MATE PREFERENCE ACROSS THE SPECIATION CONTINUUM IN A CLADE OF MIMETIC BUTTERFLIES

Richard M. Merrill,1,2 Zachariah Gompert,3 Lauren M. Dembeck,4 Marcus R. Kronforst,5 W. Owen McMillan,4,6 and Chris D. Jiggins1

1Department of Zoology, University of Cambridge, Downing Street, Cambridge, CB2 3EJ, United Kingdom
2E-mail: r.merrill@zoo.cam.ac.uk
3Department of Botany and Program in Ecology, 3165, University of Wyoming, 1000 E. University Avenue Laramie, Wyoming 82071
4W.M. Keck Center for Behavioral Biology and Genetics Department, North Carolina State University, 3510 Thomas Hall, Box 7614, Raleigh, North Carolina 27695
5FAS Centre for Systems Biology, Harvard University, Northwest Labs, 52 Oxford Street, Cambridge Massachusetts 02138
6Smithsonian Tropical Research Institute, MRC 0580-12, Unit 9100 Box 0948, DPO AA 34002-9998

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Premating behavioral isolation is increasingly recognized as an important part of ecological speciation, where divergent natural selection causes the evolution of reproductive barriers. A number of studies have now demonstrated that traits under divergent natural selection also affect mate preferences. However, studies of single species pairs only capture a snapshot of the speciation process, making it difficult to assess the role of mate preferences throughout the entire process. Heliconius butterflies are well known for their brightly colored mimetic warning patterns, and previous studies have shown that these patterns are also used as mate recognition cues. Here, we present mate preference data for four pairs of sister taxa, representing different stages of divergence, which together allow us to compare diverging mate preferences across the continuum of Heliconius speciation. Using a novel Bayesian approach, our results support a model of ecological speciation in which strong premating isolation arises early, but continues to increase throughout the continuum from polymorphic populations through to “good,” sympatric ecologically divergent species.

KEY WORDS: Assortative mating, Heliconius, mate choice, reproductive isolation, speciation.

During ecological speciation reproductive isolation evolves as a consequence of divergent natural selection (Rundle and Nosil 2005). Behavioral isolation is increasingly seen as important, and a growing body of work has revealed traits under divergent ecological selection that also affect assortative mating. For example, in Gambusia fish, female mating preferences for body shape have evolved in tandem with divergent morphologies, resulting from different predator regimes (Langerhans et al. 2007); similarly, in Timema walking-sticks, females are more likely to mate with males from populations that use the same rather than different host plants (Nosil et al. 2002). Studies such as these capture a snapshot of the speciation process, but ecological speciation likely proceeds through the accumulation of alleles that contribute to reproductive isolation and thus gradually reduce gene flow. Unlike adaptation within populations, the evolution of reproductive isolation is unlikely to be observed within a single human
life-time (Coyne and Orr 1989); however, its course might be inferred from observations of multiple stages of divergence—from polymorphic populations to “good” species, reflecting the continuum of speciation (Mallet 2008; Peccoud et al. 2009).

A number of studies now exist that review the occurrence of hybrid sterility and/or inviability across different stages of divergence (e.g., Coyne and Orr 1989; Presgraves 2002; Price and Bouvier 2002), but only a handful address premating isolation (Coyne and Orr 1989, 1997; Mendelson 2003; Berner et al. 2009). Furthermore, although a consensus has emerged that assortative mating seems to evolve early (Coyne and Orr 2004), very few detailed studies document mate preference behaviors at different stages of divergence, and in particular include divergent populations below the species level (but see Seehausen et al. 2008). As a result, we only have a limited understanding of the role such behaviors play throughout the entire speciation process.

We study male mate preferences across four stages of divergence in a clade of mimetic butterflies that have long been considered a prime example of adaptive speciation (Bates 1881). The neotropical genus *Heliconius* is famous for Müllerian mimicry, where distantly related species converge on the same bright warning pattern to more efficiently advertise their distastefulness to predators. Whereas distantly related *Heliconius* often converge on the same color pattern, sister taxa tend to belong to different mimicry rings and, in at least five species, males prefer to court females that share their own pattern over those of closely related taxa (Jiggins et al. 2001, 2004; Kronforst et al. 2006b; Mavérez et al. 2006; Muñoz et al. 2010). Female *Heliconius* often mate soon after eclosion, when they cannot reject males, so that although females that are not mated quickly may exert a choice, male preferences make an important contribution to assortative mating. Because color pattern is also under strong frequency-dependent selection due to predation (Kapan 2001; Mallet and Barton 1989), shifts in color pattern can theoretically cause both premating and postmating isolation, thereby promoting rapid speciation.

Mate preferences based on warning color pattern is well established in *Heliconius*. However, with one notable exception (Chamberlain et al. 2009), studies of mate choice among *Heliconius* taxa below the species level have used geographically distant populations, between which there is unlikely to be significant gene flow. As a result it is uncertain whether the observed preferences are maintained between populations in the early stages of divergence or whether they break down in the face of gene flow. In addition, the use of different methodologies has made it difficult to make comparisons between studies and ultimately between different levels of divergence. Finally, although previous studies have provided estimates of population-level mate preferences, the analysis methods have not taken into account interindividual variation in preference (but see Chamberlain et al. 2009). These limitations make it difficult to test predictions regarding the divergence of mate preferences during speciation. In particular, here we are interested in whether preference differences accumulate gradually or if there is any evidence of a “step,” which may suggest different evolutionary mechanisms working at different stages of divergence. Second, we are interested in how variation in preferences at the individual level may change as populations diverge. To address these questions, we present mate preference data collected using comparable methodology and analyzed using a novel Bayesian approach that accounts for uncertainty at both the population and individual levels. In particular, we revisit previously published data (Kronforst et al. 2006b, 2007; Merrill et al. 2010; Chamberlain et al. 2009) in addition to analyzing a novel dataset, which together allow us to compare diverging mate preferences across the continuum of *Heliconius* speciation.

**Methods**

We investigated male mate preferences within a single polymorphic population as well as between three pairs of sister taxa in the melpomene-cydno clade of *Heliconius*.

**WITHIN POPULATION COMPARISON—POLYMORPHIC HELICONIUS CYDNO ALITHEA**

*Heliconius cydno alithea* is polymorphic in western Ecuador, where yellow and white morphs co-occur. These two morphs mimic and track the local frequencies of *H. eleuchia* and *H. sapho*, respectively. Although there are areas in which one of the morphs, and its respective mimic, appear to be absent, the majority of sites are highly polymorphic. This includes the populations sampled for this study. Mark–release–recapture experiments have demonstrated that the local fitness of the two morphs is a function of the abundance of their respective co-mimics (Kapan 2001). Recently, it has been shown that yellow males prefer to court females of their own type, but despite this there is no significant genetic differentiation between the two morphs (Chamberlain et al. 2009).

**WARNING-COLOR RACE COMPARISON—H. m. AGLAOPE AND H. m. AMARYLLIS**

*Heliconius melpomene aglaope* and *H. m. amaryllis* share a narrow hybrid zone in northeastern Peru. To the southwest of this division *H. m. amaryllis* shares a “postman” warning color pattern with its co-mimic *H. erato favorinus*, whereas to the northeast *H. m. aglaope* displays the “rayed” pattern alongside *H. erato emma*. This is one of the best-studied hybrid zones in the *Heliconius* literature and translocation experiments have demonstrated strong frequency-dependent selection, which maintains the integrity of the two color-pattern races (Mallet and Barton 1989). Nevertheless, within the hybrid zone recombinant individuals are abundant and gene flow remains at high levels (Baxter et al. 2010). For our experiments, individuals were collected from either
side of the hybrid zone: For *H. m. aglaope*, collection sites were at Suniplaya, 9 km from Yurimaguas Suniplaya (05°57′450″S 076°09′142″W ELEVATION 138 m) and 5 km from Micaela Bastidas (05°57′327″S 076°14′505″W, elevation 170 m); *H. m. amaryllis* individuals were collected from Urahausha Trail, Tarapoto (06°28′448″S 076°20′622″W, elevation 120 m). These were transported to Panama in glassine envelopes where stock populations were maintained.

PARAPATRIC SPECIES COMPARISON—HELICONIUS CYDNO GALANTHUS AND H. PACHINUS

Heliconius cydno galanthus and *H. pachinus* are recently diverged parapatric species restricted to opposite costal drainages in Costa Rica, where they share mimetic warning color patterns with *H. sapho* and *H. heuwitsoni*, respectively. In spite of evidence for ongoing gene flow, few hybrids have been collected suggesting that the strong mate preferences recorded in these taxa prevent substantial hybridization (Kronforst et al. 2006a, b). Nevertheless, aside from differences in color pattern, there are no known ecological differences between the two taxa and it has been suggested that *H. pachinus* may better represent a more divergent color pattern race within the *cydno* clade (e.g., Mallet et al. 1998).

SYMPATRIC SPECIES COMPARISON—HELICONIUS CYDNO CHIONEUS AND H. MELPOMENE ROSINA

Heliconius cydno and *H. melpomene* are sympatric across much of Central and northern South America. There has been much debate over the exact relationship between *H. cydno* and *H. melpomene* (Beltrán et al. 2002; Flanagan et al. 2004; Beltrán et al. 2007) and the most recent analysis places *H. cydno* within the *H. melpomene* clade (Quek et al. 2010). Nonetheless, *H. cydno* remains the sister taxa to a clade of *H. melpomene* containing individuals collected in western Ecuador, Costa Rica, and Panama. Although hybrids are very rare in nature, ongoing rates of gene flow remain significant (Bull et al. 2006; Kronforst et al. 2006a) and, in this sense at least, the speciation process is not complete. Notably, however, in addition to color pattern, these species also differ in habitat and host plant use (Estrada and Jiggins 2002; Smiley 1978). In Panama, *H. c. chioneus* is normally found in closed forest habitats and mimics the black and white pattern of *H. sapho*; *H. m. rosina*, on the other hand, occurs in secondary forest and mimics the black, red, and yellow pattern of *H. erato*. In contrast to our other comparisons, interspecific female hybrids are sterile following Haldane’s rule (Naishbit et al. 2002). To quantify divergent mate preferences we collected *H. c. chioneus* and *H. m. rosina* from Gamboa (9°87′49″N 79°842′29″W, elevation 60 m) and the nearby Soberanía National Park, República de Panamá.

We used mitochondria sequence data of the genes Col1, tRNA-leu, and Col2 to estimate genetic divergence and to place each pair of taxa along the speciation continuum (see also Mallet 2008). Sequences from 111 individuals, representing all eight populations, were obtained from GenBank. We only used sequences that originated from the same geographical regions as the butterflies used in behavioral tests. Sequences were aligned, resulting in 1513 bp for analysis. Following Mallet (2008), we calculated raw% mtDNA divergence for each pair of populations using the program DnaSP v5 (Librado and Rozas 2009). These are presented alongside a summary of taxa differences in Table 1.

PREFERENCE EXPERIMENTS

Experiments for our between warning-color race (B) and sympatric species (D) comparisons were conducted in the Smithsonian Tropical Research Institute insectaries in Gamboa, Panamá (9°87′49″N 79°842′29″W, elevation 60 m), where stock populations were also maintained. Experiments were performed between January 2008 and August 2009. Males were introduced individually into experimental cages (1 × 1 × 2 m) with a virgin female of each type (i.e., either an *H. m. aglaope*/*H. m. amaryllis* pair or an *H. c. chioneus*/*H. m. rosina* pair, 0–10 days matched for age). Female pairs were reused and replaced when fresh individuals became available. Fifteen-minute trials were divided into 1-min intervals, which were scored for courtship (sustained hovering or chasing) directed toward each female as having occurred (1) or not occurred (0). Accordingly, if a male courted the same female twice within a minute interval, it was recorded only once; if courtship continued into a second minute, it was recorded twice. Where possible, trials were repeated for each male. Essentially the same methods were used to collect data for the within population (A) and parapatric species (C) comparisons (Kronforst et al. 2006b, 2007; Chamberlain et al. 2009 for experimental details). However, there was one important difference: Rather than being tested individually, males were placed in a cage as a group before female pairs were introduced. Nonetheless, by numbering individuals, preference data were collected for individual males. We assume that these two experimental designs provide the same measure of mate preference (i.e., we assume that the presence of multiple males does not affect the preference of individual males). Consequently, each of the four datasets used here comprise cumulative counts of total courtships directed toward different female types for each male tested.

STATISTICAL ANALYSES

We used a hierarchical random effects Bayesian model to estimate individual and population (i.e., wing pattern phenotype, race, or species) level preference for (A) yellow *H. c. alithea* females (within-population comparison), (B) *H. m. amaryllis* females (warning-color race comparison), (C) *H. pachinus* females (parapatric species comparison), and (D) *H. m. rosina* females (sympatric species comparison). We assumed that the number of
**Table 1. Summary of population comparison differences.**

<table>
<thead>
<tr>
<th>Population comparison</th>
<th>Respective co-mimics</th>
<th>Geography</th>
<th>Hybridization</th>
<th>Habitat-use differences</th>
<th>Host-use differences</th>
<th>Hybrid sterility</th>
<th>% mtDNA divergence</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) White/yellow Heliconius cydno alithea</td>
<td>H. sapho/H. eleuchia</td>
<td>Single polymorphic population</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>(B) Heliconius melpomene aglaope/H. m. amaryllis</td>
<td>H. erato emmal/ H. e. favorinus</td>
<td>parapatric</td>
<td>Frequent</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>0.30</td>
</tr>
<tr>
<td>(C) Heliconius cydno galanthus/H. pachinus</td>
<td>H. sapho/H. hewitsoni</td>
<td>parapatric</td>
<td>Rare</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>1.23</td>
</tr>
<tr>
<td>(D) Heliconius cydno chioneus/H. melpomene rosina</td>
<td>H. sapho/H. erato</td>
<td>sympatric</td>
<td>Very rare</td>
<td>yes1</td>
<td>yes2</td>
<td>yes3</td>
<td>3.72</td>
</tr>
</tbody>
</table>

1*Heliconius cydno chioneus* uses closed canopy forest whereas *H. melpomene rosina* is usually found in secondary habitats.

2*H. c. chioneus* is a generalist using most available species of *Passiflora* for oviposition; *H. melpomene rosina* is a specialist, and in Panama only lays on *P. menispermifolia*. However, there are no known performance differences of either species on different host plants.

3Female F1 hybrids between these two species are sterile. There are no known fitness differences for F1 males.

courtship bouts of a male directed toward yellow *H. c. alithea*, *H. m. amaryllis*, *H. pachinus* or *H. m. rosina* females relative to the total number of courtship bouts by that male followed a binomial distribution:

\[
P(X | p, n) = \prod_j \prod_i \left( \frac{n_j}{X_0} \right)^{X_j} \left( 1 - \frac{n_j}{X_0} \right)^{(p - X_j)},
\]

where \( n_j \) is the total number of courtship bouts by male \( i \) of wing pattern type \( j \), \( x_j \) is the number of bouts directed against yellow morph *H. c. alithea*, *H. m. amaryllis*, *H. pachinus*, or *H. m. rosina* females, and \( p \) is that individual’s preference for yellow morph *H. c. alithea*, *H. m. amaryllis*, *H. pachinus*, or *H. m. rosina* females. This model likelihood function allows each individual to have a distinct preference parameter, thus allowing for variation in preference among individuals within each population. We assumed that the logit-transformed preference for an individual was given by

\[
\log \left( \frac{p_j}{1 - p_j} \right) = \beta_0 + \beta_1 * w_j + e_j,
\]

where \( w_j \) was used as an indicator variable that took on the value of 0 for yellow *H. c. alithea*, *H. m. amaryllis*, *H. pachinus*, and *H. m. rosina* males and a value of 1 for white *H. c. alithea*, *H. m. aglaope*, *H. c. galanthus*, and *H. c. chioneus*. Finally, \( e_j \) was an individual-level random effect. We assumed that this parameter followed a Normal distribution (\( \mu = 0, \tau = \tau_p \)), where \( \tau \) is precision (i.e., the reciprocal of the variance). The precision parameter (\( \tau_p \), which is the inverse of the variance) provides a statistical estimate of the variation in mate preference among individuals within a population. We calculated population-level preferences (scaled between 0 and 1) for each male type as

\[
\pi_1 = \frac{1}{1 + \exp(-\beta_0)}
\]

(for yellow *H. c. alithea*, *H. m. amaryllis*, *H. pachinus*, or *H. m. rosina*) and

\[
\pi_2 = \frac{1}{1 + \exp(-\beta_0 - \beta_1)}
\]

(for white *H. c. alithea*, *H. m. aglaope*, *H. c. galanthus* or *H. c. chioneus*). We assigned uninformative normal priors to \( \beta_0 \) and \( \beta_1 \), Normal \( (\mu = 0, \tau = 10^{-6}) \), and a uniform prior for \( \tau_p \), U (0.001, 50,000). This specification yields the following full hierarchical Bayesian model:

\[
P(p, \beta_0, \beta_1, \tau_p | V, n) \propto P(X | p, n)P(p | \beta_0, \beta_1, \tau_p) \\
\times P(\beta_0)(\beta_1)P(\tau_p).
\]

We implemented this model in WinBugs version 14 (Lunn et al. 2000) and obtained marginal posterior probability distributions for our parameters using Markov chain Monte Carlo (MCMC). For each pair of taxa, we compared five models using deviance information criterion (DIC): the full model described above (full), a model with population-level preference constrained to be equal for both wing pattern types \( (\pi_1 = \pi_2) \), and models with the population-level preference of either or both wing pattern types set to 0.5 (i.e., no preference). When comparing models we follow the guidelines proposed by Spiegelhalter et al. (2002) and treat \( \Delta \text{DIC} > 3 \) as considerable support for one model relative to
another model. For each model, we ran 150,000 Markov chain Monte Carlo (MCMC) iterations (including a 5000 iteration burnin) with three independent chains to obtain samples from the posterior distribution. Post-burnin samples from each chain were combined for parameter estimation. Parameter and deviance history plots were monitored to ensure adequate mixing and convergence to stationary distributions.

In addition to comparing posterior probability distribution for population variance parameters (i.e., the inverse of the precision parameters), we contrasted interindividual variation among populations using the Brown–Forsythe Levene-type test for equality of variances (Brown and Forsythe 1974). Specifically, we asked whether variation existed in the extent to which the median of our point estimates for individual preferences (on the logit scale and based on the median of the posterior distribution) differed from the median preference for each population (based on the median of the individual preferences). Post-hoc pairwise differences in interindividual variation were determined using Tukey’s HSD.

For comparison, and following previous studies of Heliconius mate preferences (e.g., Jiggins et al. 2001; Kronforst et al. 2006; Merrill et al. 2010), we also estimated probabilities of male courtship for each of our populations using likelihood (Edwards 1992). Details of this frequentist approach and results are presented as Supporting information.

We tested for a relationship between percent mtDNA divergence and the degree of population-level preference. We did this by fitting linear models where the response variable was the absolute deviation between each population-level preference and 0.5 (i.e., no preference). Each of the population-level preferences that we have estimated are independent of the preference for the population it is compared to and so we are justified in treating each separately. We contrasted two models using Akaike information criterion with a correction for small sample size: a model with mtDNA divergence as the sole predictor and a model including both mtDNA divergence and squared mtDNA divergence.

## Results

### Within Population Comparison—Polymorphic H. c. Alithea

A total of 751 courtship events directed toward white H. c. alithea females and 893 courtship events directed toward yellow H. c. alithea females by 63 white and 62 yellow males were recorded. Both the full model and the model with white males displaying no preference explained the data well (Table 2). These models

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**Table 2. Results from Bayesian analyses of mate preference:**

<table>
<thead>
<tr>
<th>Model</th>
<th>$\bar{D}$</th>
<th>$\hat{D}$</th>
<th>$\bar{pD}$</th>
<th>DIC</th>
<th>ΔDIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Full</td>
<td>419.966</td>
<td>379.504</td>
<td>40.462</td>
<td>460.429</td>
<td>0</td>
</tr>
<tr>
<td>$\pi_1=\pi_2$</td>
<td>427.090</td>
<td>378.916</td>
<td>48.174</td>
<td>475.265</td>
<td>14.836</td>
</tr>
<tr>
<td>$\pi_1=0.5$</td>
<td>425.555</td>
<td>383.156</td>
<td>39.400</td>
<td>461.955</td>
<td>1.526</td>
</tr>
<tr>
<td>$\pi_2=0.5$</td>
<td>425.032</td>
<td>373.985</td>
<td>51.047</td>
<td>476.079</td>
<td>15.65</td>
</tr>
<tr>
<td>$\pi_1=\pi_2=0$</td>
<td>426.709</td>
<td>376.440</td>
<td>50.269</td>
<td>476.977</td>
<td>16.548</td>
</tr>
<tr>
<td>(B) Full</td>
<td>60.271</td>
<td>56.200</td>
<td>4.070</td>
<td>64.341</td>
<td>2.124</td>
</tr>
<tr>
<td>$\pi_1=\pi_2$</td>
<td>63.288</td>
<td>60.508</td>
<td>2.780</td>
<td>66.067</td>
<td>3.85</td>
</tr>
<tr>
<td>$\pi_1=0.5$</td>
<td>64.869</td>
<td>59.838</td>
<td>5.031</td>
<td>69.900</td>
<td>7.683</td>
</tr>
<tr>
<td>$\pi_2=0.5$</td>
<td>59.894</td>
<td>57.571</td>
<td>2.323</td>
<td>62.217</td>
<td>0</td>
</tr>
<tr>
<td>$\pi_1=\pi_2=0$</td>
<td>65.283</td>
<td>62.812</td>
<td>2.470</td>
<td>67.753</td>
<td>5.536</td>
</tr>
<tr>
<td>(C) Full</td>
<td>296.378</td>
<td>291.074</td>
<td>5.304</td>
<td>301.681</td>
<td>0</td>
</tr>
<tr>
<td>$\pi_1=\pi_2$</td>
<td>285.183</td>
<td>207.873</td>
<td>77.310</td>
<td>362.494</td>
<td>60.813</td>
</tr>
<tr>
<td>$\pi_1=0.5$</td>
<td>290.092</td>
<td>222.584</td>
<td>67.507</td>
<td>357.599</td>
<td>55.918</td>
</tr>
<tr>
<td>$\pi_2=0.5$</td>
<td>284.756</td>
<td>218.896</td>
<td>65.860</td>
<td>350.616</td>
<td>48.935</td>
</tr>
<tr>
<td>$\pi_1=\pi_2=0$</td>
<td>284.894</td>
<td>207.707</td>
<td>77.187</td>
<td>362.082</td>
<td>60.401</td>
</tr>
<tr>
<td>(D) Full</td>
<td>296.378</td>
<td>291.074</td>
<td>5.304</td>
<td>301.681</td>
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<td>207.707</td>
<td>77.187</td>
<td>362.082</td>
<td>60.401</td>
</tr>
</tbody>
</table>
Figure 1. Population-level mate preferences for (A) within population comparison between yellow and white morphs of *Heliconius cydno alithea*, (B) warning-color race comparison between *Heliconius melpomene aglaope* and *Heliconius melpomene amaryllis*, (C) parapatric species comparison between *Heliconius cydno galanthus* and *Heliconius pachinus*, and (D) sympatric species comparison between *Heliconius cydno chioneus* and *H. melpomene rosina*. In each case 1 would indicate a complete preference for the red or yellow female type of the pair and 0 would indicate a complete preference for the white or rayed female type of the pair. Error bars represent 95% ETPI for estimates of preference for each population. The dashed line represents a population preference of 0.5 (i.e., no preference).

had considerably more support than the alternative models examined. Under the full model, white males had a group-level preference for yellow females of 0.464 (95% equal tail probability interval (ETPI) 0.419—0.511), whereas yellow males had a group-level preference for yellow females of 0.631 (95% ETPI 0.584—0.677) (Fig. 1A). Our estimates of interindividual variance (the inverse of precision on the logit scale) were 0.1350 (95% ETPI 0.0001—0.3997) and 0.1760 (95% ETPI 0.0037—0.4483) for white and yellow *H. c. alithea* females, respectively. Individual-level preference for yellow females ranged from 0.346 to 0.621 in white males and from 0.512 to 0.728 in yellow males (Fig. 2A).

**WARNING-COLOR RACE COMPARISON—*HELICONIUS m. AGLAOPE AND H. m. AMARYLLIS***

We recorded a total of 64 courtship events directed toward *H. m. amaryllis* females and 44 courtship events directed toward *H. m. aglaope* females by 13 *H. m. amaryllis* and 10 *H. m. aglaope* males. The two best models for the data from this experiment (i.e., the models with the lowest DIC) were the full model and the model with *H. m. aglaope* males constrained to a preference of 0.5 (i.e., no preference; Table 2). Alternative models had considerably less support than these models. Under the full model *H. m. amaryllis* males had a race-level preference of 0.699 (95% ETPI 0.556—0.831), whereas *H. m. aglaope* males had a race-level preference...
BRIEF COMMUNICATION

Figure 2. Individual-level mate preferences for (A) within population comparison between white (black circles) and yellow (red circles) morphs of *Heliconius cydno alithea*, (B) warning-color race comparison between *Heliconius melpomene aglaope* (black circles) and *Heliconius melpomene amaryllis* (red circles), (C) parapatric species comparison between *Heliconius cydno galanthus* (black circles) and *Heliconius pachinus* (red circles), and (D) sympatric species comparison between *Heliconius cydno chioneus* (black circles) and *H. melpomene rosina* (red circles). In each case 1 would indicate a complete preference for the red or yellow female type of the pair and 0 would indicate a complete preference for the white or rayed female type of the pair. Error bars represent 95% ETPI for estimates of preference for each individual. The dashed line represents a population preference of 0.5 (i.e., no preference).

The model with *H. m. aglaope* male preference constrained gave nearly identical results (not shown). There was minimal variation in preference among individuals within each wing pattern race (Fig. 2B), which was consistent with our low estimates of interindividual variance for preference (*H. m. amaryllis*: 0.0112, 95% ETPI 0.00003—4.893; *H. m. aglaope*: 0.0034, 95% ETPI 0.00003—1.767).

PARAPATRIC SPECIES COMPARISON—*HELICONIUS C. GALANTHUS AND H. PACHINUS*

A total of 545 courtship events directed toward *H. pachinus* females and 676 courtship events directed toward *H. c. galanthus* females were recorded by 35 *H. pachinus* and 59 *H. c. galanthus* males. The best model for these data was the full model with different and unconstrained preferences for *H. pachinus* and *H. c. galanthus* males (Table 2). The full model had considerably more support than all other models (ΔDIC > 3; Spiegelhalter et al. 2002). Species-level preference for *H. pachinus* males under the full model was 0.884 (95% ETPI 0.853—0.914), whereas *H. c. galanthus* males had a species-level preference of 0.199 (95% ETPI 0.171—0.229) (Fig. 1C). We detected little variation in preference among individuals within each species (Fig. 2C), which again was consistent with our estimates of interindividual variance for preference (*H. c. galanthus*: 0.0127, 95% ETPI 0.00003—0.1990; *H. m. aglaope*: 0.0017, 95% ETPI 0.00003—0.2295).

SYMPATRIC SPECIES COMPARISON—*HELICONIUS C. CHIONEUS AND H. MELPOMENE ROSINA*

We recorded 264 courtship events directed toward live *H. m. rosina* females and 172 courtship events directed toward *H. c. chioneus* females by 16 *H. m. rosina* and 27 *H. c. chioneus* males. The best model for these data was the full model with different and unconstrained preferences for *H. m. rosina* and *H. c. chioneus* males (Table 2). The full model had considerably more support than all other models (ΔDIC > 3). Species-level preference for
Our Bayesian model not only represents a more accurate statistical model for mate preference data, but also provides additional information that would not be apparent based on models that assume a single preference parameter for all individuals (including our likelihood analysis presented as Supporting information). By collecting data on courtship events directed toward female types by individual males, we are able to estimate both population and individual variation in male mating preference, while appropriately accounting for uncertainty at both of these levels (Gelman et al. 2003). This is because we modeled courtship bouts by individual males as stochastic samples given their mate preference, with each individual’s mate preference modeled as a random sample from the population or gene pool. This approach contrasts with most previous approaches to estimating mate preference in Heliconius, which have assumed a single preference parameter for all individuals within a population and have treated multiple courtship bouts by a single individual in the same way as multiple courtship bouts by different individuals (McMillan et al. 1997; Jiggins et al. 2001; Kronforst et al. 2006b; but see Chamberlain et al. 2009 for a non-Bayesian mixed-model approach similar to that taken here).

Individual variation is the raw material for evolution and ultimately speciation, but relatively few studies document levels of individual variation in preference. Studies of preference or choice in speciation biology could therefore benefit from adopting a modeling approach similar to the one we have taken here. Our own results demonstrate that individuals within a single polymorphic population display considerably greater variation in mate preference for like color pattern morphs than individuals belonging to different warning color races or species. These results suggest more segregating genetic variation for mate preference in the earlier stages of speciation, perhaps resulting from weaker selection or a shorter history of selection on mate preference.

At the population level, our results show a notable and substantial increase in the degree of preference divergence between polymorphic populations and geographic races (both of which showed minimal mtDNA divergence) and species-level comparisons. This is evidenced by the fact that the model fitted with mtDNA divergence and the square of mtDNA divergence was more likely than that which only included mtDNA divergence. Of course an important caveat to consider when interpreting our data concerns the use of different methodologies while testing male preference—that is, whether males were tested simultaneously in groups or individually. However, there were no consistent differences between methodologies in the degree of individual variation...
detected as might be expected if this has an effect. Furthermore, a previous study has shown that *Heliconius* mate preferences are unlikely to be influenced by learning from other individuals, which would be the main concern (Jiggins et al. 2004). Finally, male–male competition is unlikely to affect our results because we measured courtship rather than mating outcome.

Although the full model received the most support, the group-level preference of the white *H. c. alithea* morph was very close to showing no preference (0.465, 95% ETPI 0.419—0.511), and this was only a very slight improvement over the model where the group-level preference of the white morph was set to 0.5 (i.e., no preference). As discussed previously by Chamberlain et al. (2009), this observation may reflect differing patterns of dominance for color pattern and the corresponding mate preferences. F₁ hybrids between *H. cydno galanthus* and *H. pachinus* are white (white is dominant over yellow) and demonstrate intermediate species-specific preferences; however, among F₂ hybrids, male preference segregates with alleles at the K locus, which is responsible for the white/yellow forewing color switch both between these two species and between the two *H. c. alithea* color morphs (Kronforst et al. 2006; Chamberlain et al. 2009). If tight linkage (or pleiotropy) between color pattern and preference similarly underlies divergent mate preferences between *H. c. alithea* color morphs, and it is hard to imagine an alternative, the lack of a detectable preference among white males may simply be due to the fact that many of these will be heterozygotes at the K locus (Chamberlain et al. 2009).

Notably, the asymmetry in population-level mate preferences is strikingly similar in our second comparison below the species level, where *H. m. amaryllis* shows a preference but *H. m. aglaope* does not. Previous studies have shown differences in mate preference between races of *H. melpomene*, but were conducted between allopatric forms collected at least 200 km apart (Jiggins et al. 2004). Consequently, our experimental results provide the first evidence of partial assortative mating between adjacent populations across a color pattern hybrid zone. Nonetheless, our results do parallel previous data, which showed a similarly asymmetric preference between the upland *H. m. notabilis* and lowland *H. m. malleti* from Ecuador. In both cases the upland “postman” (*H. m. amaryllis* and *H. m. notabilis*) race shows the stronger mate preference (Jiggins et al. 2004). In contrast to the two *H. c. alithea* morphs, here the asymmetry cannot be explained by differing patterns of dominance because heterozygotes have intermediate color patterns. However, *Heliconius* have a general tendency to be attracted to red objects, which might explain why the orange and yellow patterns of *H. m. aglaope* and *H. m. malleti* are not as strongly favored by those males. Such asymmetries in premating isolation are not unknown and have been observed in Hawaiian *Drosophila* (Kaneshiro 1980), salamanders (Arnold et al. 1996), and sea snakes (Shine et al. 2002) among others. In one example, an asymmetry has been observed between two recently diverged species of parasitoid wasp in the genus *Nasonia*, where *N. oneida* discriminates but its sister species *N. giraulti* does not (Raychoudhury et al. 2010). Preferences may simply not evolve in tandem, with one group of individuals acquiring preference alleles before the other. Patterns of asymmetry are commonly seen in postmating isolation, and presumably reflect the stochastic nature of the accumulation of differences as populations diverge (Coyne and Orr 2004). Such stochasticity might similarly explain the asymmetric accumulation of premating isolation seen here (pairs A and B) and elsewhere.

The hybrid zone between *H. m. amaryllis* and *H. m. aglaope* in northeast Peru is one of the best described in the *Heliconius* literature. Previous analysis of this hybrid zone has assumed that the zone is maintained entirely by frequency-dependent mimicry selection on wing pattern, with between 20% and 50% reduced survival of foreign morphs (Mallet and Barton 1989). Considerable gene flow persists across much of the genome but is reduced for loci linked to color pattern (Baxter et al. 2010). Our data suggest that mate preferences may also contribute to hybrid zone dynamics. A common feature across all our comparisons, and for that matter *Heliconius* taxa in general, is high levels of gene flow. Gene flow is expected to break down associations between alleles under ecological selection and components of assortative mating (Felsenstein 1981). Among *Heliconius*, mimetic wing color patterns are used both to warn predators and during mate choice. Nevertheless, alleles for preference cues and the preferences themselves may also become disassociated thereby impeding the evolution of assortative mating. The unusual genetic architecture of traits involved in *Heliconius* mate choice at the species level, involving physical linkage between alleles for color pattern and preference, would allow mate preferences to be maintained in the face of gene flow (Kronforst et al. 2006; Merrill et al. 2010). However genetic studies of mate choice have yet to be carried out at the intraspecific level and it will be interesting to determine whether similar associations exist between male preference alleles and color pattern loci. This might help illuminate why *H. m. aglaope* and *H. m. amaryllis* do not show stronger preferences, more comparable to that observed between *H. c. galanthus* and *H. pachinus*.

Among *Heliconius*, male mate preferences evolve early, with differences detected both within a single polymorphic population and across a narrow intraspecific hybrid zone. Similarly, below the species level, populations of *Pundamilia* cichlids from two islands in Lake Victoria reveal differences in female preferences across the transition of light environments and the differences are more pronounced where this transition less steep (Seehausen et al. 2008). We also demonstrate an increase in the strength of premating reproductive isolation across the spectrum of
Heliconius divergence and this increase is particularly striking at the species boundary. Not only are the times since divergence greater, allowing for further accumulation of preference alleles, but these observations may additionally reflect adaptive processes that can increase premating isolation. Persistent gene flow between divergent forms can lead to enhanced mate preferences during the final stages of speciation when natural selection acts against maladaptive hybrids, in a process known as reinforcement (Dobzhansky 1937; Fisher 1958). In fact, enhanced mate preferences in areas of contact are observed between divergent Heliconius taxa and in particular those used in our species-level comparisons (Jiggins et al. 2001; Kronforst et al. 2007). Although it has never been explicitly demonstrated, we expect increased predation against recombinant color patterns and this could drive the spread of alleles for enhanced mate preferences both among H. c. galanthus and H. p. chichimeca, and H. c. chionus and H. m. rosina in a reinforcement-like process. In contrast, rampant gene flow may inhibit the fixation of preference alleles in the narrow hybrid zone that separates H. m. amaryllis and H. m. aglaope. Finally, we also observe increased preference in the H. c. chionus and H. m. rosina comparison, as compared to that between H. c. galanthus and H. p. chichimeca. Importantly the former produce sterile F1 hybrid females, while the latter produce entirely fertile and viable hybrids. This is consistent with reinforcement playing a role in the very final stages of speciation, as postmating isolation increases.

Our study considers male mate preference behaviors rather than absolute premating isolation; nonetheless there are obvious comparisons to be made with previous studies. Perhaps the most extensive analysis of the speciation continuum remains Coyne and Orr’s (1989; 1997) well-known study on closely related pairs of Drosophila species. Here mating discrimination and postmating isolation increased gradually with time, and strong premating isolation evolved much earlier among sympatric species. Reinforcement is considered the most likely explanation of this pattern, and these data are therefore broadly consistent with our study in which strong mate preferences are observed between hybridizing taxa that diverged within the last million years. Interestingly, this would suggest that reinforcement drives the evolution of premating isolation in sympathy before the evolution of strong post-mating isolation, implying a role for extrinsic postzygotic isolation (Hatfield and Schluter 1999; Egan and Funk 2009). Indeed, reproductive character displacement, a signature of reinforcement, has been observed between Heliconius taxa without intrinsic incompatibilities (Kronforst et al. 2007). Selection against hybrids is likely, due to reduced mating-success (Naisbit et al. 2001) and increased predation on nonmimetic hybrids. Here, as in many other systems, sexual isolation evolves more quickly than postmating incompatibilities (Coyne and Orr 1997; McMillan et al. 1997; Mendelson 2003).

One clear limitation of our analysis is that currently we only have comparable mate preference data for four population pairs. Nevertheless, our results span the range of divergence from populations with polymorphism for ecological traits to “good” well-differentiated species showing intrinsic postmating isolation, analogous to that discussed in other emerging model systems of speciation research such as sticklebacks (Hendry et al. 2009). Furthermore, although one novel aspect of our study is the ability to directly compare preference data, our results appear to reflect a consensus in the Heliconius literature where very recently diverged taxa show some limited pre-mating isolation (Jiggins et al. 2004; Chamberlain et al. 2009), as compared to more diverged populations and “good” species that demonstrate considerable assortative mating (McMillan et al. 1997; Jiggins et al. 2001; Kronforst et al. 2006; Mavarez et al. 2006; Giraldo et al. 2008; Muñoz et al. 2010). Interestingly, although there appears to be a clear continuum of genetic divergence (Mallet et al. 2007), to date there is little evidence for “intermediate” levels of premating isolation. That is populations seem to show almost no assortative mating or almost complete assortative mating.

Our observations suggest a model of Heliconius speciation in which an initial divergence in mate preference, perhaps as a result of selection for more efficient mate finding, reduces gene flow. This in turn might permit the further accumulation of preference alleles that strengthen premating isolation, perhaps assisted by physical linkage and driven by reinforcement. If true, this implies gene flow is both destructive and constructive during the speciation process, but that its exact role at any point during the continuum depends on whether a threshold has been crossed. Whatever the exact mechanisms responsible, our results imply that the evolution of premating evolution is a cumulative process as genetic divergence increases.

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Supporting Information

The following supporting information is available for this article:

Table S1. Results from likelihood analyses of mate preference.

Supporting Information may be found in the online version of this article.

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