric 'control' population
603 and two *Heliconius numata* individuals as outgroups [42].
604
605 **Code and data availability.** Scripts and raw data used in analyses are available
606 online: DRYAD REPOSITORY XXX. f_d was computed in 100 kb windows using the
607 python script ABBABABAwindows.py, available from
608 github.com/simonhmartin/genomics_general. Sequence data used to make the
609 linkage maps have previously been submitted to the European Nucleotide Archive
610 (http://www.ebi.ac.uk/ena) [39], accession number ERP018627.
611
612 **References**

613 1. Nosil P. Ecological Speciation. Oxford University Press; 2012.

614 2. Gavrilets S. Fitness Landscapes and the Origin of Species. Princeton University Press;
615 2018.

616 3. Smadja CM, Butlin RK. A framework for comparing processes of speciation in the
617 presence of gene flow. Mol Ecol. 2011;20: 5123–5140. doi:10.1111/j.1365-
618 294X.2011.05350.x

619 4. Butlin RK, Smadja CM. Coupling, Reinforcement, and Speciation. Am Nat. 2018;191:
620 155–172. doi:10.1086/695136

621 5. Kopp M, Servedio MR, Mendelson TC, Safran RJ, Rodríguez RL, Hauber ME, et al.
622 Mechanisms of Assortative Mating in Speciation with Gene Flow: Connecting Theory
623 and Empirical Research. Am Nat. 2018;191: 1–20. doi:10.1086/694889

624 6. Servedio MR, Van Doorn GS, Kopp M, Frame AM, Nosil P. Magic traits in speciation:
625 "magic" but not rare? Trends Ecol Evol (Amst). 2011;26: 389–397.
626 doi:10.1016/j.tree.2011.04.005

627 7. Thibert-Plante X, Gavrilets S. Evolution of mate choice and the so-called magic traits in
628 ecological speciation. Ecol Lett. 2013;16: 1004–1013. doi:10.1111/ele.12131

8. Lamichhaney S, Berglund J, Almén MS, Maqbool K, Grabherr M, Martinez-Barrio A, et al. Evolution of Darwin's finches and their beaks revealed by genome sequencing. Nature. 2015;518: 371–375. doi:10.1038/nature14181

9. Albert AYK, Sawaya S, Vines TH, Knecht AK, Miller CT, Summers BR, et al. The genetics of adaptive shape shift in stickleback: pleiotropy and effect size. Evolution. 2008;62: 76–85. doi:10.1111/j.1558-5646.2007.00259.x

10. Arnegard ME, McGee MD, Matthews B, Marchinko KB, Conte GL, Kabir S, et al. Genetics of ecological divergence during speciation. Nature. 2014;511: 307–311. doi:10.1038/nature13301

11. Chung H, Loehlin DW, Dufour HD, Vaccarro K, Millar JG, Carroll SB. A single gene affects both ecological divergence and mate choice in Drosophila. Science. 2014;343: 1148–1151. doi:10.1126/science.1249998

12. Reed RD, Papa R, Martin A, Hines HM, Counterman BA, Pardo-Diaz C, et al. optix drives the repeated convergent evolution of butterfly wing pattern mimicry. Science. 2011;333: 1137–1141. doi:10.1126/science.1208227

13. Martin A, Papa R, Nadeau NJ, Hill RI, Counterman BA, Halder G, et al. Diversification of complex butterfly wing patterns by repeated regulatory evolution of a Wnt ligand. Proc Natl Acad Sci USA. 2012;109: 12632–12637. doi:10.1073/pnas.1204800109

14. Nadeau NJ, Pardo-Diaz C, Whibley A, Supple MA, Saenko SV, Wallbank RWR, et al. The gene cortex controls mimicry and crypsis in butterflies and moths. Nature. 2016;534: 106–110. doi:10.1038/nature17961

15. Bay RA, Arnegard ME, Conte GL, Best J, Bedford NL, McCann SR, et al. Genetic Coupling of Female Mate Choice with Polygenic Ecological Divergence Facilitates Stickleback Speciation. Curr Biol. 2017;27: 3344-3349.e4. doi:10.1016/j.cub.2017.09.037

16. Lewontin RC, Kojima K. The Evolutionary Dynamics of Complex Polymorphisms. Evolution. 1960;14: 458–472. doi:10.2307/2405995

17. Nosil P, Harmon LJ, Seehausen O. Ecological explanations for (incomplete) speciation. Trends Ecol Evol. 2009;24: 145–156. doi:10.1016/j.tree.2008.10.011

18. Servedio MR, Noor MAF. The Role of Reinforcement in Speciation: Theory and Data. Annual Review of Ecology, Evolution, and Systematics. 2003;34: 339–364. doi:10.1146/annurev.ecolsys.34.011802.132412

19. Felsenstein J. Skepticism towards Santa Rosalia, or why are there so few kinds of animals? Evolution. 1981;35: 124–138. doi:10.1111/j.1558-5646.1981.tb04864.x

20. Estrada C, Jiggins CD. Patterns of pollen feeding and habitat preference among Heliconius species. Ecological Entomology. 2002;27: 448–456. doi:10.1046/j.1365-2311.2002.00434.x

21. Jiggins CD, Naisbit RE, Coe RL, Mallet J. Reproductive isolation caused by colour pattern mimicry. Nature. 2001;411: 302–305. doi:10.1038/35077075

668 22. Naisbit RE, Jiggins CD, Mallet J. Disruptive sexual selection against hybrids contributes
669 to speciation between Heliconius cydno and Heliconius melpomene. Proc Biol Sci.
670 2001;268: 1849–1854. doi:10.1098/rspb.2001.1753

671 23. Mavárez J, Salazar CA, Bermingham E, Salcedo C, Jiggins CD, Linares M. Speciation
672 by hybridization in Heliconius butterflies. Nature. 2006;441: 868–871.
673 doi:10.1038/nature04738

674 24. Merrill RM, Wallbank RWR, Bull V, Salazar PCA, Mallet J, Stevens M, et al. Disruptive
675 ecological selection on a mating cue. Proc Biol Sci. 2012;279: 4907–4913.
676 doi:10.1098/rspb.2012.1968

677 25. Merrill RM, Van Schooten B, Scott JA, Jiggins CD. Pervasive genetic associations
678 between traits causing reproductive isolation in Heliconius butterflies. Proc Biol Sci.
679 2011;278: 511–518. doi:10.1098/rspb.2010.1493

680 26. Merrill RM, Dasmahapatra KK, Davey JW, Dell'Aglio DD, Hanly JJ, Huber B, et al. The
681 diversification of Heliconius butterflies: what have we learned in 150 years? J Evol Biol.
682 2015;28: 1417–1438. doi:10.1111/jeb.12672

683 27. Southcott L, Kronforst MR. Female mate choice is a reproductive isolating barrier in
684 Heliconius butterflies | bioRxiv [Internet]. [cited 3 Aug 2018]. Available:
685 https://www.biorxiv.org/content/early/2017/12/12/233270

686 28. Jiggins CD. Ecological Speciation in Mimetic Butterflies. BioScience. 2008;58: 541–548.
687 doi:10.1641/B580610

688 29. Coyne JA, Orr HA. Speciation. Sinauer; 2004.

689 30. Montgomery SH, Merrill RM, Ott SR. Brain composition in Heliconius butterflies,
690 posteclosion growth and experience-dependent neuropil plasticity. J Comp Neurol.
691 2016;524: 1747–1769. doi:10.1002/cne.23993

692 31. Naisbit RE, Jiggins CD, Mallet J. Mimicry: developmental genes that contribute to
693 speciation. Evol Dev. 2003;5: 269–280.

694 32. Wallbank RWR, Baxter SW, Pardo-Diaz C, Hanly JJ, Martin SH, Mallet J, et al.
695 Evolutionary Novelty in a Butterfly Wing Pattern through Enhancer Shuffling. PLoS Biol.
696 2016;14: e1002353. doi:10.1371/journal.pbio.1002353

697 33. Van Belleghem SM, Rastas P, Papanicolaou A, Martin SH, Arias CF, Supple MA, et al.
698 Complex modular architecture around a simple toolkit of wing pattern genes. Nat Ecol
699 Evol. 2017;1: 52. doi:10.1038/s41559-016-0052

700 34. Nosil P, Schluter D. The genes underlying the process of speciation. Trends Ecol Evol
701 (Amst). 2011;26: 160–167. doi:10.1016/j.tree.2011.01.001

702 35. Kronforst MR, Young LG, Kapan DD, McNeely C, O'Neill RJ, Gilbert LE. Linkage of
703 butterfly mate preference and wing color preference cue at the genomic location of
704 wingless. Proc Natl Acad Sci USA. 2006;103: 6575–6580.
705 doi:10.1073/pnas.0509685103

706 36. Chamberlain NL, Hill RI, Kapan DD, Gilbert LE, Kronforst MR. Polymorphic butterfly
707 reveals the missing link in ecological speciation. Science. 2009;326: 847–850.
708 doi:10.1126/science.1179141

709 37. Forstmeier W, Wagenmakers E-J, Parker TH. Detecting and avoiding likely false-
710 positive findings - a practical guide. Biol Rev Camb Philos Soc. 2017;92: 1941–1968.
711 doi:10.1111/brv.12315

712 38. Merrill RM, Gompert Z, Dembeck LM, Kronforst MR, McMillan WO, Jiggins CD. Mate
713 preference across the speciation continuum in a clade of mimetic butterflies. Evolution.
714 2011;65: 1489–1500. doi:10.1111/j.1558-5646.2010.01216.x

715 39. Davey JW, Barker SL, Rastas PM, Pinharanda A, Martin SH, Durbin R, et al. No
716 evidence for maintenance of a sympatric Heliconius species barrier by chromosomal
717 inversions. Evolution Letters. 2017;1: 138–154. doi:10.1002/evl3.12

718 40. Beavis WB. The power and deceit of QTL experiments: lessons from comparative QTL
719 studies. 1994 *In*: Wilkinson (ed) Chicago pp 250-266

720 41. Martin SH, Davey JW, Jiggins CD. Evaluating the use of ABBA-BABA statistics to
721 locate introgressed loci. Mol Biol Evol. 2015;32: 244–257. doi:10.1093/molbev/msu269

722 42. Martin SH, Davey J, Salazar C, Jiggins C. Recombination rate variation shapes barriers
723 to introgression across butterfly genomes. bioRxiv. 2018; 297531. doi:10.1101/297531

724 43. Fisher RA. The Genetical Theory of Natural Selection: A Complete Variorum Edition.
725 OUP Oxford; 1999.

726 44. Weber JN, Peterson BK, Hoekstra HE. Discrete genetic modules are responsible for
727 complex burrow evolution in Peromyscus mice. Nature. 2013;493: 402–405.
728 doi:10.1038/nature11816

729 45. Diao W, Mousset M, Horsburgh GJ, Vermeulen CJ, Johannes F, van de Zande L, et al.
730 Quantitative Trait Locus Analysis of Mating Behavior and Male Sex Pheromones in
731 Nasonia Wasps. G3 (Bethesda). 2016;6: 1549–1562. doi:10.1534/g3.116.029074

732 46. Seimiya M, Gehring WJ. The Drosophila homeobox gene optix is capable of inducing
733 ectopic eyes by an eyeless-independent mechanism. Development. 2000;127: 1879–
734 1886.

735 47. Martin A, McCulloch KJ, Patel NH, Briscoe AD, Gilbert LE, Reed RD. Multiple recent co-
736 options of Optix associated with novel traits in adaptive butterfly wing radiations.
737 Evodevo. 2014;5: 7. doi:10.1186/2041-9139-5-7

738 48. Maynard Smith J. Sympatric Speciation. The American Naturalist. 1966;100: 637–650.

739 49. Hawthorne DJ, Via S. Genetic linkage of ecological specialization and reproductive
740 isolation in pea aphids. Nature. 2001;412: 904–907. doi:10.1038/35091062

741 50. Shaw KL, Lesnick SC. Genomic linkage of male song and female acoustic preference
742 QTL underlying a rapid species radiation. Proc Natl Acad Sci USA. 2009;106: 9737–
743 9742. doi:10.1073/pnas.0900229106

744 51. Wiley C, Shaw KL. Multiple genetic linkages between female preference and male
745 signal in rapidly speciating Hawaiian crickets. Evolution. 2010;64: 2238–2245.
746 doi:10.1111/j.1558-5646.2010.01007.x

747 52. Wiley C, Ellison CK, Shaw KL. Widespread genetic linkage of mating signals and
748 preferences in the Hawaiian cricket Laupala. Proc Biol Sci. 2012;279: 1203–1209.
749 doi:10.1098/rspb.2011.1740

750 53. McNiven VTK, Moehring AJ. Identification of genetically linked female preference and
751 male trait. Evolution. 2013;67: 2155–2165. doi:10.1111/evo.12096

752 54. Pryke SR. Sex chromosome linkage of mate preference and color signal maintains
753 assortative mating between interbreeding finch morphs. Evolution. 2010;64: 1301–
754 1310. doi:10.1111/j.1558-5646.2009.00897.x

755 55. Saether SA, Saetre G-P, Borge T, Wiley C, Svedin N, Andersson G, et al. Sex
756 chromosome-linked species recognition and evolution of reproductive isolation in
757 flycatchers. Science. 2007;318: 95–97. doi:10.1126/science.1141506

758 56. Ortíz-Barrientos D, Noor MAF. Evidence for a One-Allele Assortative Mating Locus.
759 Science. 2005;310: 1467–1467. doi:10.1126/science.1121260

760 57. Chouteau M, Llaurens V, Piron-Prunier F, Joron M. Polymorphism at a mimicry
761 supergene maintained by opposing frequency-dependent selection pressures. PNAS.
762 2017; 201702482. doi:10.1073/pnas.1702482114

763 58. Darragh K, Vanjari S, Mann F, Gonzalez-Rojas MF, Morrison CR, Salazar C, et al. Male
764 sex pheromone components in Heliconius butterflies released by the androconia affect
765 female choice. PeerJ. 2017;5: e3953. doi:10.7717/peerj.3953

766 59. Naisbit RE, Jiggins CD, Linares M, Salazar C, Mallet J. Hybrid sterility, Haldane's rule
767 and speciation in Heliconius cydno and H. melpomene. Genetics. 2002;161: 1517–
768 1526.

769 60. Boggs CL. Selection pressures affecting male nutrient investment in mating in
770 Heliconiine butterflies. Evolution. 1981;35: 931–940. doi:10.1111/j.1558-
771 5646.1981.tb04959.x

772 61. Boggs CL, Gilbert LE. Male contribution to egg production in butterflies: evidence for
773 transfer of nutrients at mating. Science. 1979;206: 83–84.
774 doi:10.1126/science.206.4414.83

775 62. Kronforst MR, Young LG, Gilbert LE. Reinforcement of mate preference among
776 hybridizing Heliconius butterflies. J Evol Biol. 2007;20: 278–285. doi:10.1111/j.1420-
777 9101.2006.01198.x

778 63. Servedio MR. Beyond reinforcement: the evolution of premating isolation by direct
779 selection on preferences and postmating, prezygotic incompatibilities. Evolution.
780 2001;55: 1909–1920.

781 64. Jiggins CD, Estrada C, Rodrigues A. Mimicry and the evolution of premating isolation in
782 Heliconius melpomene Linnaeus. J Evol Biol. 2004;17: 680–691. doi:10.1111/j.1420-
783 9101.2004.00675.x

784 65. Walters JR, Stafford C, Hardcastle TJ, Jiggins CD. Evaluating female remating rates in
785 light of spermatophore degradation in Heliconius butterflies: pupal-mating monandry
786 versus adult-mating polyandry. Ecological Entomology. 2012;37: 257–268.
787 doi:10.1111/j.1365-2311.2012.01360.x

66. Seehausen O, Terai Y, Magalhaes IS, Carleton KL, Mrosso HDJ, Miyagi R, et al. Speciation through sensory drive in cichlid fish. Nature. 2008;455: 620–626. doi:10.1038/nature07285

67. Greenwood AK, Wark AR, Yoshida K, Peichel CL. Genetic and neural modularity underlie the evolution of schooling behavior in threespine sticklebacks. Curr Biol. 2013;23: 1884–1888. doi:10.1016/j.cub.2013.07.058

68. Melo MC, Salazar C, Jiggins CD, Linares M. Assortative mating preferences among hybrids offers a route to hybrid speciation. Evolution. 2009;63: 1660–1665. doi:10.1111/j.1558-5646.2009.00633.x

69. Baird NA, Etter PD, Atwood TS, Currey MC, Shiver AL, Lewis ZA, et al. Rapid SNP discovery and genetic mapping using sequenced RAD markers. PLoS ONE. 2008;3: e3376. doi:10.1371/journal.pone.0003376

70. Davey JW, Chouteau M, Barker SL, Maroja L, Baxter SW, Simpson F, et al. Major Improvements to the Heliconius melpomene Genome Assembly Used to Confirm 10 Chromosome Fusion Events in 6 Million Years of Butterfly Evolution. G3 (Bethesda). 2016;6: 695–708. doi:10.1534/g3.115.023655

71. Lunter G, Goodson M. Stampy: a statistical algorithm for sensitive and fast mapping of Illumina sequence reads. Genome Res. 2011;21: 936–939. doi:10.1101/gr.111120.110

72. Rastas P, Paulin L, Hanski I, Lehtonen R, Auvinen P. Lep-MAP: fast and accurate linkage map construction for large SNP datasets. Bioinformatics. 2013;29: 3128–3134. doi:10.1093/bioinformatics/btt563

73. Broman KW, Wu H, Sen S, Churchill GA. R/qtl: QTL mapping in experimental crosses. Bioinformatics. 2003;19: 889–890.

74. Green P, MacLeod CJ. SIMR: an R package for power analysis of generalized linear mixed models by simulation. Methods in Ecology and Evolution. 2016;7: 493–498. doi:10.1111/2041-210X.12504

Acknowledgements We are grateful to Moisés Abanto, Josephine Dessmann, Janet Scott and Bas van Schooten for help in the insectaries, to Justin Touchon for advice regarding statistics, and to Stephen Montgomery, Markus Möst, Ricardo Pereira, Alexander Hausmann and Vera Warmuth for comments on the manuscript. We are also grateful for insightful referee comments which greatly improved this manuscript. We thank the Smithsonian Tropical Research Institute for support and the Ministerio del Ambiente for permission to collect butterflies in Panama. We are also grateful to Edinburgh Genomics for sequencing support. RMM is funded by a DFG Emmy Noether Fellowship, and was also funded by a Junior Research Fellowship at King's

College, Cambridge and by the ERC grant 339873 Speciation Genetics awarded to CJ. JD was funded by a Herchel Smith Postdoctoral Fellowship. SHM was funded by a research fellowship from St John's College, Cambridge.

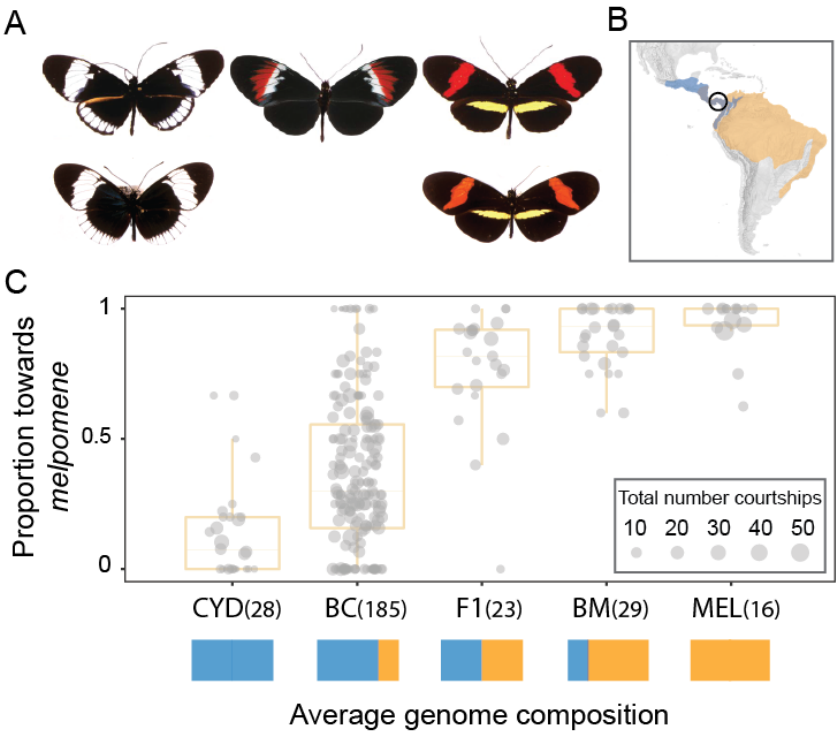
Table 1. Individual and combined QTLs for differences in relative courtship time.

Chromosome	Position (cM)	LOD score	Δ*p*LOD*a*	2ΔlnL	*P*
1	4.2 (2-9.1)	4.54	-1.04	18.54	<0.001
17	24.4 (0-48.3)	3.50	-1.03	18.50	<0.001
18	0 (0-6)	6.83	-3.87	31.60	<0.001
1+17+18	**–**	**14.90**	**(5.93)**	**–**	**–**

Position in cM (1.5 LOD interval); LOD score, log odds ratio; Δ*p*LOD*a*, penalized LOD score, change in penalized LOD score compared to the full (best supported) model incorporating all three putative QTLs (in bold); 2ΔlnL & *P* values compare the full model to reduced models in which individual QTLs were eliminated; *n* = 139.

844 **Figure 1. Divergence in warning pattern cue and corresponding preference in sympatric**
845 ***Heliconius* butterflies.** A, Wing pattern phenotypes of: top, *Heliconius cydno chioneus* (left), *H.*
846 *melpomene rosina* (right) their non-mimetic first generation hybrid (center), and bottom, their sympatric
847 co-mimics *H. sapho sapho* (left) and *H. erato demophoon* (right). B, Distribution of *H. cydno* (blue) and
848 *H. melpomene* (orange). Individuals were collected, and experiments performed in Panama (black
849 circle), where the two species co-occur in Central and northern South America. **C**, Proportion of
850 courtships directed towards *H. melpomene* (as opposed to *H. cydno*) females for *H. cydno* (CYD), *H.*
851 *melpomene* (MEL), their F1 and backcross hybrids to *H. cydno* (BC) and *H. melpomene* (BM). Values
852 in parentheses indicate total number of individuals with behavioral data. Solid colored boxes represent
853 expected average genome contribution of each generation. Note that a further 11 BC individuals were
854 tested but performed no courtship behaviors.

855



856

857

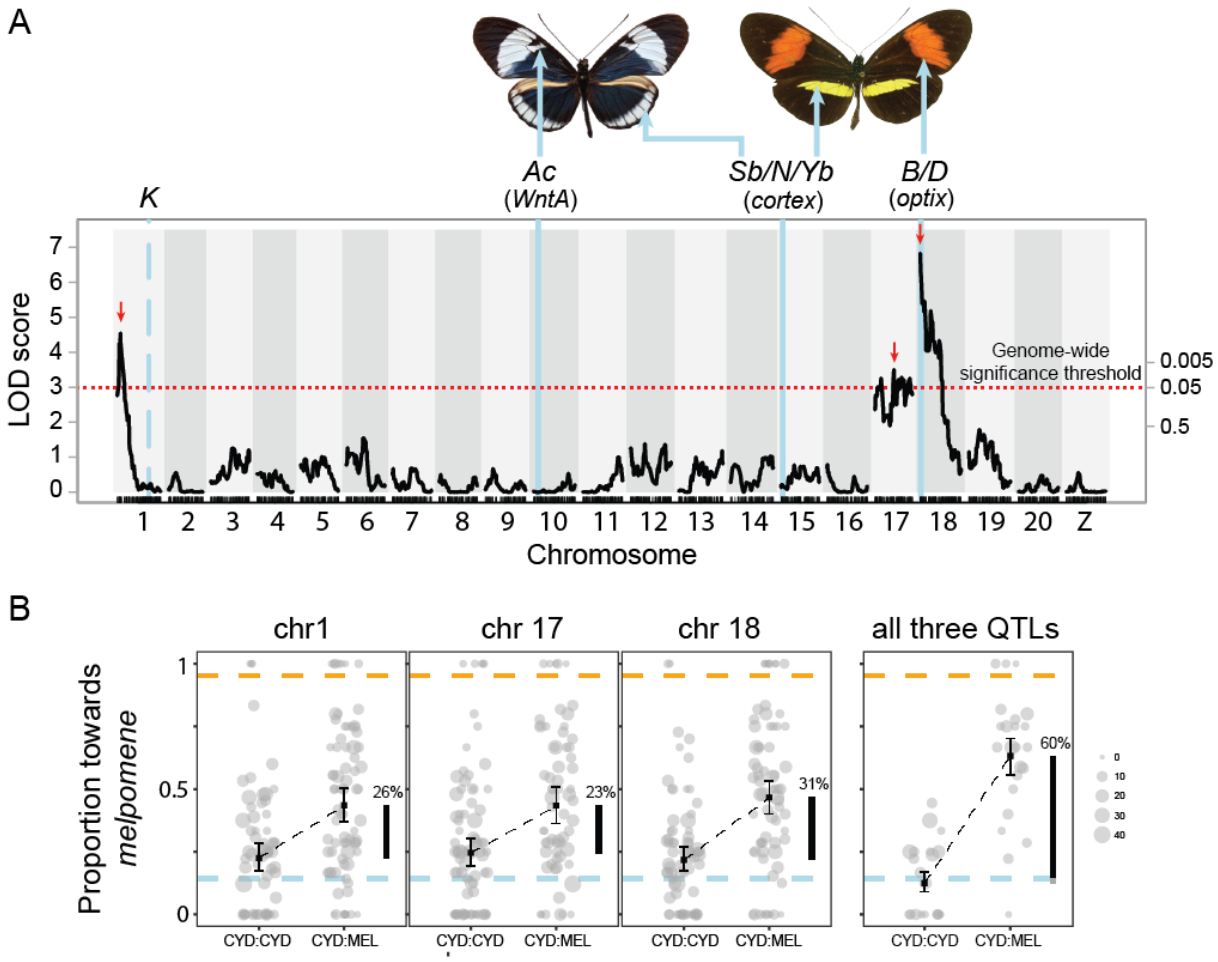
858

859

860

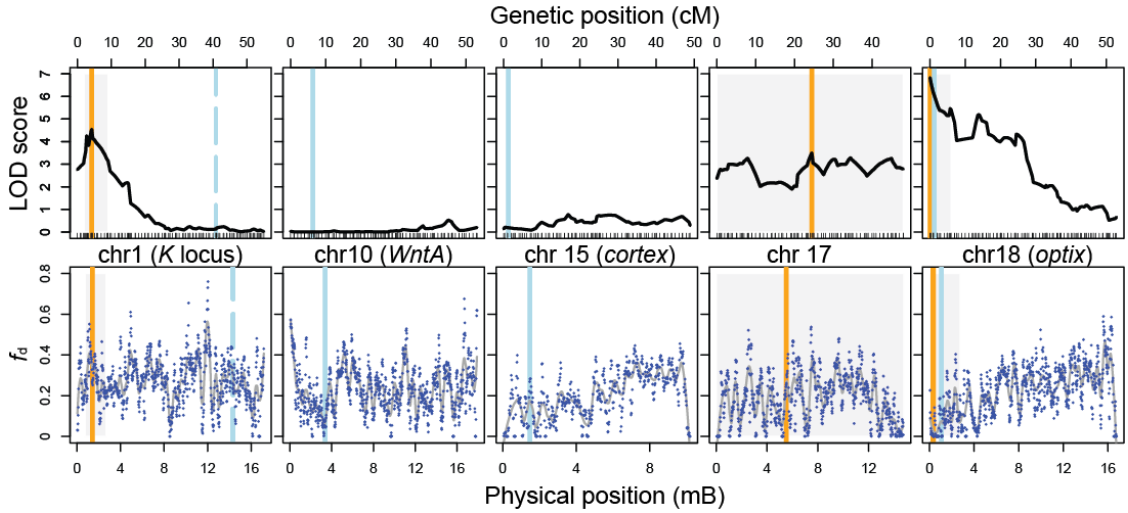
861

862 **Figure 2. QTL analysis of variation in mate preference.** A, QTLs for relative time males court *H.*
863 *melpomene* (as opposed to *H. cydno*) females on chromosomes 1, 17 and 18 (n = 139). Scale on right
864 axis depicts genome-wide significance, determined through permutation, corresponding to the LOD
865 score as shown on the left axis. Dotted red line represents log odds ratio (LOD) significance threshold
866 (genome-wide alpha = 0.05, LOD = 2.99). Dashes indicate position of genetic markers (SNPs) and red
867 arrows indicate the position of the max LOD score for each QTL (used in B). Vertical blue lines
868 represent the position of major color pattern loci, and their phenotypic effects. Note that the *K* locus
869 only has limited phenotypic effects in crosses between *H. cydno chioneus* and *H. melpomene rosina*,
870 but is responsible for the switch from yellow to white color pattern elements between other taxa within
871 the *melpomene-cydno* clade. B, Proportion of time males court *H. melpomene* (as opposed to *H.*
872 *cydno*) females for each of the two genotypes for respective QTLs (homozygous = CYD: CYD, and
873 heterozygous = CYD:MEL). Error bars represent 95% confidence intervals. Lower dashed blue and
874 upper orange bars represent mean phenotypes measured in *H. cydno* and *H. melpomene,*
875 respectively. Circle size depicts total number of 'courtship minutes' for each male. Vertical black bars
876 indicate the percentage of the difference measured in the parental species explained.



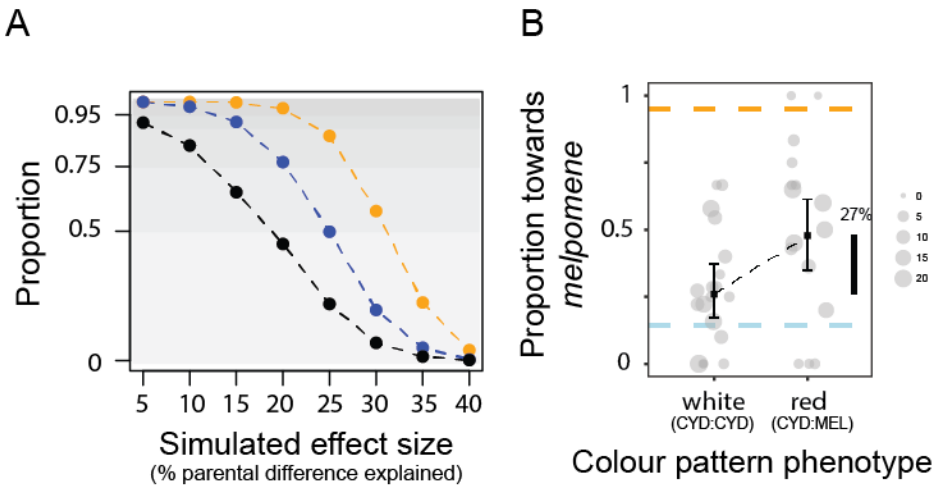
877

878 **Figure 3. Genetic and physical positions of behavioral QTL and the warning pattern loci, and**
879 **localized levels of admixture (f_d).** Vertical blue lines represent the position of major color pattern loci
880 and orange lines represent the position of peak LOD score for each behavioral QTL. Gray boxes
881 indicate the 1.5-LOD support interval for each QTL. Top panel: Dashes along the x-axis indicate
882 position of genetic markers (SNPs). Bottom panel: Blue points represent f_d values for 100kb
883 windows. f_d was measured between *H. melpomene rosina* and *H. cydno chioneus* individuals from
884 populations samples in Panama; *H. melpomene melpomene* from French Guiana, which is allopatric
885 with respect to *H. cydno*, was the 'control' population.
886

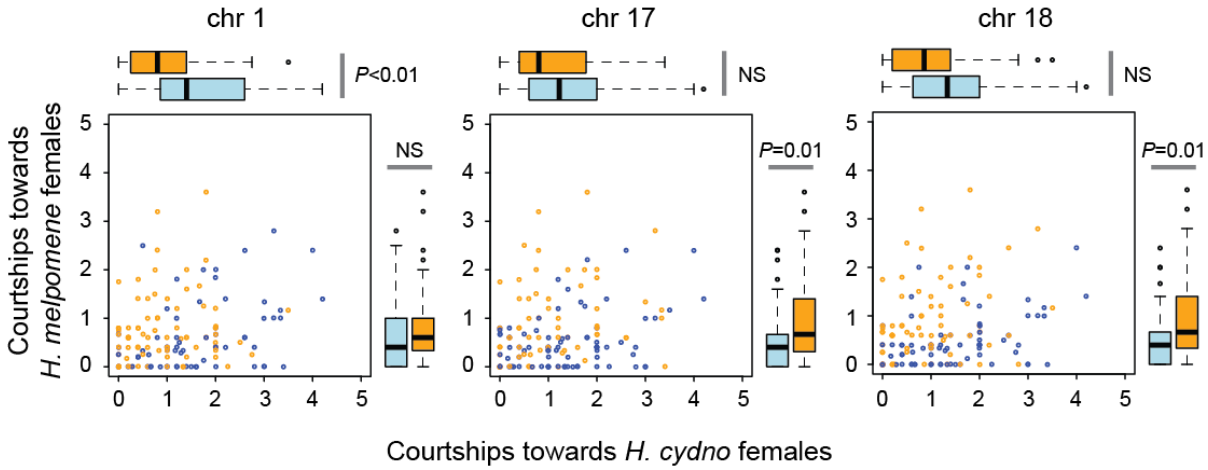


887
888
889
890
891
892
893
894
895
896
897
898

Figure 4. QTL effects in consideration of the to the Beavis effect. A, Proportion of 'significant' simulations that would be smaller than our empirically measured effects, for preference QTL on chromosome 1 (blue), chromosome 17 (black), and chromosome 18 (orange). 10000 simulations were run for effect sizes corresponding to between 5 and 40% of the difference in male preference behavior between the parental species. In each case the distribution of sample effect sizes was determined for those simulations that reached the genome-wide significance threshold determined through permutation (Fig. 2). B, Proportion of time males court *H. melpomene* (as opposed to *H. cydno*) females for each of the two genotypes for white (homozygous = CYD:CYD) and red (heterozygous = CYD:MEL) hybrid males for which we were unable to generate RAD data (and so which were not included in our initial QTL analysis). Error bars represent 95% confidence intervals. Lower dashed blue and upper orange bars represent mean phenotypes measured in *H. cydno* and *H. melpomene,* respectively. Circle size depicts total number of 'courtship minutes' for each male. Vertical black bars indicate the percentage of the difference measured in the parental species explained.



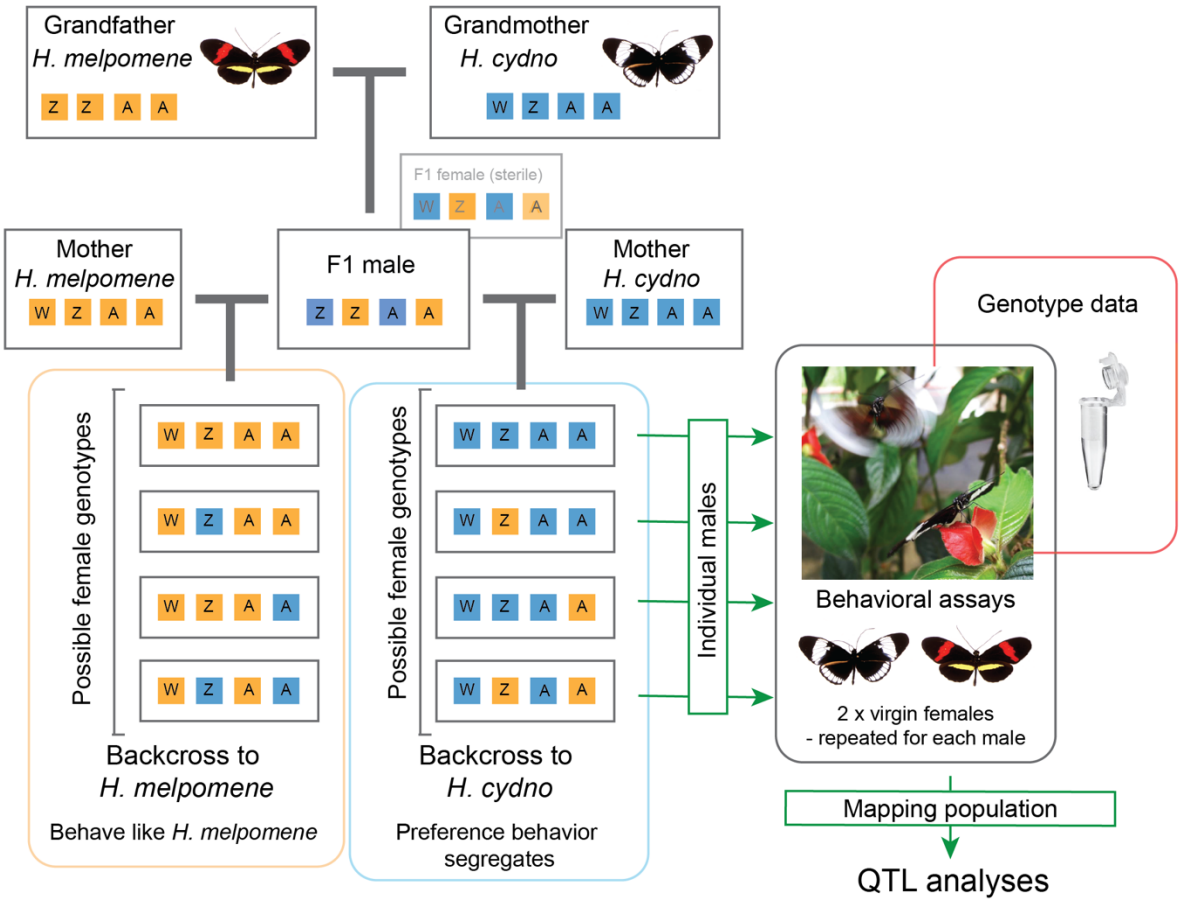
922 **Figure 5. Different QTL affect different aspects of behavior.** The QTL on chromosome 1 influences
923 courtship towards *H. cydno,* but not *H. melpomene* females. The opposite is the case for the QTLs on
924 chromosomes 17 and 18, where there is little evidence that either QTL influence courtships directed
925 towards *H. cydno* females. Data presented are for number courtship events corrected by the total
926 number of trials. Blue circles and boxplots represent data for individuals homozygous at each QTL (i.e.
927 CYD:CYD), orange circles and boxplots represent data for individuals heterozygous at each QTL (i.e.
928 CYD:MEL)



929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944

945 **Supporting Information**

946 **Figure S1. Overview of crossing design.** Colored boxes represent segregating *H. cydno* (blue) and
947 *H. melpomene* (orange) alleles; Z and W refer to the alleles on the sex-chromosomes and A to those
948 on autosomes.



949

950

951

952

953

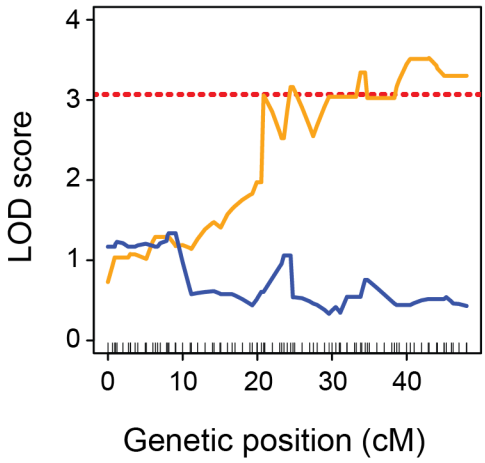
954

955

956

957 **Figure S2. QTL analysis of variation of mate preference for individuals with alternative**
958 **genotypes at LG18@0cM.** QTL associated with the proportion of time males court *H. melpomene* (as
959 opposed to *H. cydno*) females on chromosomes 17 for individuals homozygous (i.e. white, CYD:CYD
960 = blue line) and heterozygous (i.e. red, CYD:MEL = orange line) at LG18@0cM. Dashed line
961 represents log odds ratio (LOD) significance threshold (*i.e.* genome-wide alpha = 0.05) for
962 heterozygous (*i.e.* red, CYD:MEL) individuals. Dashes along the x-axis indicate position of genetic
963 markers (SNPs).

964



965

966

967

968

969

970

971

972

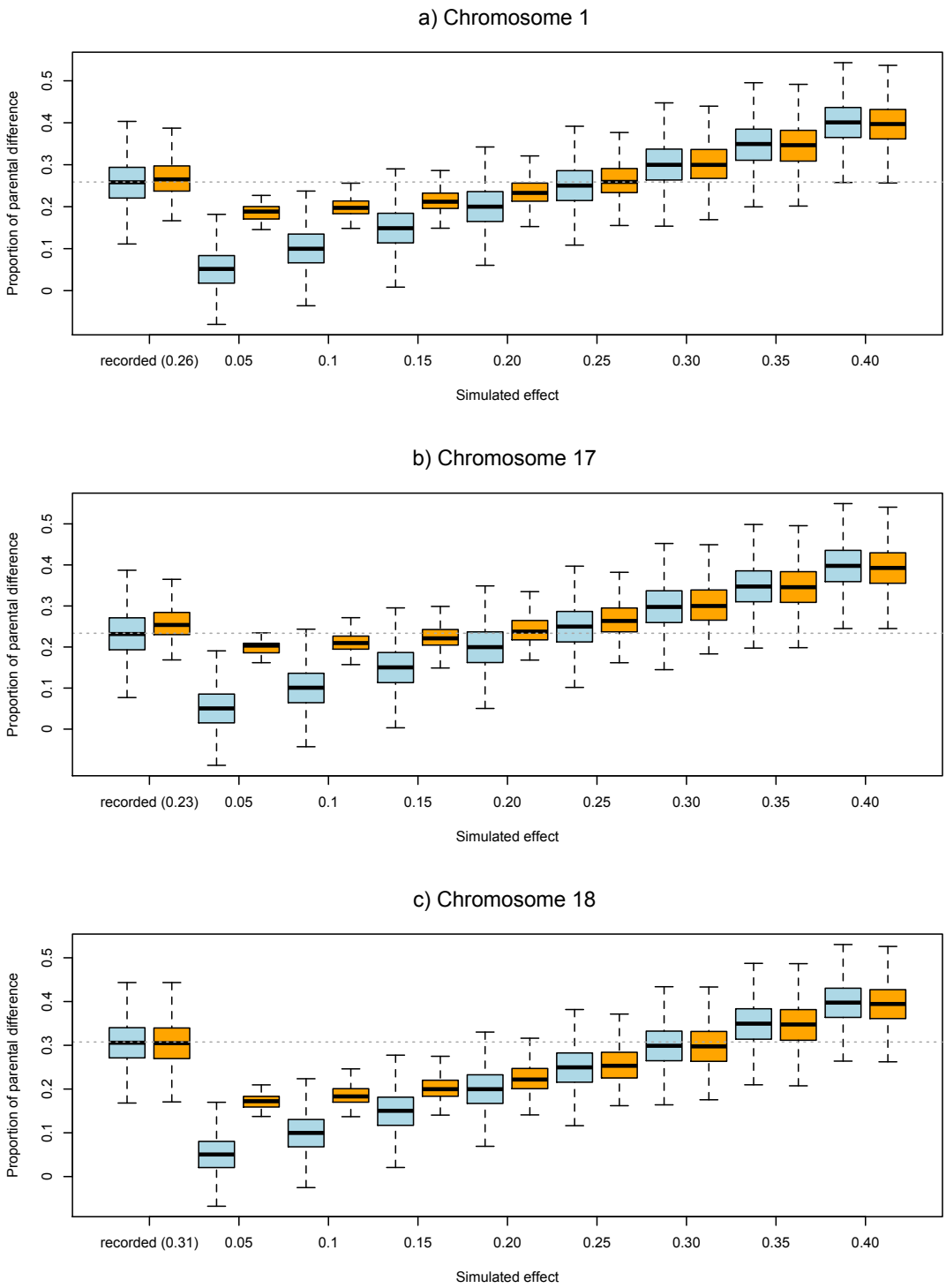
973

974

975

976

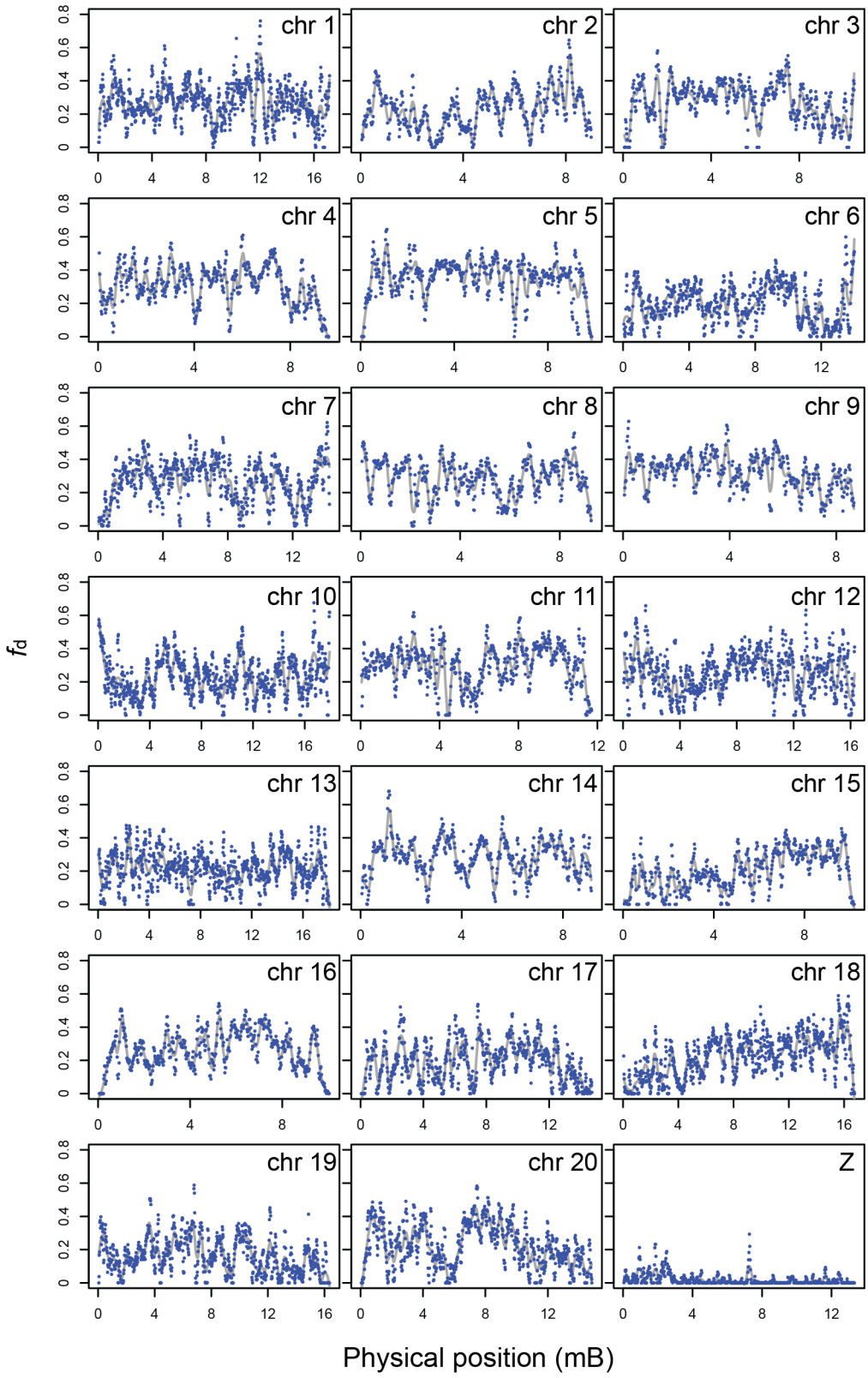
977 **Figure S3. Simulations suggest QTL effect sizes are not greatly overestimated.** For each
978 simulated effect size, the distribution of all simulated effects (blue) and those which would be
979 significant in our analysis (*i.e.* LOD ≥ 2.99) (orange) are shown. In each case, 'recorded' refers to the
980 empirically measure effect size.



981

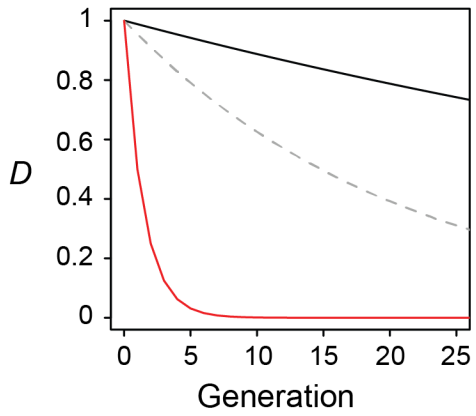
982

983 **Figure S5. Localized levels of admixture (f_d) across all 21 chromosomes.** Blue points represent f_d
984 values for 100kb windows. f_d was measured between *H. melpomene rosina* and *H. cydno chioneus*
985 individuals.
986



987

988 **Figure S5. Decline in linkage disequilibrium (LD) of linked and unlinked loci under an**
989 **assumption of random mating.** Whereas linkage disequilibrium (*D*) between unlinked loci (red solid
990 line) declines by 50% in one generation of random mating, LD between two loci that are 1.2cM apart
991 would decline by only 1.2 % per generation (black solid line), and LD between two loci that are 4.8cM
992 apart (gray dashed line) would decline by only 4.6% per generation.



993

994

995

996 **Table S1. Summary of genome-wide QTL analyses using binomial GLMM methods (reported in**
997 **main text) and non-parametric methods implemented in R/qtl.**

	Binomial GLMM			**Non-parametric**		
Chromosome	Position (cM)	LOD	*P*	Position (cM)	LOD	*P*
1	4.23	4.54	<0.001	4.23	3.6	~0.009
17	24.47	3.5	~0.013	24.47	2.89	~0.049
18	0	6.83	<0.001	0	5.34	<0.001

998 Position in cM refers to the position the peak LOD score, i.e. the most likely genetic position of the
999 putative QTL, for each QTL. *P* values are determined through permutation as described in the
1000 methods.
1001

1002

1003

1004 **Table S2. Courtship data and total trials for all 292 individuals included in the study.** Type CYD
1005 = pure *H. cydno chioneus*; MEL = pure *H. melpomene rosina*; F1 = first generation hybrids (*H. cydno*
1006 *chioneus* mother and *H. melpomene rosina* father); BC = backcross to *H. cydno chioneus*; and BM =
1007 backcross to *H. melpomene rosina.*
1008

1009 *See attached .csv file:* Table_S2.csv
1010