

## Multiple sources of reproductive isolation in a bimodal butterfly hybrid zone

A. G. MUÑOZ\*, C. SALAZAR†‡\*, J. CASTAÑO\*, C. D. JIGGINS† & M. LINARES\*

\*Instituto de Genética, Universidad de los Andes, Bogotá D.C., Colombia

†Department of Zoology, University of Cambridge, Cambridge, UK

‡Smithsonian Tropical Research Institute, Panamá

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### Abstract

An important evolutionary question concerns whether one or many barriers are involved in the early stages of speciation. We examine pre- and post-zygotic reproductive barriers between two species of butterflies (*Heliconius erato chesteronii* and *H. e. venus*) separated by a bimodal hybrid zone in the Cauca Valley, Colombia. We show that there is both strong pre- and post-mating reproductive isolation, together leading to a 98% reduction in gene flow between the species. Pre-mating isolation plays a primary role, contributing strongly to this isolation (87%), similar to previous examples in *Heliconius*. Post-mating isolation was also strong, with absence of Haldane's rule, but an asymmetric reduction in fertility (< 11%) in inter-specific crosses depending on maternal genotype. In summary, this is one of the first examples of post-zygotic reproductive isolation playing a significant role in early stages of parapatric speciation in *Heliconius* and demonstrates the importance of multiple barriers to gene flow in the speciation process.

### Introduction

The order in which different forms of reproductive isolation evolve in the early stages of speciation can be difficult to determine, as multiple mechanisms often act simultaneously (Coyne & Orr, 2004; Doebeli, 2005; Gray, 2005; Ortiz-Barrientos & Noor, 2005). Hybrid zones offer an opportunity to study the evolution of reproductive isolation in different stages of speciation (Veen *et al.*, 2001; Bridle & Butlin, 2002; Widmer *et al.*, 2009). Studies in these zones have revealed that factors such as linkage of divergent traits and multiple pleiotropic effects of loci controlling species differences can facilitate speciation (Qvarnström & Bailey, 2009). Thus, comparative analysis of hybrid zones in different stages of divergence might help to reconstruct the sequence of events leading to speciation (Jiggins & Mallet, 2000; Cianchi *et al.*, 2003; Putnam *et al.*, 2007; Carling & Brumfield, 2008; Kawakami *et al.*, 2009; Wiley *et al.*, 2009).

In particular, bimodal hybrid zones are characterized by a high number of parental genotypes and few intermediate hybrids and thus represent a stage of divergence close to complete speciation (Jiggins & Mallet, 2000; Redenbach & Taylor, 2003; Peterson *et al.*, 2005; Arntzen *et al.*, 2009). For example, the bimodal hybrid zone between two incipient species of butterflies, *Heliconius himera* and *H. erato* in southern Ecuador, is maintained by strong prezygotic isolation because of assortative mating without intrinsic incompatibilities (Jiggins *et al.*, 1996; McMillan *et al.*, 1997). In addition, frequency-dependent predation against hybrids must also contribute to reproductive isolation (Jiggins *et al.*, 1996). The lack of hybrid incompatibility between *Heliconius himera* and *H. erato* led to the suggestion that post-mating isolation is less important in the early stages of ecological speciation. Nonetheless, this may not be the case in other bimodal hybrid zones, or in other butterfly species (Presgraves, 2002; Arntzen *et al.*, 2009). Here, we study another bimodal hybrid zone involving *H. erato*, offering the opportunity to investigate the relative importance of post-mating isolation in this group.

We have recently described the bimodal hybrid zone between *H. erato chesteronii* and *H. erato venus*, which

Correspondence: Astrid G. Muñoz, Instituto de Genética, Universidad de los Andes, Carrera 1 No 18A – 70, P.O.Box 4976, Bogotá D.C., Colombia.  
 Tel.: +57 1339 4949 ext. 2750; fax: +57 1332 4069;  
 e-mail: as-munoz@uniandes.edu.co

meet and form wing pattern hybrids at a frequency of approximately 25% in the Calima river valley, Cauca Valley, Colombia (Arias *et al.*, 2008). This is higher than the 10% hybrids found in the *H. erato* × *H. himera* hybrid zone, suggesting that *H. e. chesteronii* is at an earlier stage of speciation compared to *H. himera*. Microsatellite markers showed that all *chesteronii* × *venus* wing pattern hybrids are either backcrosses or indistinguishable from pure types, such that F<sub>1</sub> genotypes are extremely rare. The strong deviation from Hardy–Weinberg frequencies between these parapatric species in this zone must therefore be mediated by strong pre- and post-mating reproductive isolation. As in *H. himera*, the divergence between *H. e. chesteronii* and *H. e. venus* is also associated with ecology. The former is found in higher altitudes, dryer habitat and mimics *H. cydno* rather than *H. mel-pomene* (Arias *et al.*, 2008). Furthermore, the narrow hybrid zone (≈ 4 Km) is located on an ecotone from dry to wet forest, consistent with the idea that adaptation to different habitats has contributed to speciation, although there is no evidence for ecological segregation within the narrow zone (Arias *et al.*, 2008). Both species collect pollen from *Psiguria* sp. and *Gurania* sp. to obtain amino acids that are used in egg production. They lay eggs on *Passiflora suberosa* and *P. rubra* host plants (Muñoz, Pers. Obs). Also these species belong to the *Heliconius* pupal mating clade, in which males detect females at this immature stage and guard them prior to emergence. Here, we quantify the relative contribution of reproductive barriers in the *H. e. chesteronii* and *H. e. venus* hybrid zone and contrast these with *H. himera*/*H. erato*.

## Materials and methods

### Specimen sampling and experimental populations

Samples of *H. e. venus* and *H. e. chesteronii* were collected in Cauca Valley and Risaralda (Colombia) in the following localities: Ladrilleros (03°57'08"N, 77°22'03"W), Montañitas (3°41'03"N, 76°31'33"W), Calima river valley – Km 15 (3°53'60"N, 76°37'57"W) and Marsella (4°55'50"N, 75°44'43"W). Stocks of these populations were established in outdoor insectaries of 2 × 3 × 2 m<sup>3</sup> in la Vega (Cundinamarca) with access to enough pollen sources (*Lantana* and *Psiguria*) and artificial nectar (10% sugar solution). Plants were provided for oviposition, mainly *Passiflora rubra* and *P. suberosa*. Individuals of each species and their hybrids were reared as previously described (McMillan *et al.*, 1999).

### Analysis of pre-mating isolation

Two designs of mating experiments were performed to investigate the strength of prezygotic reproductive isolation in the Cauca valley hybrid zone. First, the probability of males and females to mate inter-specifically was determined in experiments without choice, recreating a

natural situation in which males encounter females singly (at adult or pupal stage). Second, we carried out choice experiments to test the importance of wing pattern as a mating cue, using wing colour pattern models.

### No-choice mating experiments

A single virgin female was exposed to a group of 6–10 mature males of the same species (*H. e. chesteronii* or *H. e. venus*) in an insectary for a period of 1 day. Males were at least 5 days old before each experiment to ensure reproductive maturity. *H. erato* shows pupal mating in the wild; males patrol the forest searching for female pupae, so they can mate with unclosed or freshly emerged females (Gilbert, 1976; Sourakov, 2008). We tested both adult and pupal females in our trials (Deinert *et al.*, 1994; McMillan *et al.*, 1997). In these experiments, we used intra-specific, inter-specific and F<sub>1</sub> individuals. The success or failure of mating was recorded throughout by direct observation of coupling and/or the presence of a spermatophore in the female abdomen and post-mating female odour (Gilbert, 1976). Mating pairs were removed and not re-used. The remaining males were re-used for the subsequent experiment, with replacement of the mated male (Number of males in Table 1). Likelihood was used to calculate the probability of mating ( $P_{ij}$ ) for each cross between female type *i* and male type *j*, using the function:  $m \log_e P_{ij} + n \log_e (1 - P_{ij})$ , where *m* and *n* are the number of experiments with success or failure to mate, respectively. Subsequently, we evaluated the minimal number of mating categories (parameters) that could explain our data using likelihood. This process involves the comparison of models with different numbers of parameters, grouping mate categories when their probabilities were statistically similar. For example, a model with two parameters ( $a \times b$  and  $c \times d$ ) represented two mating categories: intra-specific and inter-specific including four types of crosses (for more details see Appendix S1). We compared hierarchical models of mating with different number of parameters using a likelihood ratio test (McMillan *et al.*, 1997).

### Colour pattern models

The role of colour pattern in maintaining reproductive isolation between *H. e. chesteronii* and *H. e. venus* was tested in experiments carried out with wing colour pattern models. Briefly, twenty-eight males of *H. e. chesteronii* and nineteen males of *H. e. venus* were isolated after emergence, during a period no < 5 days to ensure sexual maturation. These males were tested individually against colour pattern models made with dissected female wings. Previous experiments have demonstrated the importance of cuticular hydrocarbons in *H. erato* courtship thus, for comparison, colour pattern wing models were used both without any treatment or after being washed with 96% hexane for 12 h to remove pheromones (Estrada & Jiggins, 2008). Two models were

**Table 1** Summary of no-choice experiments with adult females and pupae.

Cross type	Virgin adult female				Female pupa			
	Successful	Failure	Total	Mean of males in each experiment	Successful	Failure	Total	Mean of males in each experiment
Intra-specific or Pure	87	23	110	10	17	0	17	10
Inter-specific (♀ <i>H. e. chesteronii</i> × ♂ <i>H. e. venus</i> )	2	12	14	10	10	0	10	10
Inter-specific (♀ <i>H. e. venus</i> × ♂ <i>H. e. chesteronii</i> )	0	6	6	10	4	0	4	10
Backcrosses (♀ F <sub>1</sub> × ♂ Pure)	22	5	27	10	6	0	6	10
Backcrosses (♀ Pure × ♂ F <sub>1</sub> )	3	8	11	7	3	0	3	7
Total			174				40	

presented simultaneously, one being a 'control' with the same pattern as the males being tested and the other being an 'experimental' colour pattern. Both species were tested against the other species wing pattern and against the F<sub>1</sub> hybrid phenotype. The models were hung from nylon lines in the centre of a spherical area of 60 cm as described previously (Estrada & Jiggins, 2008; Melo *et al.*, 2009). Males observed entering the sphere were scored as an 'approach' whereas sustained fluttering towards the model was recorded as 'courtship'. Male behaviour was recorded in 5 min trials, and males were not reused between trials (Estrada & Jiggins, 2008; Melo *et al.*, 2009).

We evaluated the male response to the models using a general lineal model with binomial error distribution, or quasibinomial in the case of overdispersion. We conducted this analysis with R STATISTICAL software version 2.9.0 (<http://www.r-project.org/>). For these estimations, we set the male phenotype as the response variable (*H. e. chesteronii* or *H. e. venus*) and the model type in each experiment (control or experimental) as the explanatory variable. We evaluated approaches and courtships for each treatment (hexane and without hexane) independently, and we constructed a total of eight different input files for the analysis (data for each input are in Appendix S2).

Additionally, we estimated the probabilities  $Q_{ij}$  that males of type  $j$  approached or courted models of type  $i$  relative to that of their own type  $j$ , using likelihood. Thus, for *H. e. chesteronii* males (EC) with EC versus *H. e. venus* (EV) models, the actual probabilities are  $Q_{A_{EC \times EV}}/(Q_{A_{EC \times EV}} + 1)$  that males approach EV and  $1/(Q_{A_{EC \times EV}} + 1)$  that they approach EC. The log<sub>e</sub> likelihood for this experiment is therefore  $[X_{A_{EC \times EV}} \log_e\{Q_{A_{EC \times EV}}/(Q_{A_{EC \times EV}} + 1)\} + X_{A_{EC \times EC}} \log_e\{1/(Q_{A_{EC \times EV}} + 1)\}]$ , where  $X_{A_{EC \times EV}}$  is the number of EC males approaching EV, and  $X_{A_{EC \times EC}}$  is the number approaching to EC. The probability for courtships was obtained with the same model. The summed log<sub>e</sub> likelihood was maximized over all experiments by varying the  $Q_{i \times j}$  parameters. Maximization was carried out with the Solver algorithm implemented in Excel. Support limits were obtained by

determining the parameter values that decrease the maximum likelihood by two units, equivalent to a 95% confidence interval. Hypothesis testing was carried out using likelihood ratio tests (LRT), where the significance was established using the  $\chi^2$  distribution (Melo *et al.*, 2009).

### Analysis of post-mating isolation

We took advantage of the successful matings obtained in the no-choice experiments to estimate hybrid sterility and inviability. After mating, each female was separated into an individual insectary with enough food resources and larval host plants. Eggs were collected once a week and after eclosion, larvae were reared individually in small plastic pots. New shoots of *P. rubra* and *P. suberosa* in each pot were replaced daily to ensure larval survival. Both intra-specific and inter-specific crosses were reared in the same environmental conditions. The number of eggs laid, their hatching success and the number of individuals successfully reared to adults was recorded each week. The data were analysed with the likelihood approximation implemented in BETABINO 1.1 program (<http://www.ucl.ac.uk/taxome/jim/bin/software.html>) and explained by Jiggins *et al.* (2001a). Briefly, a beta-binomial distribution is used with count data (number of eggs) to obtain the maximum probability of observing an event (success of hatching) through different classes (type of cross). This likelihood function takes into account the variability within replicates of the same type and between different types of crosses. The program calculates the maximum log-likelihood under four models considering variation in the hatch rate among classes. As earlier, likelihood ratio tests were used to differentiate among alternative models with dissimilar number of parameters (Jiggins *et al.*, 2001b; Naisbit *et al.*, 2002; Salazar *et al.*, 2005).

Larval survival was quantified as the proportion of first instar larvae reaching the adult stage. To test for variation in this proportion among intra- and inter-specific crosses, we performed a nonparametric analysis of variance (Kruskal–Wallis) in SPSS 17.0.0. software (SPSS, 2008).

We tested for deviation from a 1 : 1 sex ratio using goodness-of-fit G tests in SPSS 17.0.0. software (SPSS, 2008).

### Contributions to reproductive isolation

To determine the relative contribution of barriers involved in reproductive isolation, we estimated the absolute contribution of the reproductive barriers analysed earlier for the *H. e. chesteronii* and *H. e. venus* hybrid zone. First, individual values were estimated as  $1 - (\text{heterospecific frequency/homospecific frequency})$  for both pre- and post-mating barriers. Thus, the individual contribution of premating isolation barriers was estimated as: sexual isolation (No-choice mating experiments) =  $1 - (\text{heterospecific mating frequency/homospecific mating frequency})$ . A proportion of this total sexual isolation was because of colour pattern, where preference (colour pattern models/with hexane) =  $1 - (\text{frequency of approaches to heterospecific model/frequency of approaches to homospecific model})$ , with the remainder of the sexual isolation assumed to be because of pheromonal communication. In the case of post-mating isolation barriers we have: hybrid viability =  $1 - (\text{hybrid mean hatch rate/parental mean hatch rate})$ . Although larval survival and sex ratio could be components of viability, no significant differences were found between crosses ( $P > 0.05$ , Table 2) so they were not included in the analysis. Mimicry selection against hybrid phenotypes, although not measured directly, was also included in this analysis as a post-mating barrier. The absolute value of each barrier was then calculated as a multiplicative function of the individual components of reproductive isolation of previous barriers during the life history (Ramsey *et al.*, 2003).

These parameters can have values between  $-\infty$  and 1, where one means total isolation, 0 implies no contribution to isolation. Negative values would represent gene

flow that is exceeding random expectation (Ramsey *et al.*, 2003). The total reproductive isolation was estimated as a sum of the absolute contribution of all barriers (Ramsey *et al.*, 2003; Nosil *et al.*, 2005).

## Results

### Analysis of pre-mating isolation

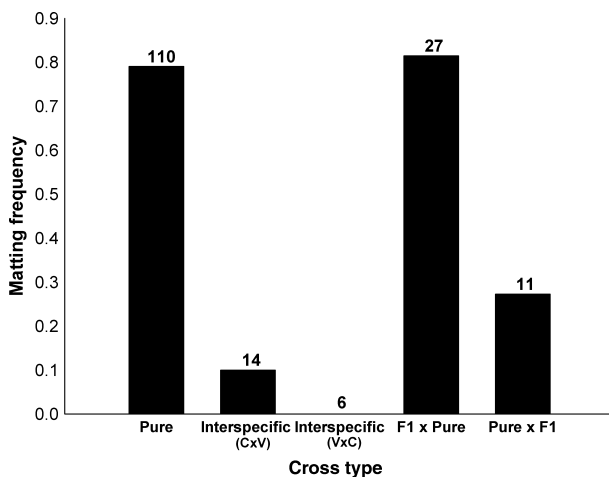
#### No-choice mating experiments

In total, 214 no-choice experiments were used to evaluate differences in the probability of mating for intra- and inter-specific crosses between *H. e. venus*, *H. e. chesteronii* and their hybrids. All the 40 pupae virgin females mated successfully irrespective of the type of males used (Table 1). In contrast, among the 174 adult virgin females, mating success was considerably lower, and there was heterogeneity in the mating probabilities with respect to the genotypes of the males and females tested. The initial twelve parameter model applied over the adult female experiments (encompassing all the types of crosses, Appendix S1) was reduced to a best fit model with five parameters: intra-specific (control), reciprocal inter-specific crosses and two types of backcrosses ( $L = -82.9070$ , Fig. 1, Table 1, Appendix S1). This model indicated a significant reduction in the mating probability in the inter-specific trials when compared to the 79% mating rate among controls. When the female was *H. e. chesteronii*, a mating success of 10% was observed. In the reverse direction, only 6 of 6 trials were completed, with no successful mating. Backcrosses to the parental species involving  $F_1$  females showed a similar frequency of successful mating (81%) compared to the controls (79%). However, there was a significant reduction in the mating probability when the  $F_1$  males were backcrossed, implying some breakdown of male hybrid mating (27%). Overall, the degree of isolation because of assortative mating is therefore likely to depend on the frequency of pupal mating in the wild.

**Table 2** Mean hatch rates, larval survival and sex ratio in control,  $F_1$ , and backcross broods.

Cross genotype				Hatch rate		Larval survival Mean (SE)	Sex ratio (female/total) Mean (SE)
Female	Male	No. experiments	No. eggs	Mean (SE)	Variance (SE)		
C	C	49	1501	0.30 (0.04)	0.0001 (0.01)	0.83 (0.8)	0.55 (0.06)
V	V	14	582	0.24 (0.06)	0.0003 (0.02)	0.82 (0.06)	0.57 (0.05)
C	V	8	492	0.14 (0.04)	0.0002 (0.01)	0.77 (0.09)	0.53 (0.07)
V	C	2	91	0.10 (0.04)	0.001 (0.002)	0.79 (0.14)	0.55 (0.05)
C × V	Pure (C or V)	16	1294	0.12 (0.02)	0.002 (0.002)	0.42 (0.08)	0.56 (0.06)
V × C	Pure (C or V)	3	98	0.35 (0.06)	0.002 (0.007)	0.82 (0.18)	0.52 (0.06)
Pure (C or V)	C × V	5	106	0.22 (0.07)	0.002 (0.01)	0.81 (0.15)	0.47 (0.06)

Hatch rate column correspond to values derived from the beta-binomial model fitted over all broods ( $P < 0.001$ ). C are *Heliconius erato chesteronii*, V are *Heliconius erato venus*, both genotypes are named as Pure. Cross types are given with the female genotype first. The standard error (SE) for each parameter is showed in brackets.

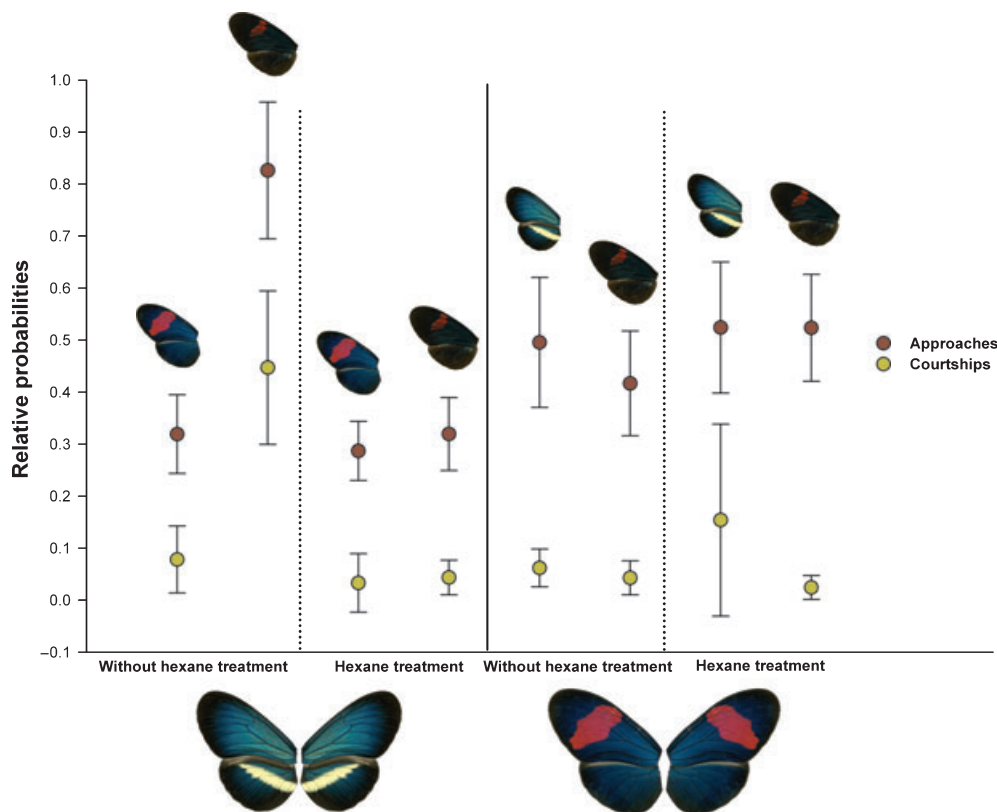


**Fig. 1** Relative probabilities in mate no-choice experiments with virgin adult females. C is *H. e. chesteronii*, V is *H. e. venus*. Cross types are given with the female genotype first. The number on each bar represents the total of experiments in the respective cross type.

We observed no assortative mating in pupal mating trials, albeit with a small sample size, but strong assortment when mating was between adults.

#### Colour pattern models

Likelihood estimates of approach and courtship probabilities were then estimated showing that in all cases males showed stronger preferences in courtships when compared to approaches (Fig. 2). Approaches and courtships of all males to experimental models were significantly different to the controls model with the same colour pattern as the male ( $P < 0.05$ , Appendix S2). In general, there was no significant effect of hexane washing of the wings, indicating that preferences are likely to be because of colour pattern rather than pheromonal cues. The only case in which we observed that treatment affected male behaviour was when F<sub>1</sub> models were exposed to male *H. e. chesteronii* (Fig. 2, Appendix S2). The F<sub>1</sub> models without hexane treatment were more attractive to *H. e. chesteronii* males. In the assays with *H. e. chesteronii* males, the probability of



**Fig. 2** Relative probabilities in wing pattern choice experiments. The males being tested are shown below the x-axis, *H. e. chesteronii* (left) and *H. e. venus* males (right). Dashed lines separate the treatments with intact dissected wings on the left and hexane-washed wings on the right. Wings above data points show the colour pattern of the experimental models (*H. e. chesteronii*, *H. e. venus* and F<sub>1</sub> in each case). The estimated probabilities of approaching (brown) and courting (green) the experimental model relative to a control with the same pattern as the male being tested are shown. The specimens in this figure were taken of Museo de Historia Natural ANDES collection: ANDES-E12320, ANDES-E12321 and ANDES-E11363.



approaching the *H. e. venus* pattern was around one-third of that to the control, and courtship probabilities were less than 10% ( $P > 0.05$ ). When the same *H. e. chesteronii* males were tested with the  $F_1$  female models a striking pattern appeared. They approached and courted models without the hexane treatment almost three times more than those washed in hexane ( $G_{approaches}$ , 27 = 158.41;  $G_{courtships}$ , 27 = 205.97;  $P < 0.05$ ). *H. e. venus* males approached *H. e. chesteronii* as well as  $F_1$  models less than half as often as to their own colour pattern. Courtships to these models were less than 20% in comparison with the controls ( $P < 0.05$ ). In these experiments, the *H. e. venus* male behaviour was not affected by hexane treatment ( $P > 0.05$ ). Overall these results show that the colour pattern has a role in mate choice, but in the case of *H. e. chesteronii*, chemical signals are also important.

### Analysis of post-mating isolation

The beta-binomial likelihood model detected significant differences in the hatch rate among all cross types ( $G$ , 8 = 46.40;  $P = 1.98 \times 10^{-07}$ , Table 2). A considerable (nonsignificant) reduction in the hatching success for both reciprocal inter-specific crosses (14% and 10%, Table 2) was observed with respect to the controls (30% and 24%, Table 2). For unknown reasons, the values for control hatch rates are low; however, experimental and controls were treated in the same way and therefore should provide comparable estimates of hatch rate. Fertility in the backcrosses was similar irrespective of the pure phenotype involved in the cross. For example, there is not a statistical difference in fertility if an  $F_1$  female mates with an *H. e. chesteronii* or an *H. e. venus* male ( $P > 0.05$ ; Table 2). The 24 backcrosses showed an

intriguing pattern whereby the hatch rate was dependent on the source of the mother. Thus, crosses of an  $F_1$  female (derived from an *H. e. chesteronii* mother and an *H. e. venus* father) to a pure male all produced a markedly reduced hatch rate (12%). In contrast, when the  $F_1$  female was derived from the reciprocal cross, the hatch rate was similar to controls (35%). In backcrosses involving  $F_1$  males, there was also a reduced hatch rate when the hybrid males were obtained from the cross *H. e. chesteronii* female by *H. e. venus* male (22%). When the survival of larvae was compared among crosses, no differences were observed (median rate equal to 0.76, Table 2) with the exception of the larvae obtained from the  $F_1$  hybrid females between an *H. e. chesteronii* female mated with an *H. e. venus* male (survival rate equal to 0.42, Table 2). Finally, none of the crosses showed any significant deviation from the expected 1:1 sex ratio ( $G$ , 1 < 3.84;  $P > 0.05$ ). Together, these experiments reveal a significant degree of post-mating isolation.

### Contributions to reproductive isolation

A summary of the reproductive barriers evaluated in this study and their contribution to reproductive isolation between *H. e. chesteronii* and *H. e. venus* are shown in Table 3. Gene flow was reduced by 97.9% between the two species (Table 3). The pre-mating barriers (colour pattern preference and pheromone preference) strongly contribute to isolation, preventing 87% of inter-specific matings. It should be noted that our experimental design does not allow a separate estimate of mating propensity and mating preference, and ideally both should be taken into account in estimating sexual isolation (Rolan-Alvarez & Caballero, 2000; Rolan-Alvarez, 2004). However, biases because of a failure to estimate mating

**Table 3** Contributions of different barriers to reproductive isolation between *H. e. chesteronii* and *H. e. venus*.

Species pair Isolation barrier	chesteronii/venus		himera/erato		melpomene/cydno	
	IC	AC	IC	AC	IC	AC
Habitat choice	–	–	–	–	0.66	0.66
Colour pattern preference	0.57	0.87	0.89	0.89	0.75	0.32
Pheromone preference	0.3				0.88	0.018
Hybrid viability (hatch rate)	0.56	0.07	0	0	–	–
Colour pattern selection	0.65?	0.039	0.65?	0.072	0.65?	0.0016
Hybrid sterility	–	–	0	0	0.7	0.0003
Total reproductive isolation		0.979		0.962		0.999

Individual contribution (IC) was estimate as  $1 - (\text{heterospecific frequency}/\text{homospecific frequency})$  in each barrier (details in Methods and Ramsey *et al.*, 2003). The absolute contribution (AC) to reproductive isolation from pre-mating effects (pheromone and colour pattern preference) is estimated from no-choice experiments with live females. This is likely to be reduced depending on the frequency of pupal mating in the wild (see text). The proportion because of colour pattern preference is estimated from model experiments, with the remainder assumed to be because of pheromonal cues. For comparison, values for the parapatric *H. himera* and *H. erato cyrba* (McMillan *et al.*, 1997) and the sympatric hybridizing species *H. melpomene* and *H. cydno* are shown (Jiggins, 2008). Colour pattern selection refers to mimicry selection against hybrid phenotypes. <sup>?</sup>This has not been estimated directly in any case, but is assumed similar to that in wing pattern hybrid zones (Jiggins, 2008).

propensity are likely to be less in controlled insectary conditions when compared to the wild (Perez-Figueroa *et al.*, 2005). Post-mating isolation barriers were also strong, although their absolute contribution was more minor because of their impact later in the life history after pre-mating barriers had taken effect (Table 3).

## Discussion

One approach to understanding speciation is to compare examples in different stages of divergence, thus assembling a broad picture of how reproductive isolation accumulates (Coyne & Orr, 2004). Hybrid zones show such a continuum of stages and are ideal places to study how barriers to gene flow evolve and their relative importance in species (Carling & Brumfield, 2008; Seehausen *et al.*, 2008; Arntzen *et al.*, 2009; Qvarnström & Bailey, 2009). Here, we have studied two taxa that form a bimodal hybrid zone in the western Cordillera of Colombia, *H. e. chesteronii* and *H. e. venus*. These are separated by partial pre- and post-mating isolation, showing that multiple sources of reproductive isolation can be important even in the early stages of speciation.

When they encounter one another as adults, *H. e. chesteronii* and *H. e. venus* show strong assortative mating. The probability of mating with a heterospecific is less than 10% that of mating with a con-specific. Nonetheless, when males of either species were presented with heterospecific females as pupae, they invariably succeeded in mating in 14 trials. *H. erato* are known to 'pupal mate' by guarding pupae and mating during or soon after female emergence (Gilbert, 1976; Deinert *et al.*, 1994), but it is unknown what proportion of matings in wild populations occur this way. Insectary populations are variable in their propensity to pupal mating (McMillan *et al.*, 1997). Larger sample sizes might reveal assortative mating even during 'pupal mating', but it is clear that the strength of premating isolation in the wild will depend on the relative frequency of matings that occur away from the pupae. This, in turn, is likely to be variable between populations depending on the density and distribution of larvae and host plants.

Nonetheless, adult males clearly show strong preferences for conspecific colour patterns as demonstrated in the experiments with wing models, leading to assortative mating preferences. They similarly are less likely to approach F<sub>1</sub> wing patterns, implying that hybrids may be selected against in mating. This is consistent with previous experiments across a wide variety of *Heliconius* taxa that have demonstrated the use of wing patterns in mate selection (Estrada & Jiggins, 2008; Melo *et al.*, 2009). In particular, a previous study of visual mate preferences showed that several races of *H. erato* were more likely to approach their own pattern relative to other patterns (Estrada & Jiggins, 2008). Furthermore, males of two races of *H. erato* were able to distinguish

wing models of their own species from the phenotypically highly similar forms of their mimic, *H. melpomene* (Estrada & Jiggins, 2008). However, this effect disappeared when the wings were washed in hexane, suggesting a likely role for chemical cues. This result prompted us to carry out the same manipulation here. Most strikingly, *H. e. chesteronii* males were around 80% as likely approach to F<sub>1</sub> wing models when compared to their own pattern, but after hexane washing this fell to around 30%. This would seem to imply that the cuticular hydrocarbons used as cues by *H. e. chesteronii* males are largely dominant and therefore expressed in F<sub>1</sub> males, but can be removed by hexane washes.

One of the most striking aspects of the hybrid zone studied between *H. himera* and *H. erato* was a complete lack of any detectable intrinsic hybrid sterility or inviability (McMillan *et al.*, 1997). In contrast, here, we have demonstrated a marked reduction in hatching rate among the offspring of both inter-specific crosses. However, this reduction was only observed in the backcrosses involving F<sub>1</sub> females from the *H. c. chesteronii* female by *H. e. venus* male cross. It remains unclear whether this is a sperm-egg incompatibility, perhaps resulting in a failure of fertilization, or alternatively an F<sub>1</sub> hybrid breakdown such that development fails in the early stages of embryogenesis prior to hatching. There was also a lack of Haldane's rule, with no deviation from parity in sex ratios.

Overall, our data provide an explanation for the deviation from Hardy-Weinberg found in the *H. e. chesteronii* × *H. e. venus* hybrid zone in the Cauca Valley (Arias *et al.*, 2008). Only around 25% of individuals collected in the zone of contact were hybrids, significantly fewer than would be expected under Hardy-Weinberg equilibrium, implying partial reproductive isolation between the taxa. Here, we have shown that this is likely a result of both pre- and post-mating barriers, representing approximately a 98% reduction in gene flow between parental forms. This is slightly higher than that estimated for *H. himera* and *H. erato cyrbia*, which is surprising as hybrids are rarer in the latter hybrid zone. However, these estimates do not include many ecological factors, and also likely include considerable error. In particular, for example, for inter-specific mate choice involving *H. e. venus* females where sample sizes were small in this study. Overall, the two pairs of taxa are similar in both showing strong pre-mating isolation, if all mating is assumed to occur between adult butterflies (Table 3). Other closely related *Heliconius* species and races also show similarly strong pre-mating isolation (Estrada & Jiggins, 2008; Melo *et al.*, 2009). In contrast, post-zygotic isolation observed here is much greater than that seen between *H. himera* and *H. erato* (McMillan *et al.*, 1997), and more similar to that between the more divergent sympatric species *H. cydno chioneus* and *H. melpomene rosina* from Panama (Naisbit *et al.*, 2001). The only other example of post-mating isolation between geographical races of *Heliconius* involves crosses

between *H. m. melpomene* from French Guiana and *H. m. rosina* from Panama, in which partial hybrid sterility was observed. However, in this case, these are geographically distant populations that may be separated through isolation by distance, rather than representing incipient parapatric species (Jiggins *et al.*, 2001b). Thus, in the Cauca valley *H. erato* hybrid zone, post-zygotic isolation has evolved faster than in other *Heliconius* species.

One of the current areas of interest in speciation research is the question of whether one strong or many weak barriers to gene exchange are important in the early stages of speciation (Coyne & Orr, 2004; Schluter, 2009). *Heliconius* butterflies are one of a number of cases where the importance of particular ecological traits has been emphasized. Butterfly wing patterns, the beaks of Darwin's Finches or the body size of lake sticklebacks are all traits involved in ecological speciation, and have been highlighted as major contributors to this kind of speciation (Podos, 2001; Jiggins, 2008; Schluter, 2009). Nonetheless, it is important to consider the contribution of all possible barriers (Coyne & Orr, 2004) to assess the relative importance of these ecological factors. Several other recent studies of incipient species have demonstrated the importance of multiple factors of more minor effect in reproductive isolation (Ramsey *et al.*, 2003; Dopman *et al.*, 2010). For example, between the E and Z strains of the European Corn Borer, seven of 12 potential sources of reproductive isolation were found to make a significant contribution (Dopman *et al.*, 2010).

The difference may relate to the relative importance of geographical isolation. Where speciation is driven by strong selection in the face of gene flow, one or a few major factors that are under strong selection are likely to play a key role, whereas speciation involving geographical separation is more likely to involve accumulation of multiple factors of more minor effect (Coyne & Orr, 2004). In the case of *H. e. chesteronii*, there are several barriers to gene exchange with *H. erato*, perhaps consistent with the fact that the Cauca valley is largely isolated from the Pacific slopes apart from a few narrow zones of contact, suggesting a history of limited parapatric contact. In the future, it will be interesting to further investigate the correlation between genetic architecture and the geographical context of speciation.

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## References

- Arias, C.F., Munoz, A.G., Jiggins, C.D., Mavarez, J., Bermingham, E. & Linares, M. 2008. A hybrid zone provides evidence for incipient ecological speciation in *Heliconius* butterflies. *Mol. Ecol.* **17**: 4699–4712.
- Arntzen, J.W., Jehle, R., Bardakci, F., Burke, T. & Wallis, G.P. 2009. Asymmetric viability of reciprocal-cross hybrids between crested and marbled newts (*Triturus cristatus* and *T. marmoratus*). *Evolution* **63**: 1191–1202.
- Bridle, J.R. & Butlin, R.K. 2002. Mating signal variation and bimodality in a mosaic hybrid zone between *Chorthippus* grasshopper species. *Evolution* **56**: 1184–1198.
- Carling, M.D. & Brumfield, R.T. 2008. Haldane's rule in an avian system: using cline theory and divergence population genetics to test for differential introgression of mitochondrial, autosomal, and sex-linked loci across the *Passerina* bunting hybrid zone. *Evolution* **62**: 2600–2615.
- Cianchi, R., Ungaro, A., Marini, M. & Bullini, L. 2003. Differential patterns of hybridization and introgression between the swallowtails *Papilio machaon* and *P. hospiton* from Sardinia and Corsica islands (Lepidoptera, Papilionidae). *Mol. Ecol.* **12**: 1461–1471.
- Coyne, J.A. & Orr, H.A. 2004. *Speciation*. Sinauer Associates, Inc. Publishers, Sunderland, Massachusetts USA.
- Deinert, E.I., Longino, J.T. & Gilbert, L.E. 1994. Mate competition in butterflies. *Nature* **370**: 23–24.
- Doebeli, M. 2005. Adaptive speciation when assortative mating is based on female preference for male marker traits. *J. Evol. Biol.* **18**: 1587–1600.
- Dopman, E.B., Robbins, P.S. & Seaman, A. 2010. Components of Reproductive Isolation between North American Pheromone Strains of the European Corn Borer. *Evolution* **64**: 881–902.
- Estrada, C. & Jiggins, C.D. 2008. Interspecific sexual attraction because of convergence in warning colouration: is there a conflict between natural and sexual selection in mimetic species? *J. Evol. Biol.* **21**: 749–760.
- Gilbert, L.E. 1976. Postmating female odor in *Heliconius* butterflies: a male-contributed antiaphrodisiac? *Science* **193**: 419–420.
- Gray, D.A. 2005. Does courtship behavior contribute to species-level reproductive isolation in field crickets? *Behav. Ecol.* **16**: 201–206.
- Jiggins, C.D. 2008. Ecological speciation in mimetic butterflies. *Bioscience* **58**: 541–548.
- Jiggins, C.D. & Mallet, J. 2000. Bimodal hybrid zones and speciation. *Trends Ecol. Evol.* **15**: 250–255.
- Jiggins, C.D., McMillan, W.O., Neukirchen, W. & Mallet, J. 1996. What can hybrid zones tell us about speciation? The case of *Heliconius erato* and *H. himera* (Lepidoptera: Nymphalidae). *Biol. J. Linn. Soc.* **59**: 221–242.
- Jiggins, C.D., Linares, M., Naisbit, R.E., Salazar, C., Yang, Z.H. & Mallet, J. 2001a. Sex-linked hybrid sterility in a butterfly. *Evolution* **55**: 1631–1638.
- Jiggins, C.D., Naisbit, R.E., Coe, R.L. & Mallet, J. 2001b. Reproductive isolation caused by colour pattern mimicry. *Nature* **411**: 302–305.
- Kawakami, T., Butlin, R.K., Adams, M., Paull, D.J. & Cooper, S.J. 2009. Genetic analysis of a chromosomal hybrid zone in the Australian morabine grasshoppers (*Vandiemenella*, *Viatica* species group). *Evolution* **63**: 139–152.



- McMillan, W.O., Jiggins, C.D. & Mallet, J. 1997. What initiates speciation in passion-vine butterflies? *Proc. Natl. Acad. Sci. USA* **94**: 8628–8633.
- McMillan, W.O., Weight, L.A. & Palumbi, S.R. 1999. Color pattern evolution, assortative mating and genetic differentiation in brightly colored butterflyfishes (Chaetodontidae). *Evolution* **53**: 247–260.
- Melo, M.C., Salazar, C., Jiggins, C.D. & Linares, M. 2009. Assortative mating preferences among hybrids offers a route to hybrid speciation. *Evolution* **63**: 1660–1665.
- Naisbit, R.E., Jiggins, C.D. & Mallet, J. 2001. Disruptive sexual selection against hybrids contributes to speciation between *Heliconius cydno* and *Heliconius melpomene*. *Proc Biol Sci* **268**: 1849–1854.
- Naisbit, R.E., Jiggins, C.D., Linares, M., Salazar, C. & Mallet, J. 2002. Hybrid sterility, Haldane's rule and speciation in *Heliconius cydno* and *H. melpomene*. *Genetics* **161**: 1517–1526.
- Nosil, P., Vines, T.H. & Funk, D.J. 2005. Perspective: reproductive isolation caused by natural selection against immigrants from divergent habitats. *Evolution* **59**: 705–719.
- Ortiz-Barrientos, D. & Noor, M.A. 2005. Evidence for a one-allele assortative mating locus. *Science* **310**: 1467.
- Perez-Figueroa, A., Caballero, A. & Rolan-Alvarez, E. 2005. Comparing the estimation properties of different statistics for measuring sexual isolation from mating frequencies. *Biol. J. Linn. Soc.* **85**: 307–318.
- Peterson, M.A., Honchak, B.M., Locke, S.E., Beeman, T.E., Mendoza, J., Green, J., Buckingham, K.J., White, M.A. & Monsen, K.J. 2005. Relative abundance and the species-specific reinforcement of male mating preference in the *Chrysomus* (Coleoptera: Chrysomelidae) hybrid zone. *Evolution* **59**: 2639–2655.
- Podos, J. 2001. Correlated evolution of morphology and vocal signal structure in Darwin's finches. *Nature* **409**: 185–188.
- Presgraves, D.C. 2002. Patterns of postzygotic isolation in Lepidoptera. *Evolution* **56**: 1168–1183.
- Putnam, A.S., Striber, J.M. & Andolfatto, P. 2007. Discordant divergence times among Z-chromosome regions between two ecologically distinct swallowtail butterfly species. *Evolution* **61**: 912–927.
- Qvarnström, A. & Bailey, R.I. 2009. Speciation through evolution of sex-linked genes. *Heredity* **102**: 4–15.
- Ramsey, J., Bradshaw, H.D., Jr & Schemske, D.W. 2003. Components of reproductive isolation between the monkeyflowers *Mimulus lewisii* and *M. cardinalis* (Phrymaceae). *Evolution* **57**: 1520–1534.
- Redenbach, Z. & Taylor, E.B. 2003. Evidence for bimodal hybrid zones between two species of char (Pisces: *Salvelinus*) in northwestern North America. *J. Evol. Biol.* **16**: 1135–1148.
- Rolan-Alvarez, E. 2004. Evolution of asymmetry in sexual isolation: a criticism of a test case. *Evol. Ecol. Res.* **6**: 1099–1106.
- Rolan-Alvarez, E. & Caballero, A. 2000. Estimating sexual selection and sexual isolation effects from mating frequencies. *Evolution* **54**: 30–36.
- Salazar, C.A., Jiggins, C.D., Arias, C.F., Tobler, A., Bermingham, E. & Linares, M. 2005. Hybrid incompatibility is consistent with a hybrid origin of *Heliconius heurippa* Hewitson from its close relatives, *Heliconius cydno* Doubleday and *Heliconius melpomene* Linnaeus. *J. Evol. Biol.* **18**: 247–256.
- Schluter, D. 2009. Evidence for ecological speciation and its alternative. *Science* **323**: 737–741.
- Seehausen, O., Terai, Y., Magalhaes, I.S., Carleton, K.L., Mrosso, H.D., Miyagi, R., van der Sluijs, I., Schneider, M.V., Maan, M.E., Tachida, H., Imai, H. & Okada, N. 2008. Speciation through sensory drive in cichlid fish. *Nature* **455**: 620–626.
- Sourakov, A. 2008. Pupal mating in Zebra longwing (*Heliconius charithonia*): photographic evidence. *News of the Lepidopterists' Society* **50**: 26–29.
- SPSS 2008. *Version 17.0.0.*, SPSS Inc., Chicago.
- Veen, T., Borge, T., Griffith, S.C., Saetre, G.P., Bures, S., Gustafsson, L. & Sheldon, B.C. 2001. Hybridization and adaptive mate choice in flycatchers. *Nature* **411**: 45–50.
- Widmer, A., Lexer, C. & Cozzolino, S. 2009. Evolution of reproductive isolation in plants. *Heredity* **102**: 31–38.
- Wiley, C., Qvarnstrom, A., Andersson, G., Borge, T. & Saetre, G.P. 2009. Postzygotic isolation over multiple generations of hybrid descendents in a natural hybrid zone: how well do single-generation estimates reflect reproductive isolation? *Evolution* **63**: 1731–1739.

## Supporting information

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

**Appendix S1** No-choice likelihood parameters.

**Appendix S2** Data for colour pattern models analysis.

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