Ecological and genetic associations across a *Heliconius* hybrid zone

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Abstract

Differences in habitat use can bridge early and late stages of speciation by initiating assortative mating. *Heliconius* colour pattern races might select habitats over which each pattern confers a relative fitness advantage because signal efficacy of wing patterns can vary by environment. Thus habitat preferences could serve to promote the evolution of mimetic colour patterns for mate choice. Here I compare colour pattern genotype and phenotype frequencies to environmental variation across the *H. erato hydara* × *H. erato erato* hybrid zone in French Guiana to determine whether races exhibit habitat preferences. I found that genotype and phenotype frequencies correspond to differences in land cover moreso than to other environmental factors. Temporal shifts in colour pattern genotypes, phenotypes and land cover also were associated at individual sample sites, which further suggests that *H. erato* races differ in habitat use and that habitat preferences may promote speciation among *Heliconius* butterflies.

Introduction

Speciation has been portrayed as a process that unfolds over a continuum, where early stages of adaptive divergence progress to later stages of reproductive isolation (Mallet *et al.*, 1998). In *Heliconius* butterflies, the adaptive divergence of mimetic wing colour patterns precedes assortative mating (Mallet *et al.*, 1998; Jiggins *et al.*, 2001, 2004). Although little is known about the specific forces that initiated *Heliconius* colour pattern radiations (Turner & Mallet, 1996), studies indicate that the evolutionary divergence of *Heliconius* butterflies is driven by natural and sexual selection (Benson, 1972; Jiggins *et al.*, 2004). Reciprocal transplant experiments have demonstrated that strong frequency-dependent selection maintains colour pattern differences among *H. erato* colour pattern races (Benson, 1972; Mallet & Barton, 1989a), and laboratory crosses indicate that colour pattern races and sister species prefer mates with whom they share a common colour pattern (McMillan *et al.*, 1997; Jiggins *et al.*, 2001, 2004; Kronforst *et al.*,

2006; Mavarez *et al.*, 2006). Jiggins *et al.* (2004) have suggested that natural selection for colour pattern fidelity among Müllerian mimics could involve strong epistasis between alleles conferring local adaptation and mate preference, and therefore could act as a mechanism for speciation among *Heliconius* butterflies (Futuyma & Mayer, 1980; Jiggins *et al.*, 2004). Alternatively, reinforcement (Dobzhanksy, 1940; Jiggins *et al.*, 2001; Kronforst *et al.*, 2007), a profound mimetic switch (Naisbit *et al.*, 2001), and habitat preferences (MacCallum *et al.*, 1998) also could lead to assortative mating following adaptive divergence of wing colour patterns.

Comparatively little consideration has been given to the possibility that habitat preferences bridge early and late stages of speciation in *Heliconius* butterflies. As *Heliconius* wing colour patterns are under natural and sexual selection, the relative fitness advantage and distribution of species and colour pattern races might be expected to conform to environmental conditions that govern the efficacy of signal emission, signal transmission through the environment and signal detection (Endler & McLellan, 1988; Endler, 1992, 1993). Pattern reflectance varies depending on ambient environmental light and pattern composition (Sweeney *et al.*, 2003; Langham, 2004). Wing colour patterns may appear differently in different light environments, such that a colour pattern may be an

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effective signal in some environments while relatively ineffective in others. The evolution of colour patterns as signals driving divergence of colour pattern races and incipient species might therefore be expected to involve preference or choice in habitat use (Endler, 1993; Endler & Thery, 1996; Seehausen *et al.*, 1997; Fleishman, 2000).

One approach to assessing whether habitat preferences could bridge early and late stages of speciation in *Heliconius* butterflies is to evaluate whether the location and width of intraspecific hybrid zones reflect environmental gradients (MacCallum *et al.*, 1998). Further assessment of ecological associations of parental and hybrid populations might also demonstrate whether phenotypes are randomly distributed or repeatedly associated with environmental variables (Cruzan & Arnold, 1993). Close correspondence between phenotypes and habitat would imply that habitat mediates mimetic signalling and mate choice recognition among closely related *Heliconius* butterflies.

In this study, I examined how spatial and temporal changes in a forest-savannah ecotone correspond to shifts in colour pattern frequencies across a *Heliconius erato* (Linnaeus, 1758) hybrid zone in French Guiana. I first characterized the contemporary structure of the hybrid zone according to a two-locus model of genotypic differences between *H. erato erato* and *H. erato hydara*, and similarly assessed the width and position of the forest-savannah ecotone from remotely sensed satellite imagery. To determine whether a relationship occurs among wing colour patterns and habitat, I compared the frequency of colour pattern genotypes and phenotypes to the distribution of forest cover across the hybrid zone. Through a GIS-based analysis of land cover change and reference to a previous study of the hybrid zone, I then examined how temporal shifts in colour pattern frequencies correspond to local and regional changes in forest cover over time. I found that the distributions of genotypes and phenotypes correspond more strongly to land cover than to other environmental factors such as elevation. Temporal frequency shifts in colour pattern genotypes and phenotypes also were associated with land cover change at individual sample sites, although conservative estimates indicate the colour pattern clines have remained stable over time. The relationships that occur between colour pattern expression and land cover nonetheless indicate that habitat heterogeneity plays an important role in structuring the distribution of colour pattern races, and suggest that ecological differences among *H. erato* races could promote critical stages of speciation.

Materials and methods

Hybrid zone structure

Heliconius erato erato and *H. erato hydara* hybridize in areas of contact along the coast of French Guiana (Benson, 1982). In 1970, Benson (1982) surveyed three north-south transects across the hybrid zone. The farthest eastern transect ran from Cayenne to the south of Roura, and the central transect ran from Sinnamary south into the upland plateau. The third transect was located along the western border of French Guiana close to Suriname, from Mana southwards to St Laurent du Maroni. Benson (1982) characterized clinal transitions at three wing colour pattern traits along each transect to describe the hybrid zone. Benson (1982) found that the three clines were coincident across each transect and that the cline widths across the transects ranged from 20 to 30 km. He proposed that differences in cline width corresponded to underlying topography that varied the width of a coastal forest-savannah ecotone (Benson, 1982).

To demonstrate the relationship among clines of wing colour pattern traits and the coastal French Guiana forest-savannah ecotone, new collections were made along the eastern Cayenne transect in May 2000 and January 2001 – henceforth referred to as the '2001' sampling period. The Cayenne transect was of particular interest because it penetrated farther into upland forest and encompassed more sampling locations and more sampled individuals than the other two 1970 transects (Table 1). In 2001, butterflies were collected at 16 locations along the transect (Table 1, Fig. 1). Nine of the 16 locations were the same sites that Benson (1982) sampled in 1970. The other 1970 sites could not be resampled because of either limited road access or localized deforestation. However, because of road building efforts since 1970, it was possible to extend the overall length of the Cayenne transect to include more northern coastal sites and additional inland locations beyond the most southern 1970 collection location (Table 1). Individual butterflies were typically sighted visiting adult food plants and caught with aerial nets.

Following a two-locus model (*Appendix 1*) for wing pattern differences among *H. erato erato* and *H. erato hydara* (Fig. 1), each individual was genotyped at the D^{RY} locus that controls the expression of a dennis-ray complex and the colour of the forewing band, and the *Sd* locus that controls the shape of the forewing band. Frequencies of genotypes and alleles were directly estimated from the individuals sampled at each site (Blum, 2002a). To be consistent with the original characterization of the hybrid zone, the frequencies were scored relative to how wing colour pattern traits are expressed by pure *H. erato hydara* (Benson, 1982). Using the maximum likelihood (ML) approach described in Dasmahapatra *et al.* (2002) and Blum (2002a), sigmoidal tanh clines for each locus were then fitted and compared to assess potential differences in width and position.

Landscape analysis of the Cayenne transect

In 1970, Benson (1982) observed that pure *H. erato erato* populations were only present in unbroken forest. To

Table 1 Sample location information along the Cayenne transect visited in 1970 and 2001, with environmental data collected for each location. Also presented: the frequency of the three different wing colour pattern traits and a trait index calculated for each location per sampling period; the frequency of the *dr* and *sd* alleles at each sample location in 2001, assuming a two locus model of colour pattern expression. All traits and the trait index are calculated in reference to *Heliconius erato hydara* trait expression.

Site no.	Location	Sample size 1970	Sample size 2001	Longitude	Latitude	Km south of Cayenne	Elevation (m)	% forest 2001	% open 2001	Dennis-ray 1970	FW band colour 1970	FW band shape 1970	index 1970	Dennis-ray 2001	FW band colour 2001	FW band shape 2001	index 2001	dr 2001	sd 2001
1	Sablance		15	52.25	4.58	0.5	2	32.43	47.49					1	1	0.88	0.96	1	0.83
2	Belle Terre		5	52.24	4.56	1	2	32.24	46.83					1	1	0.9	0.97	1	0.80
3	Montsinery		36	52.27	4.55	1.5	8	56.77	34.79					0.89	0.90	0.93	0.91	0.93	0.88
4	Mt du Tigre	6	19	52.18	4.54	2	7	16.63	55.28	1	1	1	1	1	1	0.91	0.97	1	0.90
5	Mt Mahury	6	3	52.16	4.53	5	57	16.95	56.02	1	1	0.9	0.97	1	1	0.92	0.97	1	0.83
6	Mt Grand Matoury	6	1	52.19	4.53	7	121	50.05	36.52	0.5	1	1	0.83	1	1	1	1	1	1
7	NE Mt Grand Matoury	6		52.21	4.52	8	12	54.60	22.23	1	1	0.9	0.97						
8	la Desiree/Matoury	7	18	52.22	4.51	9	12	51.05	36.90	1	1	0.9	0.97	0.89	1	0.89	0.93	0.94	0.83
9	Port Inini	2		52.28	4.50	12	4	54.00	31.10	0.5	0.75	0.8	0.68						
10	Mt Paramana	4	11	52.20	4.49	13	11	56.28	24.14	0.25	0.3	0.5	0.35	0.73	0.91	0.93	0.86	0.82	0.95
11	Stoupan/Galion	1	6	52.24	4.47	18	4	60.85	30.28	0	1	0.25	0.42	0.67	0.75	0.54	0.65	0.75	0.42
12	Roura	2	19	52.19	4.43	21.5	58	86.00	10.52	0.5	1	1	0.83	0.53	0.76	0.61	0.63	0.74	0.47
13	Mt de Chevaux/Nancibo	6	5	52.23	4.41	23.5	43	50.13	23.62	0.2	1	0.25	0.48	0	0.2	0.3	0.17	0.20	0.30
14	Roche Tablou/S. Roura	6	8	52.18	4.40	24	179	81.00	8.48	0.2	0.25	0.25	0.23	0.13	0.63	0.59	0.45	0.375	0.5625
15	Sourou Crk, Degrad Menado	4		52.22	4.38	32.5	31	41.12	5.86	0	0	0	0						
16	Fourgassie	10		52.19	4.38	34	46	76.38	6.43	0.1	0.5	0.25	0.28						
17	1–3 km past Fourgassie	5		52.19	4.37	35.5	60	86.82	2.20	0	0.1	0.35	0.15						
18	2–4 km past Fourgassie	7		52.19	4.36	37	60	88.53	3.02	0	0.1	0.35	0.15						
19	18.3 km south of Roura		7	52.16	4.35	38	268	85.66	2.00					0.14	0.36	0.36	0.29	0.43	0.29
20	3–6 km past Fourgassie	9		52.14	4.35	38.5	60	85.08	1.51	0	0.2	0.4	0.2						
21	Cacao		16	52.24	4.34	40	64	72.61	2.67					0	0	0	0	0	0
22	Camp Caiman		44	52.13	4.34	40	299	49.58	11.93					0.23	0.47	0.37	0.35	0.41	0.33
23	12 km South of Caiman		8	52.08	4.32	45	288	58.33	12.14					0.38	0.5	0.28	0.39	0.375	0.375
24	Camp Hervo		16	52.20	4.27	57	28	70.53	4.16					0	0	0	0	0	0
	Total collected	87	237																

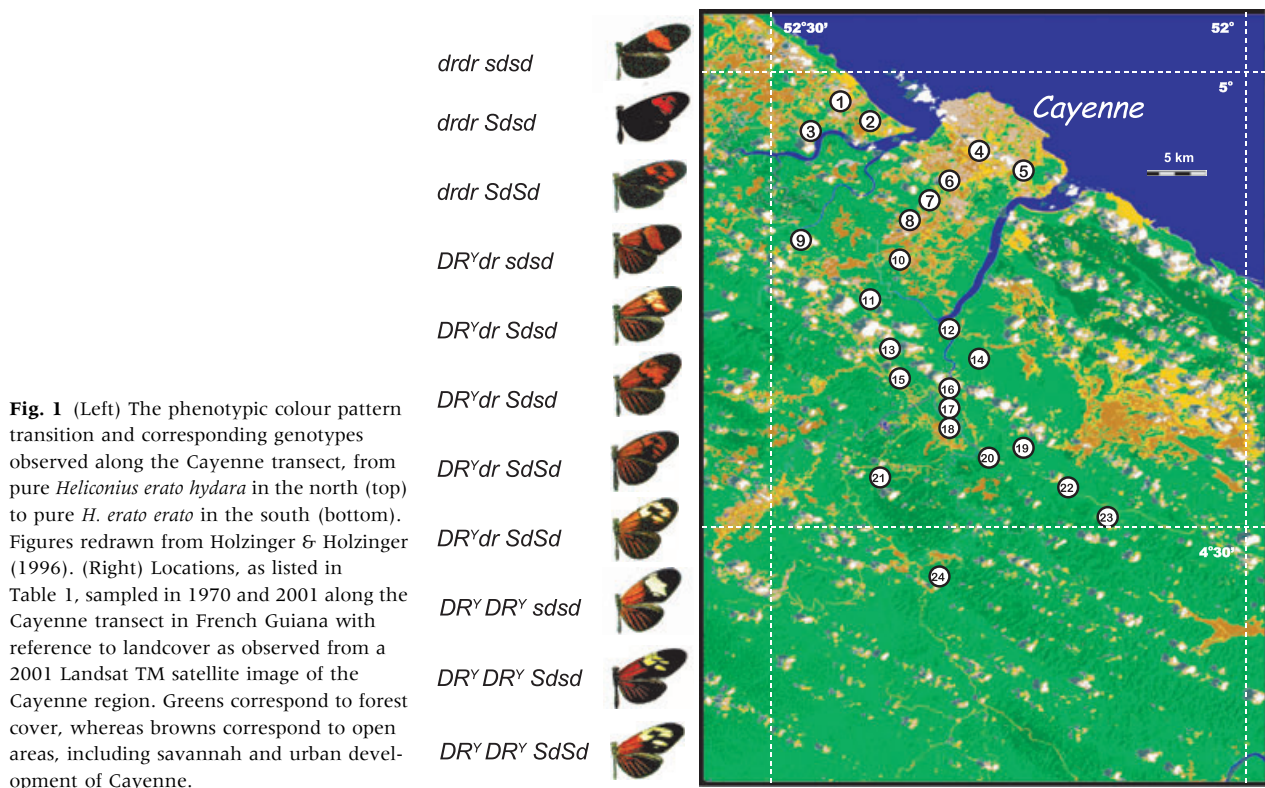Dennis-ray, dennis-ray complex; FW, forewing; index, trait index.

Fig. 1 (Left) The phenotypic colour pattern transition and corresponding genotypes observed along the Cayenne transect, from pure *Heliconius erato hydara* in the north (top) to pure *H. erato erato* in the south (bottom). Figures redrawn from Holzinger & Holzinger (1996). (Right) Locations, as listed in Table 1, sampled in 1970 and 2001 along the Cayenne transect in French Guiana with reference to landcover as observed from a 2001 Landsat TM satellite image of the Cayenne region. Greens correspond to forest cover, whereas browns correspond to open areas, including savannah and urban development of Cayenne.

better evaluate the relationship between colour pattern expression and environment, I quantified habitat heterogeneity along the Cayenne transect by calculating the spatial distribution of land cover from analysis of Landsat Thematic Mapper (TM) satellite images of the transect region using ERDAS IMAGINE v.8.6 (Leica Geosystems, St. Gallen, Switzerland) and ARCGIS v.8 (ESRI, Redlands, USA) software (*Appendix 2*). I further characterized the distribution of land cover at two time points to assess the stability of the ecotone and demonstrate whether any potential changes in hybrid zone structure correspond to land cover change over time (*Appendix 2*). Although it was possible to obtain a > 90% cloud-free 2001 TM image, the earliest available TM image without overwhelming cloud cover (> 30% cloud cover) was taken in 1986, limiting comparisons to a 15-year period. The 1986 image was therefore used as a conservative proxy for evaluating habitat conditions in 1970.

Ecological and genetic associations across the hybrid zone

The land cover data and genotype frequency data from the 2001 sampling period were used in multivariate analyses to quantify the relationship of *H. erato* wing colour pattern expression to environmental variation. Along with the site coordinates and land use data, the distance between the sample locations and a standard

reference point in Cayenne was estimated to the nearest half kilometre from 1 : 25 000 scale L'Institut Geographique National Series Blue topographic maps. I also obtained elevation data for each sample location from these topographic maps. I then ran a multiple correlation analysis as implemented in SYSTAT 10.0 (SPSS Inc., Chicago, USA) to build a Pearson correlation matrix of latitude, longitude, elevation, distance from Cayenne, land cover calculations and frequency estimates of alleles at the D^{RY} and *Sd* loci. To better identify major ordination axes, I ran a principal components analysis (PCA) using the listed environmental variables. Another PCA was run with the environmental variables and the frequency estimates of both alleles at the D^{RY} and the *Sd* loci to compare the ordination of allele frequencies relative to environmental variation.

To further demonstrate how genotype distributions reflect spatial and landscape characteristics along the Cayenne transect, SYSTAT 10.0 was used to run discriminant and canonical correspondence analyses (CCA) on environmental factors and genotype frequencies. Four separate analyses were run with the set of environmental factors as explanatory variables against response variables that reflected one of four different interpretations of individual genotypes, where each specimen was scored as: a combined D^{RY}/Sd genotype; a 'collapsed' genotype where individuals were scored as either double homozygous or as a heterozygote with a phenotype that more

closely resembled *H. erato erato* or *H. erato hydara*; as homozygous or heterozygous at the D^{RY} locus and lastly, as homozygous or heterozygous at the *Sd* locus.

Hybrid zone and ecotonal change over time

To compare the 1970 and 2001 collections, I treated the colour pattern differences among *H. erato erato* and *H. erato hydara* as three phenotypic traits. Values for the 1970 sampling period were taken from Benson (1982). For the 2001 collection, expression of the three colour pattern traits was scored by phenotype as either parental (0 or 1) or intermediate (0.25, 0.5, 0.75) states following rules set by Benson (1982). Frequencies of the different phenotypes were directly estimated from the individuals sampled at each site, relative to how the three colour pattern traits are expressed by pure *H. erato hydara* (Benson, 1982). Each of the three pattern elements was first treated individually to establish the positions and widths of the corresponding character clines. I then calculated a hybrid index that averaged over the three characters to summarize the relative amount of polymorphism observed at the different sample locations.

Using the aforementioned ML approach for fitting and comparing sigmoidal tanh clines (Blum, 2002a; Dasmahapatra *et al.*, 2002), I tested whether the 1970 clines were concordant and coincident. I also compared the 2001 clines to determine concordance and coincidence, and further compared the 1970 clines to their respective 2001 counterparts to assess whether the hybrid zone structure had changed over time. The same procedure was followed to assess the structure and stability of the forest-savannah ecotone between 1986 and 2001. SYSTAT 10.0 was also used to examine whether frequency changes at each of the three colour pattern traits covaried with changes in forest and open area cover across the Cayenne transect over time, with multiple correlation analyses limited to the nine locations sampled in both 1970 and 2001 (Table 1).

Results

Current location and width of the *H. erato hydara* × *H. erato erato* zone

A total of 237 individuals were sampled in 2000–2001 across 57 km at 16 sites distributed between Sablance to the southwest of Cayenne, and Camp Hervo near Regina (Fig. 1, Table 1). This compares to the 87 individuals collected in 1970 by Benson (1982) at 16 sites across 38.5 km (Fig. 1, Table 1). Sample size per site ranged between 1 and 44 individuals, with a mean of 14 (SD = 12) individuals collected per location. Distance south of Cayenne was found to be an excellent proxy for the observed loss of open area cover across the sampling transect (as described below), and was therefore used to parameterize the colour pattern clines into spatially

linear transitions. However, because the sampling transect ran along a south-by-southwest heading, consideration was given to the potential influence of longitudinal proximity of site locations to the coastal plain. To assess the potential influence of longitudinal proximity to the coastal plain, clinal analyses were conducted with and without two outlying southern sites (nos 22 and 23 as listed in Table 1).

Comparison of the frequency values at each location indicated a clinal transition of both the *dr* allele and *sd* allele across the 57 km transect (Table 1, Fig. 2). Reciprocal comparison of the two cline positions demonstrated that the *dr* cline was not coincident with the *sd* cline (Table 3; $G_{\text{cline } dr\text{--cline } sd}$ = 9.62, $P < 0.005$; $G_{\text{cline } sd\text{--cline } dr}$ = 5.68, $P < 0.025$). A more conservative analysis of the cline positions, where the two outlying southern sites were removed from analysis, recovered a similar result (Table 2; $G_{\text{cline } dr\text{--cline } sd}$ = 4.9, $P < 0.05$; $G_{\text{cline } sd\text{--cline } dr}$ = 3.9, $P < 0.05$), although both cline centres were estimated to occur at more northerly locations. Reciprocal comparison of the widths of the two clines indicated that the *dr* cline is also narrower than the *sd* cline ($G_{\text{cline } dr\text{--cline } sd}$ = 3.2, $0.05 < P < 0.10$; $G_{\text{cline } sd\text{--cline } dr}$ = 4.2, $P < 0.05$). More conservative analysis of the cline widths recovered a similar result ($G_{\text{cline } dr\text{--cline } sd}$ = 4.2, $P < 0.05$; $G_{\text{cline } sd\text{--cline } dr}$ = 4.8, $P < 0.05$), but both clines were estimated to be about 12 km narrower when the two outlying southern sites were excluded (Table 2).

Land cover change across the Cayenne transect

In both 1986 and 2001, the amount of forest cover increased south along the Cayenne transect, whereas the amount of open area decreased (Table 1). In 1986, the amount of forest cover varied from 28% outside of Cayenne to 85% towards the southern end of the transect. A similar but broader range was observed in 2001; forest cover was observed to vary between 23% and 98% across the transect (Table 1). In 1986, the amount of open area ranged from 41% near Cayenne, to < 8% towards the southern end of the transect (Table 1). In 2001, the amount of open area maximized at 77% outside of Cayenne, and dropped to < 2% near the southern end of the transect.

The observed patterns of forest loss and gain along the Cayenne transect indicate that the forest-savannah ecotone has narrowed and shifted northwards over time. Clinal analysis of the ecotone is consistent with this characterization. The positions and widths of the opposing forest cover and open area cover clines were similar within each time period (Table 2, Fig. 3). For example, the width of the forest cline in 1986 was not significantly different from the width of the 1986 open area cline ($G_{\text{open--forest}}$ = 0.2, $0.60 < P < 0.70$; $G_{\text{forest--open}}$ = 0.4, $0.50 < P < 0.60$). Similarly, the widths of the 2001 clines did not differ ($G_{\text{open--forest}}$ = 1.3, $0.20 < P < 0.30$; $G_{\text{forest--open}}$ = 1.2, $0.20 < P < 0.30$). However, both cline
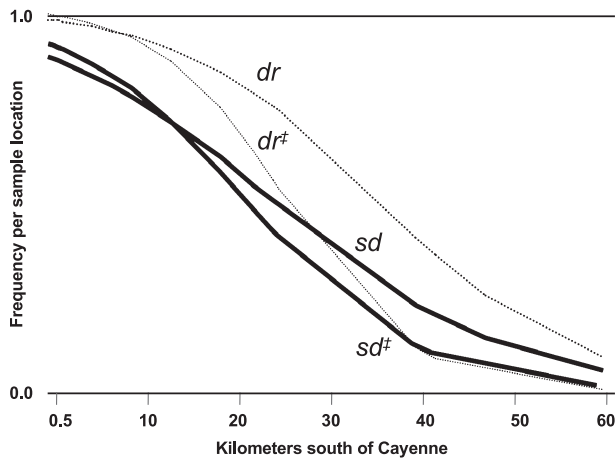
Fig. 2 Comparison of the *dr* and *sd* clines across the 57 km Cayenne transect with and without (‡) two outlying southern sites.
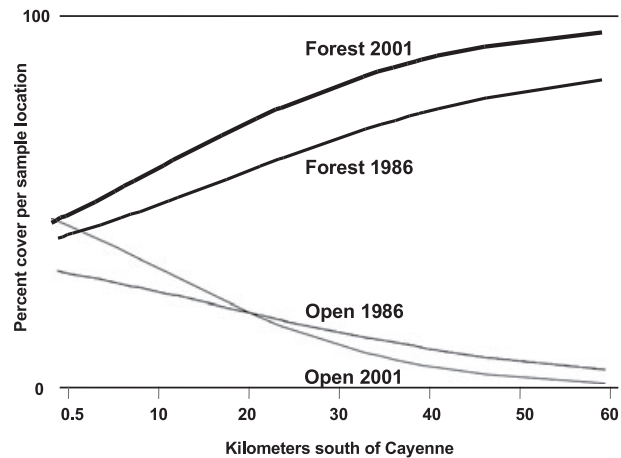
Table 2 Centre and width estimates of allele frequency clines, wing colour pattern clines and ecotones between 1970 and 2001.

Cline	Centre	CI	Width	CI
2001				
dr	31 km	27–34 km	39 km	32–49 km
sd	25 km	22–29 km	49 km	40–63 km
2001*				
dr	24 km	22–29 km	27 km	21–37 km
sd	21 km	18–24 km	37 km	28–51 km
1970				
Dennis-ray complex	16 km	13–19 km	21 km	14–31 km
Forewing band colour	27 km	23–31 km	28 km	19–43 km
Forewing band shape	25 km	20–30 km	39 km	27–65 km
Trait index	22 km	19–27 km	30 km	22–36 km
2001				
Dennis-ray complex	24 km	21–27 km	34 km	28–43 km
Forewing band colour	29 km	25–33 km	43 km	34–56 km
Forewing band shape	28 km	25–32 km	42 km	34–53 km
Trait index	28 km	25–32 km	40 km	33–49 km
2001*				
Dennis-ray complex	21 km	17–23 km	28 km	18–32 km
Forewing band colour	27 km	23–30 km	29 km	21–34 km
Forewing band shape	24 km	20–28 km	34 km	25–43 km
Trait index*	23 km	20–27 km	30 km	21–35 km
1986				
forest cover	16 km	10–21 km	65 km	48–125 km
open area cover	17 km	6–26 km	75 km	49–125 km
2001				
forest cover	11 km	5–15 km	58 km	44–81 km
open area cover	10 km	5–14 km	49 km	39–66 km

CI, 95% confidence interval.

*Also presented are estimates of cline centres and widths in 2001 excluding sites nos 22 and 23 from all analyses.

widths appear to have contracted over time (Table 2, Fig. 3), although this characterization is more robust for the open area cover cline than the forest cover cline

Fig. 3 Forest and open area land cover clines observed across the Cayenne transect in 1986 and 2001.

($G_{forest2001–1986} = 0.6$, $0.40 < P < 0.50$; $G_{forest1986–2001} = 0.3$, $0.50 < P < 0.60$; $G_{open2001–1986} = 3.3$, $0.05 < P < 0.10$; $G_{open1986–2001} = 3.9$, $P < 0.05$). Support for north-ward cline movement was also observed in comparisons of cline centres ($G_{forest2001–1986} = 15.2$, $P < 0.0001$; $G_{forest1986–2001} = 3.0$, $0.05 < P < 0.10$; $G_{open2001–1986} = 11.8$, $P < 0.001$; $G_{open1986–2001} = 1.9$, $0.10 < P < 0.20$). Exclusion of the two outlying sites had no effect on clinal estimates of the habitat transition across the transect.

Ecological and genetic associations across the hybrid zone

The PCA of environmental and spatial variation across the sample sites identified two principal components that account for 83% of the observed variance (65% and 18% respectively). The first principal component reflects strong associations among latitude, distance from Cayenne, forest cover and open area cover along the transect (Fig. 4a). The second principal component corresponds to elevation and longitudinal variation (Fig. 4a). Distance south of Cayenne was found to be negatively correlated with latitude and open area land cover along the transect (Fig. 4a, *Appendix 3*). Contrary to expectation, elevation was not correlated with distance south of Cayenne or variation in land cover. Rather, elevation was found to be negatively correlated with longitude (Fig. 4a, *Appendix 3*).

Analyses of wing colour pattern expression relative to environmental variation were consistent with Benson's (1982) observation that *H. erato hydara* most often occur in lowland open or edge forest habitat, whereas *H. erato erato* are mostly restricted to enclosed upland forested areas. However, the distributions of alleles, genotypes and phenotypes appear to more closely correspond to land cover than to elevation (Fig. 4a). Both the PCA and CCA of allelic and genotypic frequencies demonstrate stronger relationships between genetic variation and
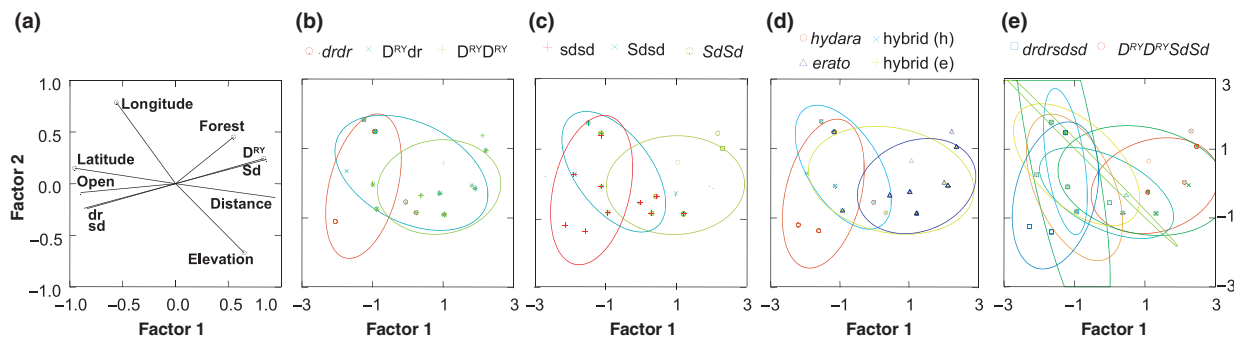
Fig. 4 (a) Principal components (PC) analysis of environmental factors and the frequency of colour pattern alleles measured at each sample location (b) Canonical correspondence analysis (CCA) plot of D^{RY} locus genotype frequencies relative to PC factors (c) CCA plot of Sd locus genotype frequencies relative to PC factors (d) CCA plot of dominant phenotypes relative to PC factors (e) CCA plot of all possible genotypes relative to PC factors, with attention given to parental genotype distributions. Circles represent the areas containing 90% of the samples.

habitat. The distributional separation of *drdrsdsd* and $D^{RY}D^{RY}SdSd$ homozygotes (corresponding to pure *H. erato hydara* and *H. erato erato* phenotypes), in particular, is driven strongly by habitat (Fig. 4d). The distributional overlap of $D^{RY}dr$ heterozygotes with $D^{RY}D^{RY}$ homozygotes shows a similar pattern (Fig. 4b). Some exceptions occur – elevation appears to restrict the overlap of *Sdsd* heterozygotes and *sdsd* homozygotes (Fig. 4c), and neither elevation nor habitat differentiates the distribution of *Sdsd* heterozygotes from *SdSd* homozygotes. Some genotypes and phenotypes also exhibit wider environmental tolerances than do others. For example, the distribution of *drdr* homozygotes appears to be more restricted by habitat than is the distribution of $D^{RY}D^{RY}$ individuals (Fig. 4b), and $D^{RY}dr$ heterozygotes occupy a narrower elevation range than *drdr* homozygous individuals.

The environmental tolerances of hybrids are generally comparable with the parental homozygotes they most closely resemble. More overlap among heterozygotes resembling *H. erato hydara* occurs with *H. erato hydara* homozygotes than with *H. erato erato* homozygotes

(Fig. 4d). Similarly, heterozygotes with phenotypes resembling *H. erato erato* overlap more broadly with *H. erato erato* homozygotes than *H. erato hydara* homozygotes (Fig. 4d). However, heterozygotes resembling *H. erato erato* are distributed more broadly than are heterozygotes resembling *H. erato hydara*. Both habitat and elevation appear to limit the distribution of intermediate genotypes resembling *H. erato hydara* (Fig. 4d). These patterns are less clear when each of the nine possible $D^{RY}dr/Sdsd$ genotypes (Fig. 1) is treated individually. Although the distribution of double homozygous individuals remains discrete, little distinction remains among other homozygous and heterozygous genotypes (Fig. 4e).

Hybrid zone and colour pattern frequency changes over time

For both 1970 and 2001, comparisons found clinal transitions across the Cayenne transect for each colour pattern trait and the trait index (Table 1, Fig. 5). In 1970, the cline describing the expression of the dennis-ray
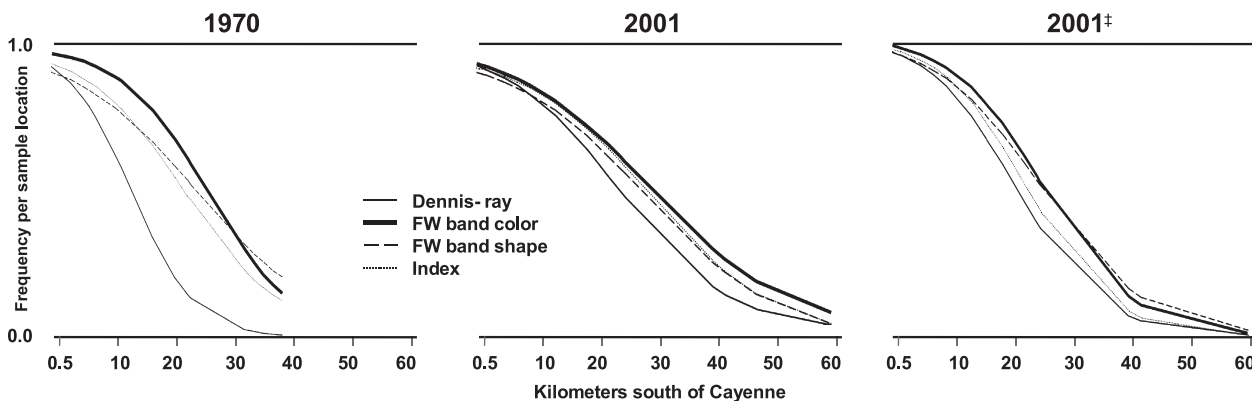

Fig. 5 Comparison of clines for colour pattern traits in 1970 and 2001 with and without (‡) two outlying southern sites. The composite cline of the trait index values is also presented. FW, forewing.

complex was notably removed from the clines describing the expression of the forewing band colour and shape (Table 2, Fig. 5; $G_{cline1-cline2} = 30.716$, $P < 0.001$; $G_{cline1-cline3} = 20.646$, $P < 0.001$; $G_{cline2-cline3} = 0.944$, $0.30 < P < 0.40$). The centre of the dennis-ray cline was located farther north than either of the other two colour pattern clines. The width of the dennis-ray cline was also narrower, particularly in comparison with the forewing band shape cline (Table 2, Fig. 5; $G_{cline1-cline2} = 2.122$, $0.10 < P < 0.20$; $G_{cline1-cline3} = 9.252$, $P < 0.005$; $G_{cline2-cline3} = 2.798$, $0.05 < P < 0.10$).

Similar patterns were observed in 2001 (Table 2, Fig. 5). The dennis-ray cline remained significantly removed from the other two clines (Table 2, Fig. 5; $G_{cline1-cline2} = 8.7$, $P < 0.005$; $G_{cline1-cline3} = 5.7$, $P < 0.025$; $G_{cline2-cline3} = 2.9$, $0.05 < P < 0.10$). A more conservative assessment involving removal of two outlying southerly sample locations (site nos 22 and 23) from clinal analyses located the centres of the 2001 colour pattern clines farther northwards, closer to Cayenne (Table 2, Fig. 5). The dennis-ray cline center nonetheless remained distinct from the centres of the two other clines ($G_{cline1-cline2} = 16.2$, $P < 0.001$; $G_{cline1-cline3} = 4.6$, $P < 0.05$; $G_{cline2-cline3} = 3.0$, $0.05 < P < 0.10$). The width of the dennis-ray cline in 2001 was also narrower than the other two cline widths, although cline widths across the Cayenne transect appear to have become more concordant since 1970 ($G_{cline1-cline2} = 4.6$, $P < 0.05$; $G_{cline1-cline3} = 3.1$, $0.05 < P < 0.10$, $G_{cline2-cline3} = 0.15$, $P = 0.70$). Removal of the two outlying southern sample locations narrowed the estimated cline widths (Table 2) but the greater concordance of cline widths remained unchanged ($G_{cline1-cline2} = 1.6$, $0.20 < P < 0.30$; $G_{cline1-cline3} = 5.5$, $P < 0.025$; $G_{cline2-cline3} = 0.6$, $0.40 < P < 0.50$).

Treating all three traits independently, the dennis-ray complex cline appears to have shifted approximately 8 km southwards between 1970 and 2001 ($G_{2001-1970} = 26.4$, $P < 0.001$; $G_{1970-2001} = 18.7$, $P < 0.001$). The positions of the two other clines also appear to have shifted southward, however the difference between the 1970 and 2001 cline centres is not significant ($G_{2001-1970} = 2.31$, $0.10 < P < 0.20$; $G_{1970-2001} = 1.84$, $0.10 < P < 0.20$; $G_{2001-1970} = 3.74$, $0.05 < P < 0.10$; $G_{1970-2001} = 1.8$, $0.10 < P < 0.20$). Comparison of the 1970 cline positions to the conservative estimates of the 2001 cline positions resulted in similar conclusions; the dennis-ray cline has shifted southwards ($G_{2001-1970} = 6.8$, $P < 0.01$; $G_{1970-2001} = 6.1$, $P < 0.025$), while the forewing band colour and band shape clines have not moved ($G_{2001-1970} = 0.8$, $0.30 < P < 0.40$; $G_{1970-2001} = 0.2$, $0.60 < P < 0.70$; $G_{2001-1970} = 0.9$, $0.30 < P < 0.40$; $G_{1970-2001} = 0.4$, $0.50 < P < 0.60$). Cline widths have remained more stable over time. The dennis-ray cline appears to have broadened by approximately 13 km since 1970 ($G_{2001-1970} = 28.2$, $P < 0.001$; $G_{1970-2001} = 6.2$, $P < 0.025$) although comparison of the 1970 cline width

to the conservative 2001 dennis-ray cline width indicates that the width of the cline has not changed ($G_{2001-1970} = 0.4$, $0.50 < P < 0.60$; $G_{1970-2001} = 0.8$, $0.30 < P < 0.40$). Comparison of the 1970 and 2001 forewing band colour clines resulted in similar conclusions. The forewing band colour cline appears to have broadened by 15 km since 1970 ($G_{2001-1970} = 8.8$, $P < 0.005$; $G_{1970-2001} = 4.1$, $P < 0.05$) but exclusion of the two outlying southern sample locations removed any signature of cline width change over time ($G_{2001-1970} = 0.4$, $0.50 < P < 0.60$; $G_{1970-2001} = 0.2$, $0.60 < P < 0.70$). All comparisons of the forewing band shape cline widths indicated that no change has occurred since 1970 ($G_{2001-1970} = 0.8$, $0.30 < P < 0.40$; $G_{1970-2001} = 0.2$, $0.60 < P < 0.70$; $G_{2001-1970} = 1.8$, $0.10 < P < 0.20$; $G_{1970-2001} = 0.4$, $0.50 < P < 0.60$).

Results from comparison of the 1970 and 2001 clines of the trait index scores largely reflect the individual cline comparisons (Table 2, Fig. 5). Comparison of the 1970 and 2001 trait index clines indicated that the cline has shifted southwards ($G_{2001-1970} = 9.8$, $P < 0.005$; $G_{1970-2001} = 6.0$, $P < 0.025$). However, this difference disappears when the 1970 trait index cline is compared with the conservative estimate of the 2001 index cline centre ($G_{2001-1970} = 0.2$, $0.60 < P < 0.70$; $G_{1970-2001} = 0.1$, $P = 0.75$). Similarly, comparison of the 1970 and 2001 trait index clines also indicated that the hybrid zone expanded by approximately 10 km ($G_{2001-1970} = 7.1$, $P < 0.01$; $G_{1970-2001} = 1.908$, $0.10 < P < 0.20$), but no difference in cline width was observed when the 1970 composite cline width was compared with the conservative estimate of the 2001 cline width ($G_{2001-1970} = 0.6$, $0.40 < P < 0.50$; $G_{1970-2001} = 0.2$, $0.60 < P < 0.70$).

Comparison of colour pattern trait values observed at the nine locations sampled in 1970 and 2001 found that colour pattern trait frequency shifts favouring *H. erato hydara* phenotypic expression were associated with open area gain between 1986 and 2001. Similarly, changes towards *H. erato erato* phenotypic expression were associated with forest gain over time. The correlation coefficients for these comparisons were low and non-significant ($0 > R < 0.37$, $P > 0.05$), although stronger associations were found between forest gain and shifts towards *H. erato erato* phenotypic expression ($R = 0.24$, $P = 0.50$), than between deforestation and shifts towards *H. erato hydara* phenotypic expression ($R = 0.11$, $P = 0.78$) as indicated by changes in trait index values.

Discussion

Hybrid zone structure along the Cayenne transect

The *H. erato hydara* × *H. erato erato* hybrid zone involves clinal transitions across colour pattern traits corresponding to the action of two loci (Mallet, 1989). Characterization of the two clines indicates that the cline corresponding to the codominant D^{RY} locus was narrower than the

dominant *Sd* locus cline in 1970 and 2001. The larger width of the dominant cline may be attributable to an asymmetry in selection relative to phenotypic expression of recessive alleles (Mallet *et al.*, 1990, 1998). If frequency-dependent selection is acting against rare phenotypes, weaker selection for recessive alleles is expected to occur on the dominant side of the cline because recessive alleles are carried by heterozygotes that exhibit the dominant phenotype. Dominant alleles carried by heterozygotes on the recessive side of the cline are under stronger selection because the heterozygote phenotype is more distinct from the phenotype of recessive homozygotes. This selection asymmetry is expected to result in longer 'tails' of the recessive allele on the dominant side of the cline (Mallet *et al.*, 1998). Similar asymmetries across clines of dominant loci may also result from selection across environmental gradients (Haldane, 1948).

The two clines were also found to differ in position. The centre of the codominant D^{RY} cline was located several kilometres to the south of the dominant *Sd* cline centre. A similar study of codominant and dominant clines across the Peruvian *H. erato favorinus* × *H. erato emma* hybrid zone found coincident clines (Mallet *et al.*, 1998). However, the observed separation of the D^{RY} and *Sd* clines across the Cayenne transect is similar to the disparity found between the D^{RY} cline and clines at colour pattern loci *Cc* and *Ss* across the Bolivian *H. erato venustus* × *H. erato phyllis* hybrid zone (Langham, 2003).

Disequilibria and epistasis are expected to draw clines together, but displacement of clines across hybrid zones may occur if selection is below a critical threshold or if there are differences in dominance among loci (Endler, 1977; Mallet & Barton, 1989b). Selection on wing colour patterns is strong (Benson, 1972; Mallet & Barton, 1989a), suggesting that the staggering of clines across the French Guiana and Bolivian hybrid zones may be attributable to differences in dominance.

It is possible that other factors are contributing to the separation of the colour pattern clines across *H. erato hydara* × *H. erato erato* hybrid zone. Mallet & Barton (1989b) noted that codominant and dominant clines may separate if selection occurs before migration. In the case of *Heliconius* butterflies, however, migration most likely occurs before selection (Mallet & Barton, 1989b). Separation of the cline centres is also inconsistent with asymmetric introgression (Barton, 1993) because asymmetric introgression is expected to result in displacement exceeding one cline width (Jaarola *et al.*, 1997). Staggered clines could alternatively reflect underlying population structure (Jaarola *et al.*, 1997), selection for or against certain gene combinations (Barton, 1993), or environmental selection (Hewitt, 1988). An analysis of microsatellite genotype data by Blum (2002b) demonstrated that populations across the Cayenne hybrid zone are weakly structured, but it is unlikely that the observed structure is sufficient to stagger the colour pattern clines. Considering that heterozygotes strongly resemble either one or the other parental phenotype, and the apparent separation of the two gross categories of heterozygotes (those resembling *H. erato erato* vs. those resembling *H. erato hydara*) by habitat, it remains possible that the observed discordance in cline positions is attributable to selection for or against certain gene combinations or selection across an environmental gradient.

Ecological and genetic associations across the Cayenne transect

Benson (1982) observed that the transition from one colour pattern race to another in French Guiana corresponded to a shift from lowland savannah to upland enclosed forest. Benson (1982) suggested that differences across cline widths among the three surveyed transects reflected shifts in underlying topography that varied the width of a coastal forest-savannah ecotone. The present study confirms the presence of a forest-savannah ecotone across the Cayenne transect, where forest cover progressively increases south of the city. Contrary to Benson's (1982) observations, elevation was not found to consistently increase across the transect, nor did elevation correspond to the amount of forest cover at sample locations. This finding could be attributable to the transect crossing at least two river valleys in a south-by-southwest heading. Analyses encompassing a broader spatial scale are therefore warranted to validate whether the results presented here are representative of the general relationship between elevation and land cover in the region.

The unstructured distribution of all genotype combinations across the hybrid zone is consistent with prior characterizations of the *H. erato hydara* × *H. erato erato* hybrid zone in French Guiana as a unimodal hybrid swarm (Benson, 1982; Jiggins & Mallet, 2000). However, dissection of the general pattern into component parts indicates that the spatial distributions of colour pattern genotypes are structured by land cover, and to a lesser degree, other environmental and spatial features. It is also noteworthy that genotypes appear to be asymmetrically restricted by habitat. The pattern of overlap among consensus genotypes, where heterozygotes were scored relative to their visual resemblance to parental phenotypes, indicates that phenotypes are also differentially distributed by habitat. As was found with individual genotypes, little overlap occurred among recessive and dominant homozygotes, and individuals resembling *H. erato hydara* (both homozygotes and heterozygotes) occurred over a narrower habitat range than did individuals resembling *H. erato erato*. This can be partially attributed to the visual dominance of the dennis-ray complex in a relatively disproportionate number of mixed genotypes, where resemblance to *H. erato hydara* occurs in only three of the nine overall genotypes (Fig. 1). The relatively greater numerical abundance and distribution of individuals exhibiting *H. erato erato* phenotypes

may therefore be largely driven by co-dominant expression of the D^{RY} locus.

Hybrid zone and ecotone stability over time

In the absence of ecological or environmental associations, the two-locus model of colour pattern expression across the *H. erato hydara* × *H. erato erato* hybrid zone suggests that dominance drive would favour the spread of *H. erato erato* and move the hybrid zone northwards (Mallet, 1986; Mallet & Barton, 1989b; Mallet *et al.*, 1990; Blum, 2002a). A conservative assessment of the hybrid zone structure over a 31-year period indicates that the colour pattern clines have not shifted northwards. This suggests that some counterbalancing process is acting to maintain the position and width of the hybrid zone. Colour pattern races favouring different habitats or environmental selection acting across the hybrid zone are two possible counterbalances.

An ecological or environmental counterbalance would also explain the sharp contrast between stasis of the French Guiana *H. erato hydara* × *H. erato erato* hybrid zone and movement of the *H. erato hydara* × *H. erato petiverana* hybrid zone in eastern Panamá (Blum, 2002a). Unlike the French Guiana hybrid zone, the Panamá *H. erato* hybrid zone does not appear to correspond to an environmental gradient (Mallet, 1986; Blum, 2002a), but widespread deforestation across eastern Panamá may have instigated a migration asymmetry favouring *H. erato hydara* (Blum, 2002a,b). Deforestation along the Cayenne transect in French Guiana has not been widespread and *H. erato hydara* has not overtaken *H. erato erato* – but the frequency of wing colour pattern genotypes and phenotypes might still be expected to shift in accordance with local environmental change if the fitness of *H. erato* races is habitat dependent (Britch *et al.*, 2001; Gee, 2004). Trends in colour pattern frequency changes observed across locations sampled in both 1970 and 2001 are consistent with this idea, where shifts towards *H. erato hydara* colour pattern expression corresponded to increasing open area cover over time, and shifts towards *H. erato erato* colour pattern expression corresponded to declining open area cover and gains in forest cover.

Habitat preferences and speciation

Speciation has been portrayed as a process that unfolds over a continuum of stages, where early stages of adaptive differentiation lead to the cessation of gene flow and reproductive isolation. The sequence of events leading to speciation among *Heliconius* butterflies remains hypothetical (e.g. Mallet *et al.*, 1998). The earliest stages of differentiation among closely related *Heliconius* likely involved adaptive divergence of warning colouration (Brower, 1994, 1996; Mallet *et al.*, 1998). By itself, mimetic divergence does not appear to be sufficient to trigger reproductive isolation given the prevalence of

intraspecific mimicry complexes (e.g. Joron *et al.*, 1999). Something in addition to mimetic divergence must initiate assortative mating and later stages of reproductive isolation (Mallet *et al.*, 1998). Reinforcement is one possible candidate for the evolution of assortative mating (Dobzhanksy, 1940; Jiggins *et al.*, 2001), as is a profound mimetic switch (Naisbit *et al.*, 2001). Behavioural preferences related to abiotic or mimetic conditions could also initiate assortative mating (MacCallum *et al.*, 1998) if groups tend to reside and breed in different habitats. This is because the likelihood that selection in a heterogeneous environment results in a stable polymorphism is increased if individuals exhibiting different genotypes choose the habitat in which they are most fit (Taylor, 1976; Powell & Taylor, 1979). Even if assortative mating is weak, habitat preferences might lead to heterozygote deficits or population subdivision. If habitat preferences lead to additional ecological adaptations, stronger assortative mating is expected to evolve to further differentiate groups and possibly trigger speciation (MacCallum *et al.*, 1998).

Evidence of habitat preferences comes from associations between genotypes or phenotypes and habitat over spatial scales smaller than an organism's dispersal potential (MacCallum *et al.*, 1998). The study presented here demonstrates that associations between habitat, genotypes and phenotypes occur across spatial scales smaller than the potential dispersal capacity of *H. erato* butterflies, which can exceed 10 km (Mallet, 1986; Blum, 2002a). Indirect evidence of preferences among *H. erato* races comes from studies demonstrating associations between habitat heterogeneity and the distribution of distinct Müllerian mimicry rings (Mallet & Gilbert, 1995; Joron *et al.*, 1999; Estrada & Jiggins, 2002; Joron, 2005). Similarly, indirect evidence also comes from studies suggesting potential fitness differences of colour patterns relative to ambient light conditions (Endler, 1992, 1993; Sweeney *et al.*, 2003; Langham, 2004). Further support for preferences could come from experimental studies on the signal efficacies of the *H. erato erato* and *H. erato hydara* phenotypes in open vs. forest light environments.

The study presented here indicates that habitat heterogeneity plays an important role in structuring the distribution of *Heliconius* colour pattern races, which suggests that ecological divergence begins earlier along the *Heliconius* speciation continuum than what prior studies have proposed (Mallet *et al.*, 1998). Consideration of other *Heliconius* hybrid zone studies and ecological comparisons of sister species also suggest that ecological divergence intensifies over time – possibly beginning with habitat preferences, followed by physiological differentiation (McMillan *et al.*, 1997; Davison *et al.*, 1999) and other complex forms of divergence such as differences in host plant use (Benson *et al.*, 1975; Benson, 1978). The pathways and trajectories of such transitions likely differ. It is also likely that the degree of ecological differences among colour pattern races varies

given that some intraspecific hybrid zones are not visibly associated with habitat differences (Mallet, 1986; Blum, 2002a) whereas others appear to fall along ecotones (Benson, 1982) and elevation gradients (Brown & Mielke, 1972; Mallet & Barton, 1989a). It might therefore be prudent to continue assessing ecological variation among *H. erato* races, and determine if any exhibit physiological differences like those that have been found between *H. erato* and its incipient sister species *H. himera* (McMillan *et al.*, 1997; Davison *et al.*, 1999) that hybridize along an ecotone and elevation gradient (Mallet, 1993; Jiggins & McMillan, 1997a; Mallet *et al.*, 1998). Such an effort would parallel studies of host plant use that have found differences among species (Benson *et al.*, 1975; Benson, 1978), but no differences among incipient sister species (Jiggins *et al.*, 1997). Even if *H. erato* races do not exhibit physiological differences relative to biotic or abiotic conditions, additional comparative studies would improve understanding of the rate and range of ecological divergence across different stages of speciation. Similarly, determining whether genetic differentiation of phenotypically undifferentiated clades of *H. erato hydara* (Brower, 1994) corresponds to an environmental transition might also demonstrate whether ecological divergence occurs in the absence of colour pattern evolution. Finding evidence of ecological divergence among populations of a *Heliconius* colour pattern race would emphasize that responses to environmental heterogeneity can promote evolutionary differentiation and speciation.

Acknowledgments

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Supplementary material