Genetic dissection of assortative mating behavior

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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. 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. Three QTLs each explain a large proportion of the differences in preference behavior observed between the parental species. Two of these QTLs are on chromosomes with major color pattern genes, including one that is tightly associated with the gene *optix*. Different loci influence different aspects of attraction, suggesting that behavioral isolation in *Heliconius* involves the evolution of independently segregating modules, similar to those for the corresponding wing pattern cues. Hybridization and subsequent sharing of wing pattern loci has played an important role during adaptation and speciation in *Heliconius* butterflies. The existence of large effect preference loci could similarly assist the evolution of novel behavioral phenotypes through recombination and introgression, and should facilitate rapid speciation.

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 mating traits [2-5]. Ecologically relevant mating cues (sometimes known as 'magic traits' [2, 4]) are now predicted to be widespread in nature [4, 6], 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 [7], body shape in sticklebacks [8], cuticular hydrocarbons in *Drosophila* [9], and wing patterns in *Heliconius* butterflies [10-12]. However, the extent to which these traits contribute to assortative mating depends on the evolution of corresponding preference behaviors. This process will be greatly influenced by the underlying genetics. Both the substitution of large effect preference alleles, and physical linkage will strengthen linkage disequilibrium between cue and preference, thereby reducing the effects of recombination that might otherwise impede the evolution of assortative mating. 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 [13]).

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). Hybrids are viable but occur at very low frequency in the wild

(estimated at ~0.001 [14]), 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* ([15, 16]; see also [17]).

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 [18], and also that males prefer live females and paper models with the same color pattern as themselves [15]. These results led Servedio and colleagues [4] to conclude that, unlike other putative examples, in *H. melpomene rosina* and *H. cydno chioneus* both criteria for a magic trait have been confirmed with manipulative experiments. Although female preferences undoubtedly contribute to assortative mating [19-22], male preferences act first in these species such that strong observed male discrimination against heterospecific females will have a disproportionate absolute contribution to overall reproductive isolation [22].

Crossing experiments have shown that the shift in mimetic warning pattern between *H. melpomene rosina* and *H. cydno chioneus* is largely controlled by three major effect loci [23]. Candidate genes underlying these loci have now been identified: the transcription factor *optix* controls red patterns [10], the *WntA* gene controls forewing shape [11] and yellow patterns map to the gene *cortex* [12]. In addition, a further locus, *K*, segregates in crosses between *H. melpomene rosina* and *H. cydno chioneus* with more minor effect [23, 24]. Further modularity occurs within these loci. For example, different regulatory elements of optix each result in distinct red pattern elements [25]. The modular nature of individual color pattern loci and their functionally sufficient enhancers means that they can be combined to produce considerable phenotypic diversity [25, 26]. These loci are large-effect ‘speciation

genes', in that they control traits that generate strong reproductive isolation. To complement our extensive knowledge of the genetics of their color pattern cues, here we explore the genetics of male preference behaviors between *H. melpomene* and *H. cydno*.

Results

We studied male mating preference among F1 and backcross hybrid families between *H. melpomene rosina* and *H. cydno chioneus*, in standardized choice trials [20]. We introduced individual males into an experimental cage and recorded courtship directed towards two virgin females, one of each species. In total, we collected data from 1347 behavioral trials, across 292 individuals. Multiple trials were performed for each backcross male, from which we determined the relative courtship time directed towards *H. melpomene* and *H. cydno* females, as well as the proportion of trials in which courtship was initiated towards each species. As reported previously [20], F1 males have a strong preference for the red *H. melpomene* females, and little segregation in mate preference is observed among the backcross to melpomene (no significant difference from pure *H. melpomene* males: $2\Delta \ln L = 1.33$, $d.f. = 1$, $P > 0.2$), implying that *melpomene* mate preference alleles are dominant. In contrast, courtship behavior segregates among *H. cydno* backcross males, permitting analysis of the genetic basis for this mating behavior (Fig. 1C).

Three loci contribute to species differences in preference behavior. We used a genome-wide quantitative trait locus (QTL) mapping approach to identify the genomic regions underlying divergence in mate attraction. Previous studies of *Heliconius* behavior have only considered individual chromosomes [24] or individual loci [20,

127 27], and may have missed important contributions made by other parts of the
128 genome. Linkage maps were constructed from genotype data of 331 backcross-to-
129 cydno individuals and their associated parents [28], including 146 individual males for
130 which we had recorded attraction behaviors. We identified three unlinked QTLs on
131 chromosomes 1, 17 and 18 associated with variation in the relative amount of time
132 males spent courting red *H. melpomene* and white *H. cydno* females (Fig. 2A). Of
133 these, one is tightly linked to the optix locus on chromosome 18, which controls the
134 presence/absence of a red forewing band. Given the sample sizes feasible in
135 *Heliconius*, our analysis lacks the power to resolve smaller effect QTLs.
136 Nevertheless, modeling supports additive effects of all three detected loci (Table 1),
137 and in our mapping population these three QTLs together explain ~60% of the
138 difference between the parental species (Fig. 2B).
139
140 **Preference QTL are of large effect.** Individually, the measured effect of each QTL
141 was large, explaining between 23 and 31% of the difference between males of two
142 parental species (Fig. 2B). However, in studies with relatively small sample sizes
143 such as ours (n = 139), estimated effects of QTL are routinely over-estimated (a
144 phenomenon known as the "Beavis effect", after [29]). This is because effect sizes
145 are determined only after significance has been determined, and QTL with artificially
146 high effect sizes – due to variation in sampling – are more likely to achieve
147 'significance'. To determine the extent to which the effects of our QTL may be over-
148 estimated, we simulated QTL across a range of effect sizes, and compared the
149 distribution of measured effects for all simulations to those which would be significant
150 in our analysis. Our simulations suggest that the reported effects of our QTL are not
151 greatly over-estimated. We first considered what proportion of 'significant' simulations

152 would be smaller than our empirically measured effects (Fig. 3A). With the possible
153 exception of the QTL on chromosome 17, this suggests that the true effect sizes of
154 our QTL are likely to be large. A highly conservative threshold of 95% would suggest
155 that the QTL on chromosome 1 and 18 explain at least 10% and 20% of the
156 difference in behavior between the parental species, respectively. Adopting the
157 median values, our simulations would suggest true effects of 25%, 15% and 30%, or
158 greater, for the QTL on chromosomes 1, 17 and 18, respectively. Given simulated
159 effect sizes similar to those measured empirically, there was little bias among
160 simulation runs that achieved the genome-wide significance threshold (though there
161 is a slight deviation for the QTL on chromosome 17) (Fig. S1).

162 Although our simulations suggest the effects we have measured are
163 reasonable, ideally we would estimate effect sizes from a population of individuals
164 that were not used to determine significance. In evolutionary biology, follow-up
165 experiments such as this are uncommon; collecting phenotypic data across a large
166 number of hybrid individuals is often a considerable undertaking, and this is similarly
167 true for *Heliconius* behaviors. Nevertheless, we were able to follow-up our results for
168 the QTL on chromosome 18, using a sample of 35 individuals for which preference
169 behavior was measured, but for which we were unable to generate genotype data
170 (and so were not included in our initial QTL analysis). As reported above, the QTL on
171 chromosome 18 is in very tight linkage with the B/D color pattern locus, so that
172 segregation of the red forewing band can be used to determine genotype at this
173 locus. This analysis supports our previous result that the QTL on linkage group 18 is
174 of large effect: among these 35 hybrid males, the B/D locus explains 27% of the
175 difference in behavior between the parental species (c.f. 31% for the larger mapping
176 population, Fig. 3B).

177 **The preference QTL in linkage with warning pattern genes influence initial**
178 **attraction**. The time *Heliconius* males spend courting a particular female might
179 depend not only on male attraction, but also on the female's response (and in turn his
180 response to her behavior) [19] . To test whether the QTLs we reported above control
181 male approach behaviors (as opposed to other traits that may influence courtship),
182 we repeated our genome-wide QTL analysis but this time considered the number of
183 trials in which courtship was initiated towards cydno, and then separately, towards
184 melpomene females. These analyses each revealed a significant QTL, one on
185 chromosome 1 associated with initial attraction towards *H. cydno* females (LOD =
186 3.44, $P \sim 0.029$), and another on chromosome 18 for initial attraction towards *H.*
187 *melpomene* females (LOD = 3.10, $P \sim 0.049$). These are coincident with QTLs on
188 chromosome 1 and 18 for relative courtship time (Fig. S2).
189
190 **Different preference QTL affect different aspects of behavior.** Our analyses of
191 courtship initiation confirmed that male attraction behavior is influenced by QTLs on
192 chromosomes 1 and 18, but we were unable to detect a significant effect of
193 chromosome 17. This is in direct contrast to our analysis of the relative courtship
194 time, in which the influence of chromosome 17 was well supported. Although we
195 found no evidence of pairwise interactions between individual QTLs in our model of
196 relative courtship time, genome scans considering individuals with alternative
197 genotypes at the QTL on chromosome 18 separately revealed a significant QTL on
198 chromosome 17 (LOD = 4.16, $P < 0.05$) for heterozygous (i.e. LG18@0cM =
199 cyd:melp), but not for homozygous (i.e. LG18@0cM = cyd:cyd) males (Fig. S3).
200 Together, these results suggest that alleles on chromosome 17 may have little effect

201 on initial visual attraction but influence subsequent behaviors after courtship has
202 been initiated.

203 The male preference QTLs we have identified must influence differences in
204 male attraction towards red *H. melpomene* females, or white *H. cydno* females, or
205 towards both females types. To further explore the influence of segregating alleles at
206 these loci we considered the influence of all three QTLs on courtships directed
207 towards each female type separately. We have already robustly established a
208 significant effect of these loci on variation in the relative amount of time males spent
209 courting each female type (see Fig. 2A). Consequently, although we corrected for
210 multiple testing arising from considering three QTL across the two data sets, in these
211 analyses we did not account for multiple segregating loci across the entire genome
212 (in contrast to the results reported above). This greatly increases our power to detect
213 any influence of the QTLs on attraction towards the two species individually, but also
214 increases the likelihood of false positives. The QTL on chromosome 1 influenced the
215 number of courtships directed towards *H. cydno* females ($F_{1,145} = 11.88$, $P < 0.01$),
216 but had no significant effect on how males behaved towards *H. melpomene* females
217 ($F_{1,145} = 1.63$, $P > 0.1$). In contrast, the QTL on chromosome 17 influenced the
218 degree of courtship directed towards *H. melpomene* ($F_{1,145} = 8.97$, $P = 0.018$), but not
219 *H. cydno* females ($F_{1,145} = 0.93$, $P > 0.1$). Similarly, the QTL on chromosome 18 had
220 a significant effect on courtships directed towards *H. melpomene* ($F_{1,145} = 10.16$, $P =$
221 0.012) females (though we note that prior to Bonferroni correction there is also some
222 support for an effect on courtships directed towards *H. cydno* females: $F_{1,145} = 6.74$,
223 $P = 0.01$).
224
225

226 ## Discussion

227 We have shown that the genetic architecture of mate preference behaviors
228 can facilitate speciation in *Heliconius*. Specifically, we demonstrate that just three
229 regions of the genome are largely responsible for an important component of
230 behavioral isolation. For a behavior, presumably including a large environmental
231 component, and measured in outdoor insectaries, these results are striking. Both our
232 simulations and replication support the existence of large effect QTLs controlling this
233 important interspecific difference in behavior. Even if we adopt an especially cautious
234 approach, the QTLs on chromosomes 1 and 18 would explain *at least* 10% and 20%
235 of the difference in male preference behavior, respectively. However, a follow-up
236 analysis, exploiting individuals that were not used to determine significance (thereby
237 evading the Beavis effect), suggests that these estimates are overly conservative;
238 these data explicitly reinforce our initial estimate for the QTL on chromosome 18,
239 which explains ~30% of the difference between parents. Additional smaller effect loci
240 undoubtedly also contribute to variation in male preference, which we would be
241 unlikely to detect without very large sample sizes (a caveat shared with many QTL
242 studies of ecologically relevant behaviors e.g. [13, 29, 30]). Regardless, our results
243 suggest that during speciation, divergence between populations in both mating cue
244 and the corresponding preference behaviors can have a surprisingly simple genetic
245 architecture. This architecture will facilitate the evolution of reproductive isolation in
246 the face of gene flow by both evading the effects of drift and strengthening genetic
247 associations (*i.e.* linkage disequilibrium) between cue and preference traits, even if
248 the two are not physically linked [2, 6, 31].

249 Two of the behavioral QTL we have identified are situated on chromosomes
250 with major color pattern loci (chromosome 1 includes the *K* locus, and chromosome

251 18 includes the *optix* locus). By further strengthening the genetic associations
252 between these key components of behavioral isolation, physical linkage between
253 cues and preferences is expected to facilitate speciation with gene flow [32]. Both
254 *optix* and the *K* locus have previously been associated with variation in *Heliconius*
255 courtship behaviors [20, 24, 27]. Nevertheless, we have not previously been able to
256 robustly estimate the position of QTLs along the chromosome. The QTL we identify
257 on chromosome 1 is not tightly linked to the *K* locus. It remains to be seen whether
258 this QTL underlies the association between male preference behavior and the *K*
259 locus phenotype (a shift between white and yellow color pattern elements) previously
260 observed in crosses between *H. cydno* and *H. pachinus* [24], and within a
261 polymorphic population of *H. cydno* [27]. In contrast, our results reveal that the QTL
262 for male attraction on chromosome 18 is tightly linked to the *optix* locus, which
263 controls presence/absence of a red forewing. The mechanistic basis for linkage of
264 trait and preference loci remains unclear. There is no evidence for an inversion at this
265 locus [28]; it also seems unlikely that the same mutations control both wing pattern
266 and the corresponding attraction behavior. However, *optix* is known to function during
267 eye and neural development in *Drosophila* [33], and is expressed in the optic lobe
268 and medulla of pupal *Heliconius* [34], so it is plausible – if not likely [35] – that the two
269 traits could be controlled by different regulatory elements of the same gene. Ours
270 joins a handful of examples where genetic linkage between cue and preference has
271 been reported [13, 20, 24, 27, 36, 37], which is expected to facilitate speciation with
272 gene flow [3, 32].

273 In addition to a very simple genetic architecture of male preference behavior,
274 different aspects of preference behavior are controlled by different QTL. Differences
275 associated with QTL1 and QTL17 in the relative amount of time spent courting each

276 female type are driven by differences in attraction to either *H. cydno* or *H.*
277 *melpomene*, respectively, rather than both species. QTL18 also influences attraction
278 to *H. melpomene* much more strongly than to *H. cydno* females. This genetic
279 modularity can facilitate evolutionary change and innovation by providing a route for
280 rapid evolution of novel phenotypes [29, 38]. In *Heliconius*, the observed modularity
281 should allow different aspects of mating behavior to evolve independently. It might
282 also allow novel composite behavioral preferences to arise through hybridization and
283 recombination. Indeed, there is some evidence that this has occurred during hybrid
284 speciation in *Heliconius*, where hybrids between *H. melpomene* and *H. cydno* have a
285 novel preference similar to that of the natural hybrid species *Heliconius heurippa* [39].
286 Others factors, including habitat-use, female choice and intrinsic and extrinsic
287 hybrid incompatibilities, contribute to reproductive isolation between *H. cydno* and *H.*
288 *melpomene*. Each of these can impose a strong barrier to gene flow in their own
289 right; however, because they act early in the life cycle, habitat choice and male
290 preferences probably represent the most important contemporary barriers to gene
291 flow between *H. cydno* and *H. melpomene* (with absolute contributions estimated at
292 0.66 and 0.32, respectively [22]). Of course, the current importance of isolating
293 barriers may not reflect a barrier's historical importance, but in *Heliconius*, there are
294 many examples of within-species divergence in color pattern and corresponding male
295 preference (e.g. [27, 40, 41]), suggesting that behavioral isolation due to male
296 preferences evolves early during divergence.
297 Divergence in male preferences is likely driven by initial divergence in female
298 wing pattern. In *Heliconius*, female re-mating is a rare event [42], and males must
299 compete to find virgin females within a visually complex environment [21].
300 Divergence in female (and male) wing patterns is driven primarily by strong selection

301 for mimicry, and is likely to impose divergent sexual selection on male preferences to
302 improve their ability to find receptive females. Color pattern therefore effectively acts
303 as a female 'preference' trait (sensu [43], p.17) in that it 'biases … fertilization
304 success toward certain male phenotypes" (in this case particular behavioral
305 phenotypes). This is important because strong ecological selection on female traits,
306 which biases mating towards particular male phenotypes, will rapidly drive
307 diversification of the corresponding male trait [5]. This can provide an effective route
308 to speciation, as has been widely appreciated in examples of assortative mating
309 driven by sensory drive, such as in cichlid fishes [44]. However, it is perhaps less well
310 appreciated, that morphological traits under ecological selection (such as *Heliconius*
311 wing patterns) might impose divergent sexual selection on male preferences in a
312 similar fashion.

313 The male preferences observed here may have additionally been
314 strengthened as result of reinforcement. Non-mimetic hybrids between *H.*
315 *melpomene* and *H. cydno* suffer increased predation [18], as well as reduced mating
316 success [16] and fertility [45]. Males make a considerable reproductive investment by
317 donating a nutrient-rich spermatophore during mating [46, 47], so indirect selection
318 against poorly adapted hybrids could strengthen divergent male preferences.
319 Consistent with this, *H. melpomene* males from French Guiana, outside the range of
320 *H. cydno*, are less choosy than males from Panama, where the species co-occur and
321 are known to occasionally hybridise [15]; and similar patterns of reproductive
322 character displacement have been observed elsewhere in the *melpomene-cydno*
323 clade [48]. However, male preferences are also observed between *Heliconius*
324 populations with few opportunities for hybridization e.g. [40]. It seems likely therefore

that both direct and indirect selection contribute to the evolution of male preferences in *Heliconius*.

Under either selective regime, the genetic architecture we demonstrate here will promote the evolution of behavioral isolation by strengthening genetic associations between cue and preference. Dissasociation of alleles at loci that are physically close on the chromosome is less likely compared to that between alleles at more distant loci (due to reduced crossing over), or at loci on different chromosomes. Similarly, the substitution of large effect alleles will also increase linkage disequilibrium between cue and preference, even if they are not physically linked, because preference alleles of larger effect will more often find themselves in the same genome as alleles for the corresponding cue, compared to preference alleles with smaller effects. We cannot currently distinguish whether preference QTL result from single adaptive mutations, or represent multiple functional loci that have built up during the course of speciation. Nevertheless, the genetic basis of *Heliconius* mate preferences is remarkably similar to that for differences in the wing pattern cue. Differences in individual color pattern elements probably do involve multiple, sequential mutations (which target the same gene(s)), but 'ready-made' alleles of large phenotypic effect can be brought together in new combinations through adaptive introgression, a process we have termed 'enhancer shuffling'. This can generate a multitude of novel wing pattern phenotypes. In this way, mimicry in *Heliconius* can be achieved through hybridization and subsequent sharing of key loci between closely related species [25, 49]. The existence of large effect preference loci, potentially influencing different aspects of behavior, could similarly facilitate the

349 origin of novel phenotypes through recombination and introgression, and facilitate
350 rapid speciation.
351

352 ## Methods

353 **Butterfly collection, rearing and crossing design**. All butterfly rearing, genetic
354 crosses and behavioral experiments were conducted at the Smithsonian Tropical
355 Research Institute in Panama between August 2007 and August 2009.
356 We collected wild *Heliconius cydno chioneus* and *Heliconius melpomene rosina* from
357 Gamboa (9°7.4'N, 79°42.2' W, elevation 60 m) and the nearby Soberania National
358 Park, Panama. These were used to establish stocks maintained in insectaries in
359 Gamboa, which were further supplemented with wild individuals throughout the
360 experimental period. We established interspecific crosses by mating wild caught *H.*
361 *melpomene* males to *H. cydno* females from our stock population. In interspecific
362 crosses between *Heliconius cydno* females and *Heliconius melpomene* males, F1
363 hybrid females are sterile, restricting us to a backcrossing design. We generated
364 backcross broods to *H. cydno* and *H. melpomene* by mating F1 males to virgin
365 females from our stock populations. Brood mothers were kept individually in cages
366 (approx. 1 or 2 x 2x2m), and provided with ~10% sugar solution, a source of pollen
367 and Passiflora shoots for oviposition. Eggs were collected daily and caterpillars
368 raised individually in small pots until 4th or 5th instar, and then either in groups or
369 individually until pupation. Caterpillars were provided with fresh Passiflora leaves and
370 shoots daily.
371

372 **Behavioral assays.** We measured male attraction to *H. melpomene* and *H. cydno*
373 females in standardized choice trials [20]. Males were allowed to mature for at least 5

374 days after eclosion before testing. Males were introduced into outdoor experimental
375 cages (1x1x2m) with a virgin female of each species (0 – 10 days matched for age).
376 Fifteen-minute trials were divided into 1 min intervals, which were scored for
377 courtship (sustained hovering or chasing) directed towards each female as having
378 occurred or not occurred. Accordingly, if a male courted the same female twice within
379 a minute interval, it was recorded only once; if courtship continued into a second
380 minute, it was recorded twice. Where possible, trials were repeated for each male
381 (median = 5 trials). This resulted in three related datasets used in subsequent
382 analyses: i) the proportion of 'courtship minutes' directed towards *H. melpomene* as
383 opposed to *H. cydno* females (= "the relative amount of time males spent courting red
384 *H. melpomene* and white *H. cydno* females" = "relative courtship time"); ii) the
385 proportion of trials in which courtship was initiated towards *H. melpomene*; and iii) the
386 proportion of trials in which courtship was initiated towards *H. cydno*. In total we
387 conducted 1347 behavioral trials, and collected data from 28 *H. cydno*, 16 *H.*
388 *melpomene*, 23 F1 hybrid, 29 backcross-to-*melpomene* hybrid and 196 backcross-
389 to-*cydno* hybrid males (of which 11 performed no courtship behaviors).
390
391 **Genotyping and linkage map construction.** Genotyping and construction of
392 linkage maps has been described elsewhere [28]. In brief, backcross hybrids and
393 associated parents were preserved in 20% DMSO and 0.25M EDTA (pH 8.0) and
394 stored at -20°C. DNA was extracted with Qiagen DNeasy Blood & Tissue Kits
395 following the manufacture's protocol for animal tissue. Individuals were genotyped
396 using a RAD-sequencing approach [50] and sequenced by BGI using the Illumina
397 HiSeq 2500. Sequences were then aligned to version 2 of the *H. melpomene*
398 genome [51] using Stampy v1.0.23 [52] . Duplicates were removed with Piccard tools

399 v1.135 (http://broadinstitute.github.io/picard/), and genotype posteriors called using
400 SAMtools v1.2. Interspecific linkage maps were constructed using Lep-MAP2 [53]
401 and modules from Lep-MAP3 as described in [28]. To obtain the genotypic data for
402 QTL mapping, the parental-phased data was obtained using Lep-MAP3
403 option outputPhasedData=1. This option imputes data based on input genotypes and
404 the map order. These data were then compared to the subset of markers in which
405 grandparents could be used to phase the data for each family and chromosome
406 using custom scripts. Family and chromosome was inverted when required to obtain
407 matching phases. Finally, the non-informative markers between inferred
408 recombinations were masked (i.e. set to missing) to account for the fact the exact
409 recombination position was not known for these regions.
410
411 **Data analysis.** We were able to generate genotype data for 146 of the 196
412 backcross-to-*cydno* hybrid males for which we recorded behaviors in our choice
413 trials. The remaining 50 individuals include males from which we were unable to
414 extract sufficient DNA, were poorly sequenced, or were lost in the insectaries most
415 often due to ants or other predators. For each backcross individual, we calculated the
416 probabilities of the two alternative genotypes at every marker and centiMorgan (cM)
417 position along the chromosomes, conditional on the available marker data, using
418 R/qtl package [54]. R/qtl uses a hidden Markov model to calculate the probabilities of
419 the true underlying genotypes given the observed multipoint marker data. We then
420 tested for an association between phenotype and genotype at each position using
421 generalized linear mixed models (GLMMs) with binomial error structure and logit link
422 function (implemented with the R package lme4). We first considered the relative
423 time males courted *H. melpomene* as opposed to *H. cydno* females. For each

position along the genome we modeled the response vector of the number of ‘courtship minutes’ towards *H. melpomene* vs ‘courtship minutes’ towards *H. cydno* with the genotype probability as the independent variable. LOD scores were obtained by comparing this to a null model in which genotype probability was not included. An individual level random factor was included in all models to account for over-dispersion. This approach is analogous to the to the Haley-Knott regression implemented in R/qtl [54, 55], but more appropriately accounts for the non-normal structure of our data and for differences in total courtship data recorded for each individual [56]. Seven individuals were excluded from these analyses for which, although tested in multiple trials, no courtship towards either female type was recorded. Using permutation [57], we determined the genome-wide significance threshold for the association between marker genotype and phenotype (alpha = 0.05, n = 1000 permutations) as LOD = 2.99. By using our GLMM approach we had more power to detect QTL than would be permitted by adopting non-parametric methods. Nevertheless, we repeated all genome-wide QTL analyses using non-parametric interval mapping in R/qtl, using the ‘scanone’ and ‘model = “np” ’ commands. Results of non-parametric analyses are reported in the supplementary materials (Table S1). To consider all three QTL identified in our initial genome scans together, we again modeled the number of ‘courtship minutes’ towards H. melpomene vs ‘courtship minutes’ towards H. cydno but with the genotype at the max LOD score for each QTL as explanatory variables. The fully saturated GLMM, including all three pairwise interactions, was simplified in a stepwise manner using likelihood ratio tests, but always retaining individual id as a random factor. To further test for effects of each QTL we compared the penalized LOD scores of the full model (including all three QTL as additive effects) to reduced models in which each QTL was eliminated in

449 turn. The penalized LOD score is calculated as: pLODa(γ) = LOD(γ) –T| γ |, where γ
450 denotes a model, | γ | is the number of QTL in the model and T is a penalty
451 determined through permutation (i.e. the genome-wide significance threshold = 2.99)
452 [58] .

453 To identify QTL associated with courtship initiation towards each species we
454 again used GLMMs with binomial error structure and logit link function. This was
455 done in exactly the same way as our initial genome scan looking at relative time
456 males courted *H. melpomene* as opposed to *H. cydno* females except that the
457 response variables were i) trials in which courtship was initiated towards *H. cydno*
458 females vs trials in which courtship towards *cydno* females did not occur, and ii) trials
459 in which courtship was initiated towards *H. melpomene* females vs trials in which
460 *courtship* towards melpomene females did not occur. Here we determined the
461 genome-wide significance thresholds for the association between marker genotype
462 and phenotype (alpha = 0.05, n = 1000 permutations) as LOD = 3.08 (*cydno*) and
463 LOD = 3.02 (*melpomene*). We included all 146 backcross males for which we had
464 genotype data in this analysis.

465 Finally, to determine the contribution of each QTL to variation in courtship time
466 towards *H. cydno* and *H. melpomene* females separately, we considered the total
467 number of 'courtship minutes' directed to each female type, correcting for the number
468 of trials. We included all 146 backcross males for which we had genotype data in this
469 analysis. We square-root transformed courtship minutes/trial and then used the
470 makeqtl() and fitqtl() functions in R/qtl [54] to guide model simplification . Model
471 residuals were inspected visually for an approximate normal distribution, and we
472 tested for homogeneity of variance with Levene's tests (*H. cydno* females: F3,142 =

473 0.25, P > 0.02; *H. melpomene* females: F3,142 = 0.29, P > 0.02). We corrected P
474 values to account for the 6 tests (i.e. 3 loci x 2 species) [59].
475
476 **Simulations**. We used simulations to estimate potential inflation of measured effect
477 sizes due to the Beavis effect. We generated 10000 simulated data-sets for each of a
478 range of 'true' effect sizes for each significant QTL (i.e. on chromosomes 1, 17 and
479 18), using the R package simr [60]. For each of these we determined the LOD score
480 and compared it to our genome-wide significance threshold (i.e. LOD = 2.99). This
481 allowed us to compare i) the entire range of simulated effects (where the mean is
482 expected to equal the 'true' effect size), with those that would be significant given our
483 sample size (n = 139) and linkage map (figure S1), and ii) the empirically measured
484 effects with simulated effects that would be significant (Fig. 3A).
485
486 **Code and data availability.** Sequence data used to make the linkage maps have
487 previously been submitted to the European Nucleotide Archive
488 (http://www.ebi.ac.uk/ena) [28], accession number ERP018627.
489
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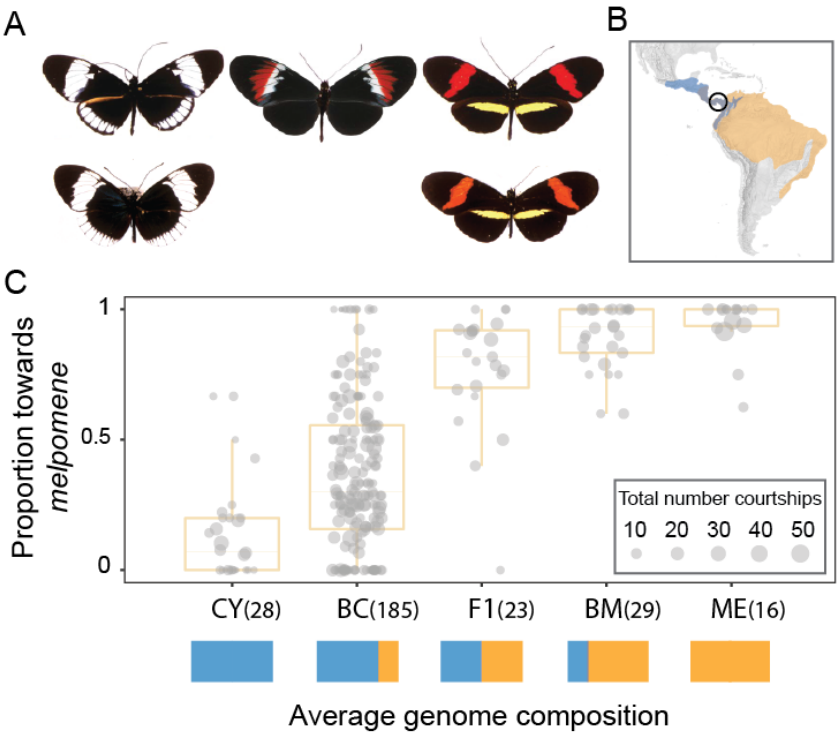
491
492
493 **Table 1. Individual and combined QTLs for differences in relative courtship time.**

Chromosome	Position (cM)	LOD score	$\Delta pLOD_a$	2ΔlnL	*P*
1	4.2 (1.8-9.1)	4.54	-1.04	18.54	<0.001
17	24.4 (0-48.3)	3.50	-1.03	18.50	<0.001
18	0 (0-13.6)	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; $\Delta pLOD_a$, 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.

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



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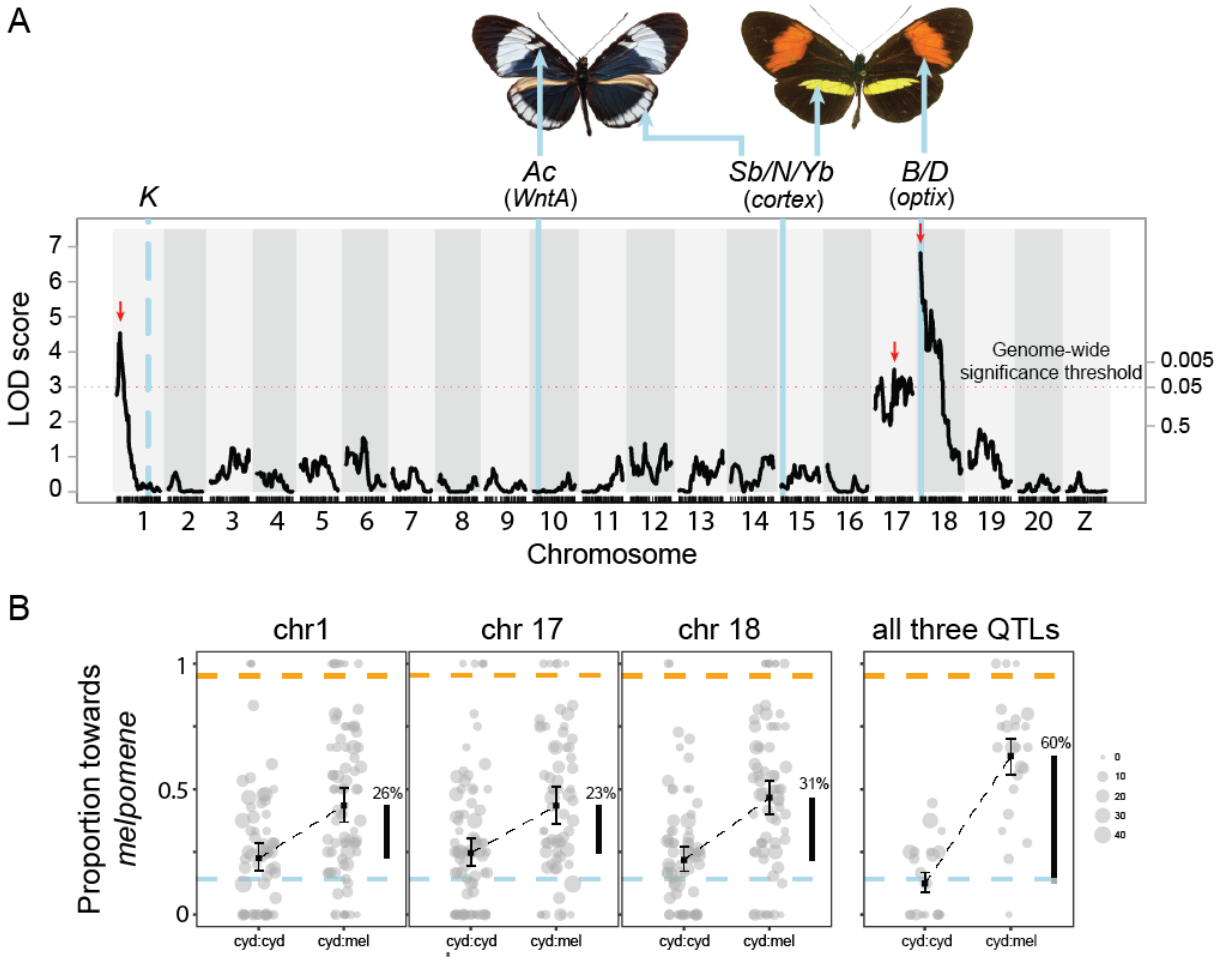
512 **Figure 2. QTL analysis of variation in mate preference.** A, QTLs for relative time males court *H.*
513 *melpomene* (as opposed to *H. cydno*) females on chromosomes 1, 17 and 18 (n = 139). Scale on right
514 axis depicts genome-wide significance (P_{perm}), determined through permutation. Dotted red line
515 represents log odds ratio (LOD) significance threshold (genome-wide alpha = 0.05, LOD = 2.99).
516 Dashes indicate position of genetic markers (SNPs) and red arrows indicate the position of the max
517 LOD score for each QTL (used in B). Horizontal blue lines represent the position of major color pattern
518 loci, and their phenotypic effects of major color pattern loci. Note that the *K* locus only has limited
519 phenotypic effects in crosses between *H. cydno chionius* and *H. melpomene rosina*, but is responsible
520 for the switch from yellow to white color pattern elements between other taxa within the *melpomene-*
521 *cydno* clade. B, Proportion of time males court *H. melpomene* (as opposed to *H. cydno*) females for
522 each of the two genotypes for respective QTLs (homozygous = cyd:cyd, and heterozygous = cyd:mel).
523 Error bars represent 95% confidence intervals. Lower dashed blue and upper orange bars represent
524 mean phenotypes measured in *H. cydno* and *H. melpomene,* respectively. Circle size depicts total
525 number of 'courtship minutes' for each male. Vertical black bars indicate the percentage of the
526 difference measured in the parental species explained.



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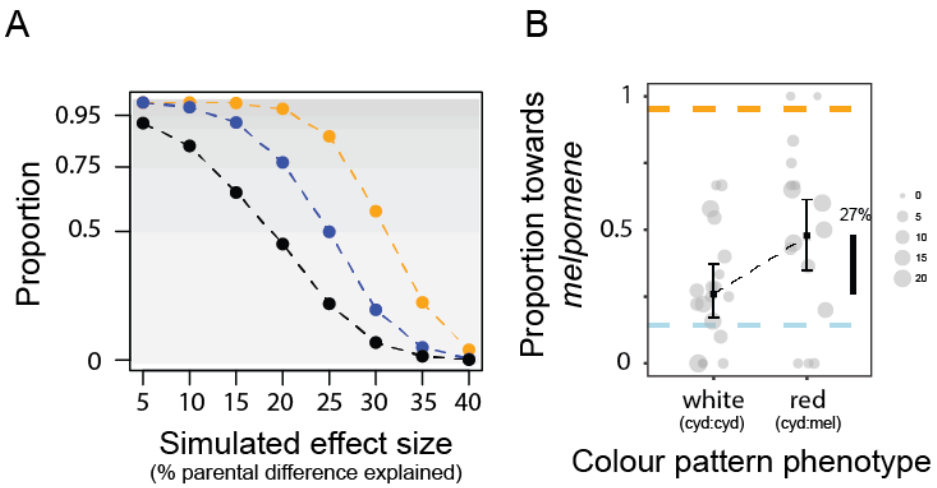
529 **Figure 3. QTL effects in consideration of the to the Beavis effect.** A, Proportion of 'significant'
530 simulations that would be smaller than our empirically measured effects, for preference QTL on
531 chromosome 1 (blue), chromosome 17 (black), and chromomsome 18 (orange). 10000 simulations
532 were run for effect sizes corresponding to between 5 and 40% of the difference in male preference
533 behavior of the parental species difference between parental species behavioral. In each case the
534 distribution of sample effect sizes was determined for those simulations that reached the genome-wide
535 significance threshold determined through permutation (figure 2). B, Proportion of time males court *H.*
536 *melpomene* (as opposed to *H. cydno*) females for each of the two genotypes for red (homozygous =
537 cyd:cyd) and white (heterozygous = cyd:mel) hybrid males for which we were unable to generate
538 RADdata (and so which were not included in our initial QTL analysis). Error bars represent 95%
539 confidence intervals. Lower dashed blue and upper orange bars represent mean phenotypes
540 measured in *H. cydno* and *H. melpomene,* respectively. Circle size depicts total number of 'courtship
541 minutes' for each male. Vertical black bars indicate the percentage of the difference measured in the
542 parental species explained.



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676

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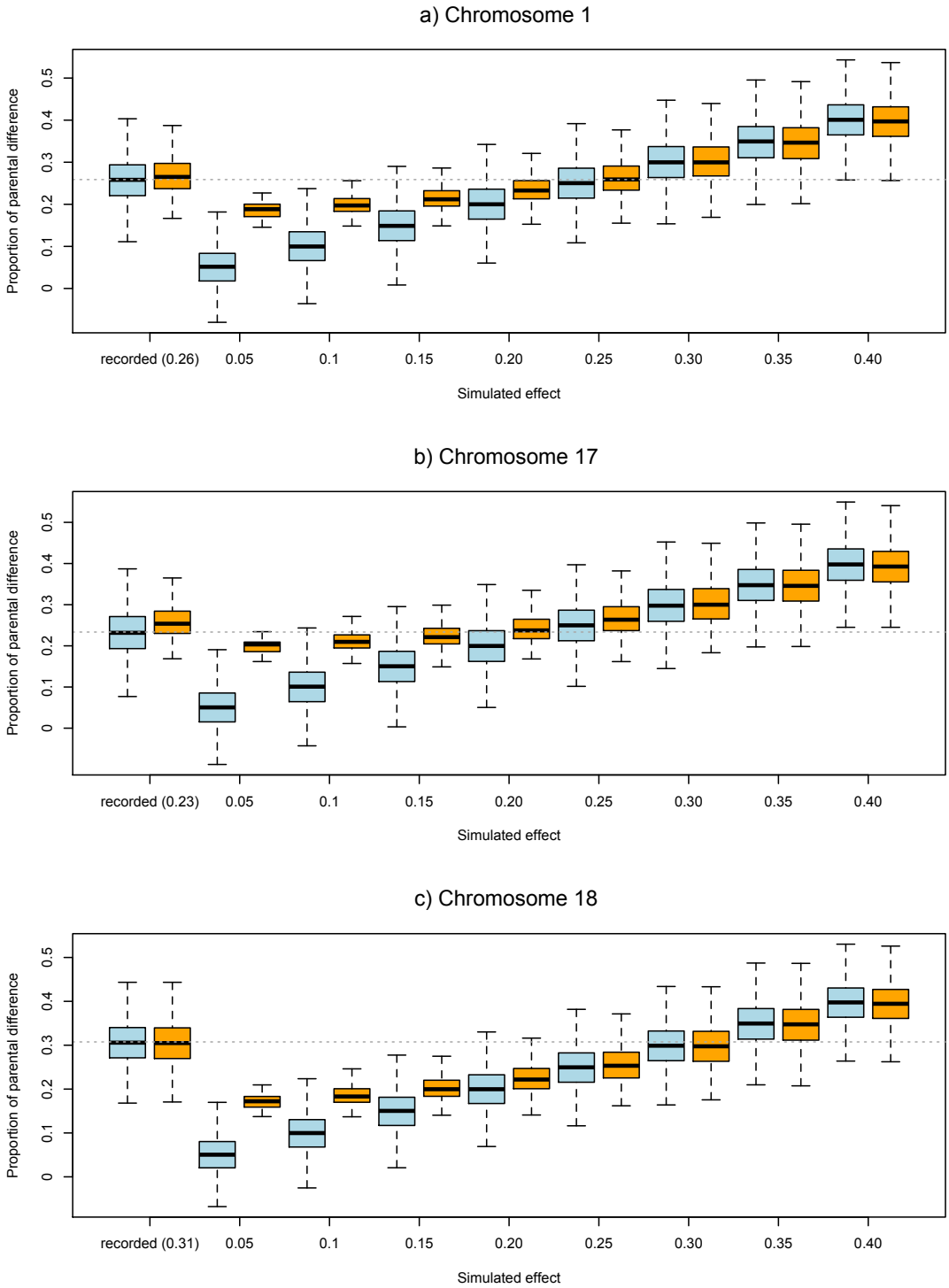
Supporting Information

Figure S1. Simulations suggest QTL effect sizes are not greatly overestimated. For each simulated effect size, the distribution of all simulated effects (blue) and those which would be significant in our analysis (*i.e.* lod ≥ 2.99) (orange) are shown. In each case, 'recorded' refers to the empirically measure effect size.



704 **Figure S2. QTL analysis of courtship initiation.** QTL associated with the proportion of trials males
705 initiated courtship towards *H. cydno* females (blue) and *H. melpomene* females (orange). Dotted blue
706 and orange lines represents log odds ratio (LOD) significance threshold (genome-wide alpha = 0.05)
707 for *cydno* (LOD = 3.08) and *melpomene* (LOD = 3.02) females, respectively. Gray boxes indicate 1.5
708 LOD interval for QTL associated with proportion of time males court *H. melpomene* (as opposed to *H.*
709 *cydno*) females on chromsomes 1, 17 and 18 (see Fig. 1a). Horizontal bars represent 1.5 LOD interval
710 for significant QTL associated with the proportion of trials males initiated courtship towards *H. cydno*
711 females (chr. 1) and *H. melpomene* females (chr. 18).

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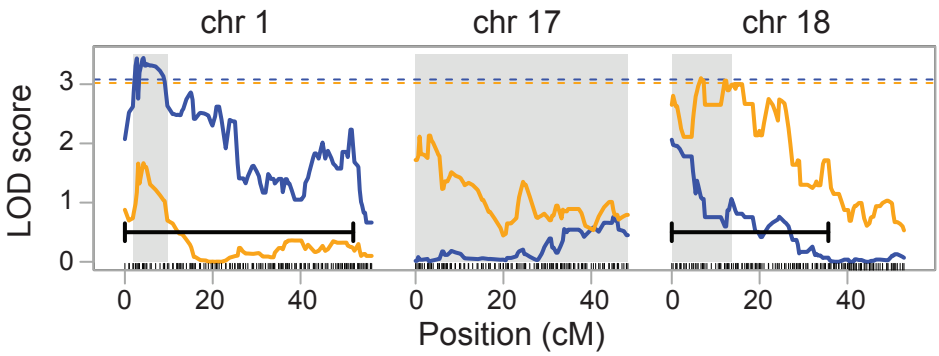

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715 **Figure S3. QTL analysis of variation of mate preference for individuals with alternative**
716 **genotypes at LG18@0cM.** QTL associated with the proportion of time males court *H. melpomene* (as
717 opposed to *H. cydno*) females on chromosomes 17 for individuals homozygous (i.e. white, cyd:cyd =
718 blue line) and heterozygous (i.e. red, cyd:mel = orange line) at LG18@0cM. Dashed line represents
719 log odds ratio (LOD) significance threshold (genome-wide alpha = 0.05, LOD =) for heterozygous
720 (i.e.red, cyd:mel) individuals. Horizontal black bars represent 1.5 LOD interval of significant the QTL.

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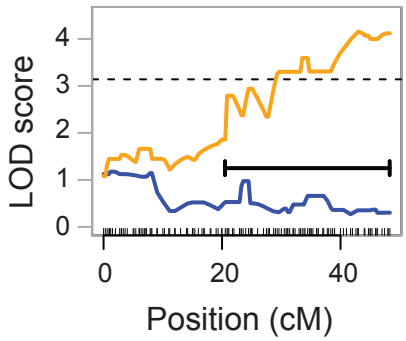

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723 **Table S1. Summary of genome-wide QTL analyses using binomial GLMM methods (reported in**
724 **main text) and non-parametric methods implemented in R/qtl.**

725
726 a) Courtship (*n* = 139)
727

	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	2.8935341	~0.049
18	0	6.83	<0.001	0	5.3432172	<0.001

728
729
730
731 b) Courtship initiation (proportion of trial in which courtship occurred) (*n* = 146)
732

		Binomial GLMM			Non-parametric		
Chromosome	Female	Position (cM)	LOD	*P*	Position (cM)	LOD	*P*
1	CP	4.23	3.44	~0.029	4.23	3.86	~0.005
	MP	4.23	1.66	~0.682	3.02	1.30	~0.871
18	CP	0	2.06	~0.371	0	2.19	~0.243
	MP	6.65	3.10	~0.049	6.65	2.98	~0.038

733
734 CP = *H. cydno chionius*; MP = *H. melpomene rosina*; *P* values are determined through permutation as
735 described in the methods. QTL are reported only for chromosomes where the genome-wide
736 significance threshold was achieved. For comparison, for (b) courtship initiation, non-significant peaks
737 are reported for attraction towards MP and CP females for chromosomes 17 and 18, respectively.
738
739