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Patterns of pollen feeding and habitat preference among *Heliconius* species

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Abstract. 1. The ecological circumstances that precipitate speciation remain poorly understood. Here, a community of *Heliconius* butterflies in lowland Panama was studied to investigate patterns of pollen use, and more specifically the ecological changes associated with the recent divergence of *Heliconius melpomene* (Linnaeus) and *H. cydno* (Doubleday).

2. Considering the seven commonest *Heliconius* species in the community, 32 types of pollen or spore were encountered in pollen loads but only five pollen species were common. Systematic exploitation of pollen was therefore confined to a small proportion of the flowers visited.

3. Most of the variation in pollen load composition between individuals was explained by differences in collecting locality. The exception was *Psiguria*, which was used in all habitats by the *melpomene*/*hecale* clade far more than by the *erato*/*sapho* clade. This may suggest an ancestral switch within *Heliconius* towards increased reliance on *Psiguria* pollen.

4. *Heliconius cydno* and *H. melpomene* differed significantly in pollen load composition for three of the five most commonly collected pollen species. This is most probably explained by differences in habitat preference; *H. melpomene* and its co-mimic *H. erato* are found in open habitat while *H. cydno* and its co-mimic *H. sapho* are found in closed-canopy forest.

5. As *melpomene* and *cydno* are known to hybridise occasionally, such differences in adult microhabitat contribute to pre-mating isolation. Habitat divergence between *H. cydno* and *H. melpomene*, which is associated with changes in mimicry, must have played a role in their recent speciation.

Key words. Co-evolution, ecological isolation, Lepidoptera, pollen feeding, speciation.

Introduction

There is increasing evidence that the early stages of speciation are driven by ecological divergence (McMillan *et al*., 1997; Orr & Smith, 1998; Schluter, 1998; Dieckmann & Doebeli, 1999; Jiggins & Mallet, 2000). In phytophagous insects, host plant shifts are considered to be a driving force in speciation, however although sympatric *Heliconius* species almost invariably have divergent patterns of host plant use (Benson *et al*., 1975; Benson, 1978), speciation can occur in parapatry without a host shift (Jiggins *et al*., 1997). This suggests that partitioning of the host-plant niche allows co-existence of *Heliconius* species but is not necessarily involved in speciation. In fact, disruptive selection on Müllerian mimicry is more likely to play a role in the origin of *Heliconius* species (Mallet *et al*., 1998; Mallet & Joron, 1999; Jiggins *et al*., 2001).

Closely related *Heliconius* species frequently differ in their mimetic colour patterns, suggesting that adaptive radiation of mimetic pattern has occurred. Furthermore, different mimicry rings tend to be found in different microhabitats. Indeed, such habitat differences are thought to play a key role in the co-existence of multiple Müllerian mimicry rings (Mallet & Gilbert, 1995; DeVries *et al*., 1999). Hence, divergence in both mimicry and microhabitat

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probably plays a role in generating reproductive isolation between incipient species. The work reported here was designed to investigate differences in pollen use and microhabitat between *Heliconius melpomene* and *H. cydno*. These closely related sister species, which are sympatric and hybridise occasionally, diverged around 1.5 million years ago (Brower, 1996; Mallet *et al.*, 1998; Beltrán *et al.*, 2001), offering an opportunity to investigate how ecological divergence can cause reproductive isolation in the early stages of speciation.

Heliconius butterflies are unique in their systematic exploitation of pollen. Adults spend long periods on a single flower collecting pollen and occupy home ranges based largely on a network of pollen plants (Gilbert, 1991). Pollen grains stick to the proboscis (Gilbert, 1972; Krenn & Penz, 1998) and are mixed with nectar to dissolve out amino acids, which are then ingested in solution. Pollen feeding provides a regular supply of amino acids to adult butterflies, which is essential for reproduction and defence (Dunlap-Pianka *et al.*, 1977; Brown *et al.*, 1991). Toxicity of *Heliconius*, for instance, is due primarily to cyanogenic glycosides stored in larval and adult tissues, which are manufactured using pollen amino acids as precursors (Nahrstedt & Davis, 1985; Brown *et al.*, 1991; Engler *et al.*, 2000).

As well as being distasteful to predators, *Heliconius* are well known for their bright colour patterns and extraordinary mimetic diversity. It has been suggested that the acquisition of pollen feeding behaviour may have stimulated the evolution of aposematism and mimicry. Indeed, pollen-feeding Heliconiines are more distasteful to predators, more brightly coloured, and show far greater mimetic diversity than non-pollen feeding species (Gilbert, 1991). There is therefore good evidence to support the contention that the evolution of pollen feeding behaviour opened an adaptive zone to primitive Heliconiines and played a key role in allowing the extensive diversification observed within the group (Gilbert, 1991).

Collected pollen remains on the proboscis for several hours, so pollen loads carried by adult *Heliconius* can be collected and used as indicators of flower visitation rates (Boggs *et al.*, 1981; Murawski, 1986). Pollen load composition therefore provides direct evidence for patterns of pollen exploitation, but also gives an indirect estimate of the degree to which adult butterflies overlap in microhabitat. Butterflies might be flying in the same microhabitat but visiting different flowers, in which case pollen load composition would be expected to vary between *Heliconius* species rather than between collecting localities. Alternatively, if butterflies exploit all of the pollen species available in their respective habitats, variation would be expected primarily due to locality rather than species. Previous studies have shown that different *Heliconius* species have divergent patterns of pollen use, but this has been explained variously as either a result of differences between major taxonomic groups in *Heliconius* or habitat partitioning (Boggs *et al.*, 1981; Murawski, 1986).

Pollen feeding was studied in Pipeline Road, Soberanía National Park, Panama. The effects of habitat and taxonomy on pollen feeding could be investigated independently, as representatives of both major taxonomic groups in *Heliconius* are found in both edge and closed-canopy forest habitats. Specifically, the following questions were considered: Do sympatric *Heliconius* species have divergent patterns of pollen exploitation? Can any differences observed be explained by habitat or do different butterflies prefer different flowers? Are there differences in pollen use between major taxonomic groups within *Heliconius*, as has been suggested (Boggs *et al.*, 1981)?

Materials and methods

Fieldwork was performed between January and May 1999 along Pipeline Road, a tropical lowland rainforest in the Panama Canal Zone (Parque Nacional Soberanía, 9°7′33′N, 79°42′90′W). Pipeline Road forms a fairly straight transect running through the forest, with marked habitat heterogeneity. Most notably, the first 5 km have many open sunny areas and a broken canopy rarely exceeding 10 m in height. This region also lies close to nearby edge habitats around the village of Gamboa and along the Panama Canal. In contrast, the more distant part of the road runs through mainly closed forest with a canopy height of 10–15 m. All species of *Heliconius* encountered were caught and their pollen loads were collected before the butterflies were marked and released. Pollen was prepared on a microscope slide (Murawski, 1986) and identified to species, genus, or family (in the case of Compositae), using a guide to the pollen and spores of nearby Barro Colorado Island (Roubik & Moreno, 1991). Pollen grains were counted, either in total counts or in transects across the slide, depending on the size of the sample, in order to estimate relative proportions of each pollen species. These proportions were analysed after arcsin-square-root transformation of the data (Sokal & Rohlf, 1981).

Each plant species was considered separately using a two-way ANOVA, in order to examine the relative importance of collection locality and phylogenetic relationship for the *Heliconius* species (*H. melpomene rosina*, *H. cydno chioneus*, *H. sapho sapho*, *H. erato* cf. *petiverana*, *H. hecale*, *H. ismenius*, and *H. sara fulgidus*). For analysis, the Pipeline Road transect was divided into three sections, corresponding approximately to habitat, 0–3 km being open secondary forest and edge habitat, 3–7.5 km being mixed habitat, and 7.5+ km being closed-canopy forest. *Heliconius* butterflies were divided into two phylogenetic groups, with *H. melpomene*, *H. cydno*, *H. hecale*, and *H. ismenius* in group 1 and *H. erato*, *H. sapho*, and *H. sara* in group 2 (Brown, 1981; Brower & Egan, 1997). *Melpomene group* is used to refer to the clade that includes the *H. cydno*, *H. melpomene*, and silvaniform (*H. ismenius*, *H. hecale*) species, while *erato group* refers to the clade including *H. erato*, *H. sapho*, and *H. sara* (Brown, 1981). *Heliconius charithonia* and *Laparus doris* were also found in Pipeline Road but were excluded from this analysis, the former

because only one individual was collected, the latter because its phylogenetic position is not well resolved.

ANOVAs were carried out with the complete data set and excluding pollen of butterflies recaptured with a second pollen load, however only analysis of the entire data set is reported, as significance levels were the same in both tests and recaptures comprised $<15\%$ of the samples. Composition of pollen load by sex was compared using a *t*-test for each pollen type within each *Heliconius* species.

Results

Collection data

The collection data show marked microhabitat segregation between some of the mimicry rings studied (Fig. 1). The mimetic pair *H. erato* and *H. melpomene* was found in open habitats and young second growth, primarily along the first 5 km of the transect, while *H. cydno* and *H. sapho* were found in mature second growth at 5–6 km and 8–13 km (Fig. 1). *Heliconius hecale*, *H. ismenius*, and *H. sara* were found at a lower density along the whole transect. These collections were made mainly in the dry season (from February to May 1999). In the wet season, there was similar segregation but with greater overlap in intermediate areas (C. D. Jiggins, pers. obs.). Fieldwork experience in Colombia, Ecuador, and Panama suggests that microhabitat differentiation between the erato/melpomene and the cydno/sapho mimicry rings is common throughout their range (see also Smiley, 1978; Linares, 1989).

Pollen data

In total, 131 pollen loads were examined from nine *Heliconius* species. Thirty-two different types of pollen or spore were encountered, of which 16 were identified to at least genus level, including all but two of those encountered in three or more samples (Table 1). Despite this diversity, pollen loads collected from all *Heliconius* were dominated by just five plant species (Table 1): *Cephaelis tomentosa* (Rubiaceae), *Lantana camara* (Verbenaceae), *Psiguria sp.* (Cucurbitaceae), *Manettia reclinata* (Rubiaceae), and *Tournefortia* sp. (Boraginaceae). Observations in the field suggest that *Psiguria* pollen was predominantly from the commoner *P. warcsewiczii*, but in the samples it was impossible to distinguish this species from the much rarer *P. bignoniacea*. The flowers of these five species are tubular and, with the exception of *Tournefortia*, which has white flowers, all have bright yellow to red corollas. Pollen size and colour differ, with *Psiguria* having large white tetrad grains (110–120 μm long), *Manettia* and *Cephaelis* having grains of an intermediate size (yellow, 44–54 × 51–61 μm,

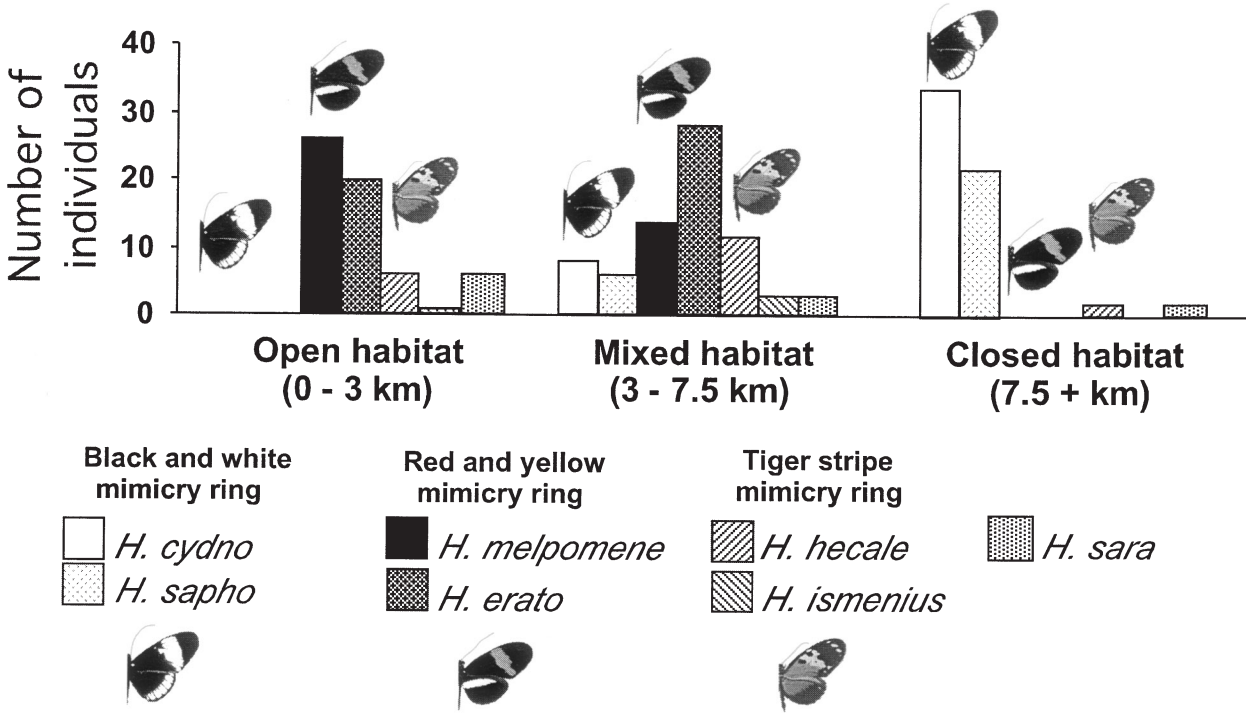

Fig. 1. Distribution of butterflies collected along the Pipeline Road transect. Individuals collected without pollen are also counted. There is marked segregation of habitat between the two mimetic species pairs (*melpomene*/*erato* and *cydno*/*sapho*), with *H. hecale*, *H. ismenius*, and *H. sara* somewhat intermediate. The *cydno*/*sapho* habitat is closed-canopy forest while *melpomene*/*erato* are found primarily in open forest and edge habitat.

Table 1. Proportions of pollen species collected from *Heliconius* pollen loads.

		Mimicry ring						
		Black and white		Red and yellow		Tiger stripe		Blue and yellow
Heliconius species		*cydno*	*sapho*	*melpomene*	*erato*	*hecale*	*ismenius*	*sara*
Pollen species/ ID no.	No. of samples	36	14	27	31	10	3	6
Cephaelis tomentosa (Rubiaceae)	85	**0.655** (0.361)	**0.647** (0.442)	0.184 (0.331)	0.236 (0.358)	0.322 (0.437)	**0.6381** (0.554)	0.002 (0.005)
Psiguria sp. (Cucurbitaceae)	56	0.215 (0.313)		0.291 (0.421)	0.004 (0.016)	**0.456** (0.471)	0.3619 (0.554)	0.335 (0.473)
Lantana camara (Verbenaceae)	52	0.012 (0.042)	0.052 (0.138)	**0.342** (0.405)	**0.609** (0.422)	0.019 (0.059)		**0.468** (0.517)
Tournefortia (Boraginaceae)	40	0.048 (0.181)	0.165 (0.326)	0.009 (0.031)	0.037 (0.166)	0.148 (0.328)		0.050 (0.082)
Manettia reclinata (Rubiaceae)	33	0.003 (0.010)		0.145 (0.283)	0.056 (0.187)	0.051 (0.112)		
Gurania makoyana (Cucurbitaceae)	7	0.023 (0.085)	0.001 (0.002)	+				
Spiracantha sp. (Compositae)	5	0.013 (0.078)			0.003 (0.014)			
Pentagonia macrophylla (Rubiaceae)	4	0.003 (0.015)	0.001 (0.004)	+				
Maripa sp. (Convolvulaceae)	3		0.001 (0.004)		0.003 (0.019)	+		
Polypodiaceae (spore)	3	0.001 (0.006)	+					
Tabebuia sp. (Bignoniaceae)	3	0.001 (0.004)	0.029 (0.094)					
Miconia sp. (Melastomataceae)	2	+	+	+				
Compositae sp. 3	2			+	+			
5	2	0.008 (0.046)			0.017 (0.095)			
25	2			+	+			
31	2		0.061 (0.176)					
Compositae sp. 1	1			0.027 (0.141)				
Citrus sp. (Rutaceae)	1				0.026 (0.168)			
Guazuma sp. (Sterculiaceae)	1					0.003 (0.010)		
Posadaea sp. (Cucurbiataceae)	1		0.033 (0.123)					
Psicotria sp. (Rubiaceae)	1	0.016 (0.092)						
Compositae sp. 2	1	+						
Cassia sp. (Leguminosae Caesalpinioidea)	1							0.146 (0.357)
9	1	0.001 (0.007)						

Table 1. Continued

		Mimicry ring						
		Black and white		Red and yellow		Tiger stripe		Blue and yellow
Heliconius species		*cydno*	*sapho*	*melpomene*	*erato*	*hecale*	*ismenius*	*sara*
Pollen species/ ID no.	No. of samples	36	14	27	31	10	3	6
12	1		0.002 (0.009)					
19	1				0.001 (0.005)			
20	1				0.002 (0.012)			
22	1	+						
27	1	+						
29	1	+						
32	1		0.006 (0.002)					
33	1				+			
Total number of species present		18	13	11	14	7	2	5

Mean proportion and standard errors (in parentheses) of each pollen species across all pollen loads are given for each butterfly. Unidentified pollen morpho-types are identified by an ID number and were encountered in only one or two samples. + indicates presence of pollen grains with a mean proportion <0.001. The commonest pollen type for each *Heliconius* species is shown in bold.

and white, 46–73 × 44–73 μm), and *Tournefortia* and *Lantana* small grains (white, 25–40 μm, and yellow, 23–25 × 25–30 μm) (Roubik & Moreno, 1991). Previous studies have shown sex differences in pollen use (Boggs *et al.*, 1981; Murawski, 1986). Here, males and females were compared using *t*-tests within each species, but in most cases no significant effects were found, most probably due to the small number of females collected for most species (data not shown).

The butterfly species encountered in closed-canopy forest habitats, *H. cydno* and *H. sapho*, collected mainly *Cephaelis tomentosa* pollen, while the species from open areas and secondary forest, *H. erato* and *H. melpomene*, collected mainly *Lantana camara*. *Heliconius hecale* and *H. ismenius* used mainly *Psiguria* and *Cephaelis* pollen. When samples from the seven butterfly species were analysed together, *Cephaelis*, *Lantana,* and *Manettia* pollen showed highly significant variation due to collecting locality (Table 2). *Tournefortia* showed no significant variation according to either locality or species (Table 2).

In marked contrast, *Psiguria* pollen was present in samples collected throughout the transect and showed no significant variation due to collecting locality, however there were highly significant differences in the proportion of *Psiguria* pollen among species, dependent on the phylogenetic associations of the species concerned. *Heliconius erato* and *H. sapho* collected very little *Psiguria* pollen, while *H. melpomene*, *H. cydno*, *H. hecale*, and *H. ismenius* all had on average 21–49% *Psiguria* pollen. The ANOVA between these two phylogenetic groups was highly significant for *Psiguria* and *Lantana* but not for *Cephaelis* or *Manettia* (Table 2). It should be noted that the gap and secondary forest specialist *Psiguria warcsewiczii* was the dominant species at Pipeline Road, but there are many other *Psiguria* species that are more common in old-growth forest in the neotropics. It is therefore impossible to be certain whether the patterns described here are specific to *Psiguria warcsewiczii* or apply to the genus *Psiguria* as a whole.

One individual of *H. charithonia* was collected with only *Lantana* pollen while *H. doris* ($n=3$) exploited mainly *Psiguria* (65%) and *Cephaelis* (21%) as well as *Lantana* and *Manettia*. These species were excluded from the analysis (see above).

The sister species *H. melpomene* and *H. cydno* differed strongly in their patterns of pollen use. Across all habitats, *H. melpomene* collected significantly less *Cephaelis* and more *Lantana* and *Manettia* pollen than did *H. cydno* (*Cephaelis*, $t_{61}=5.04$, $P<0.001$; *Lantana*, $t_{61}=4.61$, $P<0.001$; *Manettia*, $t_{61}=2.75$, $P<0.05$; Table 1). For *Cephaelis* and *Lantana*, these differences remained significant even in the area between 3.3 and 6.6 km where *H. melpomene* and *H. cydno* overlapped ($n=8$ *H. cydno*, $n=11$ *H. melpomene*; *Cephaelis*, $t_{17}=8.39$, $P<0.001$; *Lantana*, $t_{17}=3.42$, $P<0.01$; data not shown). In contrast, there were no significant differences in the proportions of *Psiguria* and *Tournefortia* pollen collected by *H. melpomene* and *H. cydno* (*Psiguria*, $t_{61}=1.09$, NS; *Tournefortia*, $t_{61}=1.10$, NS; Table 1).

Table 2. Analysis of variance among the proportions of different pollen types in each sample.

	Group	*n*	Mean proportion	Sum of squares	d.f.	*F*-ratio	*P*
Cephaelis							
Locality	0–3 km	30	0.352				
	3–7.5 km	56	0.227				
	7.5+ km	41	0.661	9.310	2	15.439	0.000
Phylogeny	melpomene	76	0.443				
	erato	51	0.328	0.005	1	0.150	0.901
Locality × phylogeny				1.458	2	2.418	0.093
Psiguria							
Locality	0–3 km	30	0.183				
	3–7.5 km	56	0.193				
	7.5+ km	41	0.177	0.079	2	0.174	0.841
Phylogeny	melpomene	76	0.279				
	erato	51	0.043	4.405	1	19.503	0.000
Locality × phylogeny				0.159	2	0.352	0.704
Lantana							
Locality	0–3 km	30	0.260				
	3–7.5 km	56	0.412				
	7.5+ km	41	0.020	7.452	2	17.349	0.000
Phylogeny	melpomene	76	0.129				
	erato	51	0.429	3.315	1	15.436	0.000
Locality × phylogeny				1.040	2	2.420	0.093
Tournefortia							
Locality	0–3 km	30	0.008				
	3–7.5 km	56	0.095				
	7.5+ km	41	0.043	0.452	2	2.635	0.076
Phylogeny	melpomene	76	0.046				
	erato	51	0.075	0.049	1	0.576	0.449
Locality × phylogeny				0.003	2	0.020	0.980
Manettia							
Locality	0–3 km	30	0.168				
	3–7.5 km	56	0.022				
	7.5+ km	41	0.001	1.350	2	11.075	0.000
Phylogeny	melpomene	76	0.060				
	erato	51	0.034	0.105	1	1.721	0.192
Locality × phylogeny				0.099	2	0.813	0.446

Proportion data were arcsin-square-root transformed for analysis. Samples are grouped by collecting locality measured along Pipeline Road (see methods). Taxonomic groups within *Heliconius* are melpomene (*H. melpomene*, *H. cydno*, *H. ismenius*, *H. hecale*) and erato (*H. erato*, *H. sara*, *H. sapho*). The data show that the proportions of *Cephaelis*, *Lantana*, and *Manettia* pollen are strongly dependent on locality, while *Psiguria* and *Lantana* show a strong effect dependent on phylogeny.

Discussion

Pollen use by a Heliconius *community*

There is considerable heterogeneity in pollen use between the *Heliconius* species found along Pipeline Road, Panama, however most of this variation can be explained as a result of habitat heterogeneity along the transect. Butterflies collected in open and secondary forest habitats are most likely to be found with *Lantana camara* pollen and to a lesser extent *Cephaelis tomentosa* and *Manettia reclinata*. In contrast, butterflies from closed-canopy forest are more likely to collect pollen from *Cephaelis tomentosa*. This distribution of pollen use corresponds to the observed distribution of flowering plants during the study period (C. D. Jiggins and C. Estrada, pers. obs.). The very high diversity of pollen types encountered on the butterflies (32 species) contrasts with the small number of species that dominate all the samples (five species), implying that *Heliconius* are visiting many flowers for purposes other than collecting pollen (presumably for nectar; see also Murawski, 1986).

In contrast, one genus, *Psiguria*, shows a striking difference in patterns of use between *Heliconius* species that cannot be attributed to microhabitat segregation. Indeed, the proportion of *Psiguria* pollen shows no significant variation due to collecting locality (Table 2). There is a highly significant difference between the two main phylogenetic groups within *Heliconius*, with species in the melpomene group (*H. hecale*, *H. ismenius*, *H. melpomene*, *H. cydno*) feeding on a far higher proportion of *Psiguria* than species in the erato group (*H. erato*, *H. sapho*, *H. sara*). This change is associated with a decrease in the proportion of *Lantana*

pollen collected (Table 2). Melpomene group species also tend to collect a greater total amount of pollen than erato group species (Murawski, 1986; C. D. Jiggins, pers. obs). This suggests that the differences in pollen proportions observed here are probably explained in part by the melpomene group collecting greater quantities of large-grained *Psiguria* pollen, in addition to the other pollen species collected by all *Heliconius*. As both of the phylogenetic groups have representatives in both edge and closed-forest habitats, this difference in pollen use cannot be explained by habitat differences alone.

Previous studies have also found differences in pollen use between major taxonomic groups of *Heliconius*. In Corcovado National Park, Costa Rica, melpomene group species (*Heliconius pachinus*, *H. hecale*, *H. ismenius*) fed almost exclusively on *Psiguria warcsewiczii* pollen, while erato group species (*H. erato*, *H. hewitsoni*) fed on a much higher diversity of pollen, including *Lantana camara*, *Cissus* spp., *Tournefortia* spp., and *Psiguria warcsewiczii* (Murawski, 1986). Similarly in La Selva, Costa Rica, an earlier study used pollen colour to distinguish primarily large-grained white pollen such as *Psiguria* from small-grained yellow pollen such as *Lantana* (Boggs *et al.*, 1981). There was a similar division with melpomene group species (*H. melpomene*, *H. hecale*, *H. cydno*) using primarily large pollen grains and erato group species (*H. erato*, *H. sara*) using more small pollen grains. These results mirror the present study in which differences were found between phylogenetic groups in the use of both *Psiguria* (large grained) and *Lantana* (small grained) pollen.

Based on host plant acceptability, it has previously been proposed that the erato group represents the derived strategy (Smiley, 1985), having evolved to use Plectostemma group *Passiflora* species and small-grained pollen such as *Lantana*, both of which occur in more open habitats. Similarly, Boggs *et al.* (1981) suggested that the erato group is adapted specifically to exploit small-grained pollen. The results presented here, however, suggest that the switch in pollen use may have occurred in the opposite direction. Along Pipeline Road, all the butterfly species studied used a wide range of pollen grain sizes. In contrast to the pattern for *Psiguria* and *Lantana*, there was no effect of phylogenetic group on the use of *Cephaelis* (large grained), *Tournefortia* or *Manettia* (small grained) pollen. Therefore, the phylogenetic patterns cannot be explained by grain size alone. The observations of Penz and Krenn (2000) further contradict the idea that *H. erato* is specialised on small-grained pollen, as *H. hecale* (melpomene group) was more efficient than *H. erato* at collecting *Lantana* pollen under experimental conditions. As there are only two major clades in *Heliconius*, and the outgroup species do not feed on pollen, it is difficult to determine whether the melpomene or the erato group represents the derived strategy; however because all *Heliconius* can use small-grained pollen, but only the melpomene group is observed to specialise on *Psiguria*, it seems reasonable to speculate that the latter represents the derived trait.

Differentiation in daily foraging behaviour between taxonomic groups may explain the observed differences in pollen use. Anthesis occurs in *Psiguria warcsewiczii* flowers around sunrise (Murawski, 1986), and butterflies in the melpomene group take advantage of this by foraging earlier in the morning than other *Heliconius* and defending flowers against other butterflies (Murawski, 1986). Thus, the melpomene and erato groups might differ primarily in competitive ability, leading to differences in *realised niche*. If this effect alone were to explain the pattern, it is possible that erato group species may have lost competitive ability for pollen, perhaps in a trade-off with some other trait such as finding host plants. The observation that *H. erato* and *H. sapho* commonly visit *Psiguria warcsewiczii* flowers in insectaries but fail to collect pollen loads as large as those of *H. melpomene* and *H. cydno* (C. D. Jiggins and C. Estrada, pers. obs.), however, suggests that other adaptations are involved. Further insectary experiments on different pollen species, such as those of Penz and Krenn (2000) with *Lantana camara*, are needed to resolve this question.

The speciation of H. melpomene *and* H. cydno

The sister species *H. melpomene* and *H. cydno* differed significantly in their patterns of pollen use for three of the five common pollen species. For *Cephaelis* and *Lantana* pollen, these differences remained significant within the area where these two butterflies overlap. The data show marked differences in microhabitat between *H. melpomene* and *H. cydno*, which are evident on two scales. First, there is broad segregation along the Pipeline Road transect between the closed-canopy forest habitat of *H. cydno* and the more open habitats of *H. melpomene* (Fig. 1). Second, there are differences in pollen composition between samples collected where *H. melpomene* and *H. cydno* overlap, suggesting that even on a fairly fine scale the two species are segregated. Insectary experiments with *H. melpomene* and *H. cydno* showed no significant differences in exploitation of either *Lantana camara* or *Psiguria warcsewiczii* between the species (C. Estrada and C. D. Jiggins, unpublished), suggesting that differences in pollen load composition are due to microhabitat segregation rather than flower preferences or differences in competitive ability.

Heliconius cydno and *H. melpomene* are known to hybridise in the wild (Mallet *et al.*, 1998), although hybrids occur at very low frequency ($< 0.1\%$). Indeed, one hybrid is known from Pipeline Road (Mallet *et al.*, 1998). These species mate after the female has emerged from the pupa and within the normal home range of the male. Hence, the microhabitat segregation observed will reduce potential mating encounters between *H. melpomene* and *H. cydno* and probably plays a significant role in reducing gene flow between the species. Nonetheless, both species make extensive use of *Psiguria warcsewiczii* pollen and also overlap to some extent in their host plant use (Smiley, 1978), and both of these overlaps are likely to provide occasional opportunities for inter-specific mating.

The segregation of microhabitat between *H. melpomene* and *H. cydno* is associated with mimicry. In the study site, *H. melpomene* mimics *H. erato* while *H. cydno* mimics *H. sapho*. Each mimetic pair has a distinct microhabitat preference (Fig. 1). There is growing evidence that the sympatric co-existence of Müllerian mimicry rings in the neotropics is dependent on microhabitat differences. In other words, butterflies with similar patterns tend to be found in similar habitat (Mallet & Gilbert, 1995; Beccaloni, 1997; DeVries *et al*., 1999). In the case of *H. melpomene* and *H. cydno*, speciation was probably triggered by an initial change in either mimicry or habitat. Whichever came first, however, selection would rapidly have favoured changes in the other trait to ensure efficient mimicry.

Heliconius melpomene rosina has a red forewing band and yellow hindwing bar, while *H. cydno chioneus* has a black and white pattern. These colour patterns play a role in species recognition, leading to assortative mating in insectary experiments (Jiggins *et al*., 2001). Furthermore, F1 hybrids have a distinct non-mimetic pattern and are likely to be selected against due to predator attacks (Benson, 1972; Linares, 1989; Gilbert, 2000). Thus, the correlated shift in habitat and mimetic association has led to assortative mating due to a reduced encounter probability as a result of habitat separation, further pre-mating isolation due to mate recognition using colour pattern cues, and disruptive selection against hybrids due to selective predation. Such associations between traits under disruptive selection and pre-mating barriers to gene flow are a key component in many models of sympatric and ecological speciation (Orr & Smith, 1998; Via, 2001).

Conclusions

The speciation of *H. melpomene* and *H. cydno* is associated with changes in mimicry and habitat, not pollen preferences *per se*. Nonetheless, patterns of pollen exploitation provide a useful means of measuring habitat segregation and suggest that the encounter probability between the butterfly species is likely to be greatly reduced in nature, generating pre-mating isolation. The results support recent studies in *Heliconius* (McMillan *et al*., 1997) and other species (Orr & Smith, 1998; Schluter, 1998), suggesting that divergence in ecology plays a key role in the early stages of speciation.

At a deeper taxonomic level, the use of *Psiguria* pollen shows marked heterogeneity between species that cannot be explained by habitat; it is suggested that an evolutionary switch towards increased reliance on *Psiguria* has occurred in the melpomene group species. This perhaps provided the stimulus for the complex co-evolutionary traits that have evolved between *Psiguria* and *Heliconius* (Murawski, 1986; Gilbert, 1991).

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