Ecological divergence and speciation in *Heliconius cydno* and *H. melpomene*

by

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A thesis submitted for the degree of
Doctor of Philosophy of the University of London

September 2001

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To my family,

for their support and encouragement

throughout this crazy endeavour

"It is hardly an exaggeration to say, that whilst reading
and reflecting on the various facts given in this Memoir,
we feel to be as near witnesses,
as we can ever hope to be,
of the creation of a new species on this earth."

Charles Darwin,
Natural History Review: Quarterly Journal of Biological Science, 1863.
From a review of "Contributions to an Insect Fauna of the Amazon Valley,"
in which Henry Walter Bates
gave an adaptive explanation for mimicry in Amazonian butterflies
and argued that variation in mimicry
might cause speciation

Abstract

We are in the midst of a renaissance in speciation research. There is a return to Darwin's belief in the role of natural selection in driving speciation, after a lengthy focus on geographic isolation and hybrid sterility. Here I describe the ecological, behavioural, and genetic bases of speciation in *Heliconius cydno* and *Heliconius melpomene* (Lepidoptera: Nymphalidae). The two species are sympatric in tropical rainforest across most of Central America and the foothills of the Andes. Ecological differentiation allows coexistence of these sister species despite rare hybridisation. Divergence in microhabitat and larval host plant use has reduced both the potential for gene flow and for competition. In Panama *H. cydno* uses most *Passiflora* species in closed canopy forest, whilst *H. melpomene* is restricted to disturbed habitats, and to *Passiflora menispermifolia.* This ecological differentiation probably generated selection for the key step in their speciation: divergence in warning colour pattern. Both species are unpalatable but are members of different Müllerian mimicry rings that segregate between the two habitats. In Panama *H. cydno* is black and white and mimics *H. sapho*, while *H. melpomene* is black, red and yellow and mimics *H. erato*. This shift in mimicry reduced both the survival and production of hybrids, due to selection against their non-mimetic pattern, and coevolution of mate choice with colour pattern. Major genes are involved in the control of colour pattern differences, including several homologous with inter-racial variation within each species. Two other postmating barriers affect the reproductive success of hybrids. Disruptive sexual selection acts against them, as both sexes of F_1 have poor mating success with the parental species. Following Haldane's rule, female F_1 hybrids are completely sterile, but males are fully fertile and backcross offspring include fertile females. In this and several recent examples, ecological adaptation has been sufficient to catalyse speciation.

Acknowledgements

None of these words or caterpillars would have made it through the larval stage without the help of many people in Colombia, Panama and the UK.
I am forever indebted

to Jim Mallet, my supervisor, for having faith initially, being concerned in the middle, encouragement at the end, and for guidance throughout;
to Jim and Chris Jiggins, for their endless re-reads of re-writes, and for nurturing this fledgling tropical biologist while he was floundering about in the undergrowth and getting lost in swamps;

to Mauricio Linares for welcoming me into his family during my first weeks in Colombia, for help throughout our stay, and together with Camilo Salazar for data from Colombian *H. melpomene*;

to Maria Teresa Almanza, Luis Beltrán, Margarita Beltrán, Catalina Estrada, Gonzalo Fajardo, Adriana Rojas, Clara Saldamando, and Roxana Yockteng, for making us feel instantly at home in Colombia;
to Enrique Bustos, Emigdio Güiza, Marina Ordoñez, Cielo Rocío de Oro, and Norma Pineda, for their help in the Instituto de Genética in Bogota;

to Mathieu Joron for history's most tragicomic short field visit, it was the best of times, it was the worst of times (a cliché that never was so true);

to Marcela Porras for making Colombia such a difficult place to leave;

to Bernard Hermier for revealing the exact spot at which to catch *H. melpomene melpomene* in French Guiana, after we'd spent more than a week feeding the mosquitoes;

to Richard Woods, Camilla Paresce, Luis Alberto Taylor R., Catalina Estrada, Margarita Beltrán, Lianne O'Donovan, Rebecca Coe, Vanessa Bull, Cris Thompson, and Yaneth Sánchez for saving caterpillars from starvation and humans from boredom on many occasions in Panama;

to Catalina Estrada for help in hunting eggs, and for identifying the parasitoids that all too often came out of them;

to Vanessa Bull and Cris Thompson for being good friends during my final onslaught down on the farm, and for enduring my brief foray into tropical forestry;

to Ziheng Yang for the use of his betabinomial program to analyse sterility data;

to the past and present inhabitants of room 407, Alan Bolton, Sam Cotton, Angela Gouge, Claire Grant, and Imogen Hurley, for their friendship and for putting up with my absent-minded wandering;

to the Autoridad Nacional del Ambiente (ANAM) for permission to work in Panama;

to the Smithsonian Tropical Research Institute for making fieldwork in Panama so simple and rewarding, and especially to Dr Eldredge Bermingham for help during our early days there;

to the Biotechnology and Biological Sciences Research Council for my studentship, and the Natural Environment Research Council for the grant to Jim Mallet;

and most of all to my family, for their encouragement and support, and for bearing the worry of this adventure during its exotic and desk-bound phases.
It is to them that I dedicate this thesis.

Declaration

The chapters include data from collaborative work, in which I performed all or the major part of the experimental work. All the chapters were written by me, and some may form the basis of co-authored publications of which I will be the first author. Chapter 4 has already been published as:

Naisbit, R. E., Jiggins, C. D. & Mallet, J. 2001 Disruptive sexual selection against hybrids contributes to speciation between *Heliconius cydno* and *Heliconius melpomene*. *Proc. R. Soc. Lond.* B **268**, 1849-1854.

The appendices contain two papers senior authored by Dr Chris Jiggins, but to which I contributed. Appendix 1 describes the effect of divergence in mimetic colour pattern on mate choice in *Heliconius cydno* and *H. melpomene*:

Jiggins, C. D., Naisbit, R. E., Coe, R. L. & Mallet, J. 2001 Reproductive isolation caused by colour pattern mimicry. *Nature* **411**, 302-305.

Appendix 2 describes a novel example of sterility and its genetic basis, in crosses between geographic races of *H. melpomene* from French Guiana and Panama or Colombia:

Jiggins, C. D., Linares, M., Naisbit, R. E., Salazar, C., Yang, Z. H. & Mallet, J. 2001 Sex-linked hybrid sterility in a butterfly. *Evolution* **55**, 1631-1638.

Russell Naisbit	Prof. James Mallet
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Contents

List of tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

List of figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

1

Introduction

Ecological divergence and speciation

The study of speciation is a challenge of historical reconstruction. In the Origin of Species, Darwin (1859) demonstrated the value of adopting a comparative approach in explaining biological diversity. Using a combination of comparison and extrapolation from biogeography, palaeontology, embryology and artificial selection, he provided evidence for evolution and natural selection. He stressed the role of natural selection, not only as a mechanism of adaptation, but as the means of speciation. Competition would favour the most distinct individuals and cause extinction of intermediate forms, leading to the formation of new species. Natural selection was stressed as the prime force in divergence. Whilst conceding a role for geographic isolation in preventing interbreeding with individuals adapted to different circumstances, more important was exposure to the variety of ecological conditions present in a large continuous area. The gradual nature of speciation was evident in the continuum between varieties and species, with no fundamental distinction on the basis of hybrid sterility.

Since that time, we have made surprisingly little progress. With the Modern Synthesis the emphasis shifted from the role of ecological divergence to the means by which genetic distinctness is maintained (Dobzhansky 1937). This prompted a focus on the role of geographical isolation in allowing divergence, and on reproductive isolation by mate choice and hybrid sterility or inviability (Wu & Palopoli 1994; Coyne & Orr 1998). However, more recent studies of hybridising taxa have led to a realisation that

ecological divergence can drive speciation without geographic isolation (Jiggins & Mallet 2000; Schluter 2001; Via 2001).

The modern approach to speciation has been to identify the "reproductive isolation mechanisms" or "barriers to gene flow" that allow coexistence of distinct taxa. Table 1.1 is a summary of the factors found to act as barriers to gene flow between natural populations. Examples are taken from "model taxa" for research in speciation, in which there is data on their ecological relationships and a variety of possible barriers. The degree of differentiation between taxa varies, but in all cases they have diverged to the extent that distinct taxa are recognisable in sympatry, at least over part of their range. It includes studies from the host race and hybrid zone literature, as well as more conventional studies of sympatric species. A comparative approach can then determine which factors tend to be important in the early stages of divergence, and which processes might be important in driving speciation.

A glance at table 1.1 shows the variety of processes that can act to reduce the level of genetic exchange between populations. These have usually been divided into barriers that reduce the probability of formation of a hybrid zygote leading to a degree of prezygotic isolation, and barriers acting afterwards to reduce the fitness of hybrid zygotes, grouped under postzygotic isolation. Despite the bewildering variety of combinations of barriers, a number of generalisations can be made.

Isolation by premating barriers alone

In some cases, species appear to be isolated by mate choice alone. In Lake Victoria cichlid fish, sympatric closely related species tend to possess male coloration at opposite ends of the spectrum, and isolation is solely by female preference for

conspecific male colour (Seehausen *et al*. 1997). This situation is echoed in the monkeyflowers *Mimulus lewisii* and *M. cardinalis*, which differ in floral colour, morphology and nectar concentration involved in the attraction of either bee or bird pollinators. Hybrids are vigorous and fertile, yet are not found in nature (Bradshaw *et al*. 1995). In the ground crickets *Allonemobius fasciatus* and *A. socius*, the assortment occurs after mating but before fertilisation. Females will mate with heterospecific males and produce hybrids, but when allowed to mate with several males as they do in the wild, the majority of offspring are fathered by the conspecific male. This barrier due to conspecific sperm precedence is sufficiently strong that hybrids make up only 1-7% of mixed populations (Howard *et al*. 1998).

Postmating barriers

More commonly, the species boundary consists of multiple, incomplete barriers to gene flow. Usually a degree of assortative mating is accompanied by some form of selection against hybrids, resulting from incompatibilities between the species that lead to hybrid sterility or inviability, environment-dependent selection against hybrids, or sexual selection against hybrids.

In the European flycatchers *Ficedula hypoleuca* and *F. albicollis* the selection against hybrids takes the form of intrinsic genomic incompatibilities. Hybrids have very low fertility, with just 26% egg-hatch (Sætre *et al*. 1997). However, they are largely isolated by assortative mating, with only 2.6% of matings in the wild being heterospecific and the same number involving one hybrid.

The Darwin's ground finches *Geospiza fuliginosa, G. fortis, and G. scandens* illustrate a second form of selection against hybrids, dependent upon the environment. Despite strong assortative mating using beak morphology, body size, and song as cues, the species will hybridise (Ratcliffe & Grant 1983; Grant 1993). Hybrids show no sterility

or inviability (Grant & Grant 1992). However, there is selection on beak size due to differences in feeding efficiency on the available seed size distribution. In many years this results in environment-dependent selection against hybrid finches, which are intermediate and fall between the parental niches (Grant & Grant 1993).

There is a third possible form of selection against hybrids arising where they have low success gaining mates. In the wolf spiders, *Schizocosa ocreata* and *S. rovneri*, male hybrids have low success because they fail to perform the courtship display, while female hybrids will not accept males of any genotype as mates (Stratton & Uetz 1986).

One striking feature of table 1.1 is the lack of intrinsic hybrid dysfunction for most pairs of species. This is in stark contrast to the focus of most studies of the genetics of speciation on hybrid sterility and inviability in *Drosophila* (Wu & Palopoli 1994; Coyne & Orr 1998). Speciation can clearly occur in the absence of this form of selection against hybrids (Grant & Grant 1992; McMillan *et al*. 1997). In fact, sterility and inviability are likely to be among the later barriers to arise. Some degree of separation, due to either geographic or intrinsic barriers, is required for the accumulation within each species of genetic differences that will ultimately cause hybrid dysfunction. Both *Triturus* newts and *Helianthus* sunflowers, the taxa for which there is evidence of intrinsic incompatibility and a measure of genetic distance, have high Nei's D ($D > 0.8$), suggesting a long period of divergence. In any case, although the effect of sterility or inviability can be very strong, in recently diverged species it is rarely complete in both sexes. For example, in *Triturus* newts there is complete male sterility but female fertility of 27% (Arntzen & Wallis 1991), while in the lacewings *Chrysopa slossonae* and *C. quadripunctata* the incidence of fertile pairing varies geographically between 0 and 40% (Albuquerque *et al*. 1996). In the sunflowers *Helianthus annuus* and *H. petiolaris*, there is around 1% seed set and 14% pollen fertility in F_1 hybrids (Heiser 1947), but

fertility is recovered rapidly in backcross generations and there is evidence of introgression in the wild (Rieseberg *et al*. 1996; Rieseberg *et al*. 1999). Even such a strong barrier is not impermeable to gene flow.

So if hybrid sterility and inviability are generally unimportant in catalysing speciation, what does drive the origin of species? A general feature of the barriers in table 1.1 is the role of selection in their origin.

Natural selection and speciation

Divergent natural selection causing the adoption of different ecological niches, whether in sympatry or allopatry, can lead to reproductive isolation as an incidental by-product (Rice & Hostert 1993; Schluter 2001). Perhaps most intuitive are the cases of host race formation in phytophagous insects, in which assortative mating is a by-product of increased host fidelity (Bush 1969; 1994). Mate choice is often absent, and assortative mating is the product of a reduced encounter rate between populations, due to host-associated mating and differences in host phenology (Wood 1980; Wood & Guttman 1982; Katakura *et al*. 1989; Craig *et al*. 1993). For example, host races of the apple maggot fly *Rhagoletis pomonella* on apple and hawthorn display host fidelity leading to assortative mating in the wild, as a result of the tree under which they eclose, allochrony arising from the different fruiting times of their hosts, and genetic host preference (Feder *et al*. 1994). Adaptation to differences in host chemistry and phenology will also lead to selection against hybrids and against migrants between hosts, creating a stronger barrier to gene flow (Katakura *et al*. 1989; Via *et al*. 2000; Hawthorne & Via 2001).

Divergent natural selection can also drive speciation if the traits under selection are important in mate choice. Pleiotropic effects of ecological traits on mate choice are seen

in the threespine sticklebacks (*Gasterosteus* sp.) of the coastal lakes of British Columbia. They coexist as sympatric limnetic and benthic forms, foraging in open water and the lake bottom respectively. Body size is under strong selection through its association with foraging efficiency, with each parental type dominant in its respective habitat, and hybrids falling near or below the average (Hatfield & Schluter 1999). Mate choice on the basis of body size forms a partial barrier to gene flow, and hybridisation is most likely between the individuals most similar in body size (Nagel & Schluter 1998; Rundle *et al*. 2000). Similarly, in Darwin's finches assortative mating is dependent on beak and body morphology which are under strong disruptive selection, and even song is correlated with beak morphology (Ratcliffe & Grant 1983; Grant & Grant 1993; Grant & Grant 1997; Podos 2001).

Reinforcement of premating isolation

Natural selection may also promote assortative mating as an adaptation in itself in a process known as reinforcement. Low fitness of hybrids between two partially differentiated populations will generate selection for greater discrimination to avoid interspecific mating. Although promoted as a means of closure of allopatric speciation following secondary contact (Dobzhansky 1937; Liou & Price 1994; Butlin 1995; Hostert 1997), reinforcement is really a special case of the selection involved from the start in driving correlation between mating traits and ecological characters in recent models of sympatric speciation (Dieckmann & Doebeli 1999; Kondrashov & Kondrashov 1999). In fact, in both ecological speciation and reinforcement, speciation is most likely if the same trait governs assortative mating and fitness (Dieckmann & Doebeli 1999; Kondrashov & Kondrashov 1999; Kirkpatrick 2000).

The expected result of reinforcement is greater discrimination against heterospecifics by individuals from sympatric populations than by those from allopatry, as is seen in *Gasterosteus* sticklebacks (Rundle & Schluter 1998), between *Drosophila pseudoobscura* and *D. persimilis* (Noor 1995), and in large surveys of *Drosophila* species pairs (Coyne & Orr 1989; Coyne & Orr 1997; Noor 1997). However, perhaps most convincing are cases where selection to reduce the probability of heterospecific mating has reversed the usual mating preference of the species. Males from allopatric populations of the pied and collared flycatchers, *Ficedula hypoleuca* and *F. albicollis*, are black and white, whereas in sympatry their plumage is divergent; brown and white in the former species, and with more white in the latter. This promotes species recognition, for females from sympatric populations show stronger assortative mating when presented with the divergent sympatric males than with the similar allopatric males (Sætre *et al*. 1997). For the pied flycatcher, the preference for less conspicuous males in sympatric populations is the opposite of that in allopatry, so that selection to avoid hybridisation appears to have reversed sexual selection (Sætre *et al*. 1997). A similar pattern is seen in a freshwater stickleback population, where it is in contact with a larger-bodied anadromous species (Borland 1986, cited in Rundle and Schluter 1998). In allopatric areas the freshwater resident males prefer larger females, due to a correlation between female size and fecundity. This preference is reversed in the lower reaches of the river, where the largest females present are those of the other species.

Sexual selection and speciation

Sexual selection can therefore act to oppose the evolution of sexual barriers, but it could also form an important agent of speciation (Panhuis *et al*. 2001). It may play a role in the origin of genomic incompatibility and Haldane's rule, the tendency for sterility or inviability to affect preferentially the heterogametic sex of F_1 hybrids (Haldane 1922).

Sexual selection, in addition to promoting divergence in male phenotype, is thought to drive the elevated rates of accumulation of loci that will produce male sterility in a hybrid genetic background in male heterogametic taxa (Wu & Davis 1993; Turelli 1998). Sexually antagonistic coevolution could lead to hybrid infertility, and the origin of postmating-prezygotic barriers such as those in *Allonemobius* crickets and cactophilic *Drosophila* (Howard *et al*. 1998; Rice 1998; Gavrilets 2000; Knowles & Markow 2001). Divergent processes of sexual selection in isolated populations could also lead them to adopt different bases for mate choice. In fact, since most theories of sexual selection are in effect simply a means by which genetic correlations are generated between female choice and male traits, divergence could occur in sympatry (Turner & Burrows 1995; Payne & Krakauer 1997; Higashi *et al*. 1999). Certainly something similar has occurred in the cichlids of Lake Victoria, where sympatric species pairs and colour morphs tend to have male coloration at opposite ends of the spectrum and in which female preference disappears when tested under monochromatic light (Seehausen *et al*. 1997). The same process has been suggested for the diversification of Lake Malawi cichlids (Deutsch 1997).

Viewed as a whole, these studies suggest that speciation is often a product of divergent natural or sexual selection. Ecologically distinct populations may have a reduced probability of encounter and so reduced potential to interbreed, or differ in mate choice so that these opportunities are less readily taken. If they do interbreed, hybrid offspring may be intrinsically inviable or infertile, or suffer reduced fitness in their interaction with the environment. This multicomponent view of speciation, with many of the barriers dependent on ecological interactions, has several consequences.

Species coexistence despite hybridisation

Firstly, species can retain differences whilst in contact despite the potential to hybridise. Several of the cases described lack intrinsic barriers to interbreeding yet maintain morphological and genetic distinctness. A seemingly fragile barrier such as pollinator recognition can lead to the complete absence of hybrids, as in *Mimulus lewisii* and *M. cardinalis* (Bradshaw *et al*. 1995), but more commonly hybrids between closely related sister species are known from areas of sympatry. Species distinctness can be the result of a balance between hybridisation and selection, rather than the product of a complete lack of hybridisation.

The semipermeable species boundary

The presence of multiple incomplete barriers to gene flow creates the possibility of a semipermeable species boundary. Some hybrids will survive to reproduce, and amongst the genotypes segregating in backcross and F_2 generations, certain genotypes are likely to escape the selection against hybrids. Selection will prevent introgression of alleles that cause dysfunction on the foreign genetic background, but will only strongly impede those that are very tightly linked to loci under selection, and will offer little resistance to neutral and universally beneficial alleles (Barton & Hewitt 1985; Ting *et al*. 2000). For example, introgression studies across hybrid zones of *Helianthus annuus* and *H. petiolaris* show some nuclear markers of *petiolaris* to be over-represented in *H. annuus* and others under-represented, the latter mostly associated with reduced pollen fertility and chromosomal arrangements (Rieseberg *et al*. 1999). Hybrid zones will often filter introgression between species (Martinsen *et al*. 2001), but selection can drive traits across, such as the plumage and behavioural traits of golden-collared manakins probably favoured by sexual selection in a hybrid zone with white-collared manakins (Parsons *et al*. 1993; McDonald *et al*. 2001). Differential introgression has implications

for phylogeny reconstruction, for it will create discordant genealogies for loci experiencing different levels of gene flow (Wang *et al*. 1997; Ting *et al*. 2000; Wu 2001). More fundamentally, this reticulate evolution destroys the concept of a single true phylogeny for the species.

Reversible speciation

Since the ability to hybridise is retained, speciation need not irrevocably commit lineages to different evolutionary paths (contrast Bush 1994). Two species can independently develop coadapted gene pools, but universally beneficial alleles will be capable of crossing the species boundary. Also, where barriers to gene flow depend in part on ecological factors, a change in ecological conditions can alter the strength of the overall barrier. For instance, selection against hybrids in Darwin's finches varies with the changing distribution of available seeds, and some cohorts of hybrids display greater fitness than the parental species (Grant & Grant 1993). Cichlid mate choice according to male colour in Lake Victoria is dependent on the local light regime: in areas with greater turbidity species coloration is less distinct and fewer species and colour morphs are able to coexist (Seehausen *et al*. 1997).

Intermediate stages and the possibility of sympatric speciation

The fact that we can identify a variety of intermediate stages, where selection maintains differences between taxa despite hybridisation, suggests that speciation can occur in the presence of gene flow. Taxa at intermediate levels of differentiation can coexist, and we can identify selective processes that would drive further divergence, suggesting that sympatric or parapatric speciation is plausible (Jiggins & Mallet 2000; Via 2001). This does not mean that all speciation is non-allopatric, but it does argue that complete allopatry is at least unnecessary for diversification.

The rate of speciation

The dependence on ecology may in part explain the differences in the rate at which speciation has occurred in different taxa. This will be determined by the rate at which behavioural or ecological divergence occurs, which in turn will be influenced by specific aspects of the ecology of the taxon. The rate may be limited by intrinsic factors such as the strength of sexual selection, dispersal ability, the importance of recognition cues in mating behaviour, or aspects of genetic architecture, or by extrinsic factors such as the availability of accessible niches, or the degree of division into allopatry. There is little data available on this question, beyond the observation that speciation rates are elevated in novel environments such as islands and lakes (Schluter 1998).

Finally, the importance of ecology suggests that generalisations may be best sought within a taxon. A comparison of pairs of populations at a variety of levels of differentiation, from hybridising races through to sympatric species, can then determine which forms of divergence are important in the early stages of speciation.

Heliconius as a model system for speciation

Heliconius butterflies and their allies (Lepidoptera: Nymphalidae: Heliconiini) offer an ideal system for the study of speciation, for we have detailed background to the ecology and evolution of their radiation in Central and South America (Brown 1981).

They are best known for their Müllerian mimicry, where two or more unpalatable species of *Heliconius* share almost indistinguishable coloration, often together with the related genera *Neruda* and *Eueides*, and other Lepidoptera especially ithomiines. They are protected by cyanogens, both sequestered from their host plants and synthesised *de*

novo (Engler *et al*. 2000). Much is known about geographic variation in their use of *Passiflora* host plants, and the patterns of host partitioning by sympatric heliconiine communities through different use of host species, age of vegetation, and microhabitat (Benson *et al*. 1975; Benson 1978; Smiley 1978a; Smiley 1978b; Waage *et al*. 1981; Gilbert 1984; Mallet & Gilbert 1995). Perhaps uniquely among butterflies, the adults feed on pollen of *Psiguria*, *Gurania* and *Lantana*, allowing increased longevity and continued reproduction (Gilbert 1972; Gilbert 1975). Details are also available of biogeography (Brown 1979), phylogeny and systematics (Brown 1979; Brower 1994; Penz 1999; Beltrán *et al*. 2002), and the genetic basis of colour pattern variation (Sheppard *et al*. 1985; Mallet 1989; Jiggins & McMillan 1997; Linares 1997).

They offer populations with the full range of degrees of differentiation (Mallet 1993; Mallet *et al*. 1998b), from polymorphic populations (Linares 1996; Joron *et al*. 2001; Kapan 2001; Mallet 2001), through geographic races separated by narrow hybrid zones (Mallet 1986; Mallet 1993; Linares 1997), parapatric species with contact zones in which hybrids are rare (Jiggins *et al*. 1997a), and sympatric pairs of sister species (Mallet *et al*. 1998b).

Geographic variation in colour pattern is seen within most species of *Heliconius* (Brown 1979). Perhaps the most extreme variation is seen in *Heliconius erato* and its comimic *H. melpomene*, which have radiated to form almost 30 subspecies across Central and South America. These parapatric races are usually separated by narrow hybrid zones, maintained by frequency-dependent selection on warning coloration. Where two races of *H. erato* meet in Peru, selection against foreign individuals transferred across the hybrid zone was shown to be 52% (Mallet and Barton 1989). This is sufficient to maintain a narrow cline between the races, despite random mating and full fertility and

viability of hybrids (Mallet 1989). Warning colour has diverged in the absence of other genetic differentiation, with no strong allozyme frequency differences (Mallet unpubl.), and the races have the same host plant preferences (Mallet 1989).

This contrasts with the situation in the parapatric species, *Heliconius himera* and *H. erato*. Hybrids between the two species form around 10% of overlapping populations in Ecuador and Peru, yet strong allozyme frequency differences are maintained, with Nei's D = 0.278 (Jiggins *et al*. 1997a; Mallet *et al*. 1998a). The presence of strong assortative mating is the critical difference between this and inter-racial hybrid zones (McMillan *et al*. 1997; Mallet *et al*. 1998a). In other respects the two types of hybrid zone are similar: selection against hybrids results from predation, rather than sterility or inviability (McMillan *et al*. 1997). In this case the position and width of the zone may also be maintained by their divergent habitat adaptation at the transition between wet and dry forest (Jiggins *et al*. 1996). The two do not differ in larval host plant use, so that competitive exclusion and strong habitat association probably prevents them from entering sympatry (Jiggins *et al*. 1997b; McMillan *et al*. 1997; Davison *et al*. 1999).

Heliconius cydno and *H. melpomene*

In this thesis, I have performed work on a different pair of species: *Heliconius cydno* (Doubleday) and *Heliconius melpomene* (Linnaeus). This pair of species are either sister taxa (Brown 1981; Beltrán *et al*. 2002) or *H. melpomene* is paraphyletic relative to *H. cydno* (Brower 1996). They represent a third level of divergence, being sympatric in tropical rainforest across most of Central America and the foothills of the Andes below 1500m (Brown 1979). Both are unpalatable and have diverged in colour pattern as Müllerian mimics of other *Heliconius* species, *H. melpomene* as a co-mimic of *H. erato*, and *H. cydno* usually of *H. sapho* or *H. eleuchia* (figure 1.1) (Brown 1979; Linares

1997). They coexist despite a low level of hybridisation (perhaps 1 in 1000 individuals in the wild) and molecular evidence for recent gene flow (Beltrán *et al*. 2002; Bull *et al*. 2002; Mallet *et al*. 2002).

Layout of the thesis

Here I investigate the ecological, behavioural and genetic factors that allow coexistence between this closely related pair of species. Given those details, and by comparison with other pairs of taxa in *Heliconius*, it will be possible to determine what forms of divergence are critical during speciation in the genus. Chapter 2 describes their divergence in microhabitat and host-plant use. *H. cydno* is found in closed canopy forest and uses most available *Passiflora* host species, while *H. melpomene* is restricted to disturbed habitat and is a specialist on *P. menispermifolia*. This differentiation may have reduced competition and gene flow between the two incipient species, and provided a selection pressure for further divergence.

In chapter 3 I describe the genetics of colour pattern in *H. melpomene rosina* and *H. cydno chioneus*. Colour pattern differences have a relatively simple genetic basis: ten loci can be identified, including genes of major effect. Several of the loci are homologous with those controlling geographic variation in colour pattern within each species. There is also clustering of several loci into linked blocks, suggesting that evolution uses pre-existing linked elements that arose by tandem duplication.

In chapter 4 I present evidence for strong assortative mating between the species, and disruptive sexual selection against F_1 hybrids. Hybrids mate readily with one another but both sexes show a reduction in mating success with the parental species, of 50% on average. This provides an additional postmating barrier between the two species, blurring the distinction between pre- and post-mating isolation.

There are Haldane's rule effects on hybrid sterility in crosses between the two species: my analysis of this situation forms the subject of chapter 5. Female F_1 hybrids between *Heliconius melpomene* and *H. cydno* are completely sterile, while males have normal to mildly reduced fertility. In backcrosses of male F_1 hybrids, female offspring range from completely sterile to fully fertile.

In chapter 6 I summarise the factors that allow ecological and genetic coexistence of *H. cydno* and *H. melpomene*, and examine the role of mimetic divergence in speciation in *Heliconius*.

In the appendices, I present two additional pieces of work to which I have contributed, but which are senior-authored by Chris Jiggins.

Appendix 1 describes the role of colour pattern in the origin of reproductive isolation between the two species. Divergence in mimicry would have led to selection against intermediate non-mimetic hybrid phenotypes. It would also have created premating isolation because colour pattern is important in mate choice.

Finally, appendix 2 describes a novel example of female hybrid sterility in crosses between races of *H. melpomene* from French Guiana and Panama or Colombia.

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Species	Prezygotic isolation				Hybrid fitness reduction			Level of distinctness		
	Assortment by encounter rate		Assortment by mate choice	Assortment before fertilisation	Intrinsic	Environment -dependent	Sexual selection	Form of differentiation	Genetic distance (Nei's D)	% hybrids in the wild (% cross-mating)
	Spatial	Temporal								
Acyrthosiphon pisum host races (pea aphid)	Y[1]		N[1]		N[2]	Y[2]		E[2]		
Epilachna niponica x *yasutomii* (ladybirds)	Y[3]	N[3]	N[3]	N[3]	N[3]			E M[3]		
Eurosta solidaginis host races (tephritids)	Y[4]	Y[4]	weak[4]		N[5]	Y[5]		E[5]		
Rhagoletis pomonella apple/haw host races (tephritids)	Y[6]	Y[6]	N[7]		N[7]			E[8]		(6%)[6]
Rhagoletis pomonella x *mendax* (tephritids)	Y[9]	N[9]	N[9]	N[9]	Y[7]			E[7]		0/907[9]
Enchenopa sp. complex (treehoppers)	Y[10]	Y[11]	Y[10]					E[12] M[10] A[13]	D = 0.013-0.255[13]	
Zeiraphera diniana host races (larch budmoth)	Y[14,15]	N[14]	Y[14]					E[16]	D = 0.039[16]	3%[15]

Table 1.1 Barriers to gene flow between species
Examples were chosen where several possible barriers have been examined, and where the ecological relationship between the taxa has been studied.

Y indicates that the factor acts as a barrier to gene flow; N that it does not. A question mark indicates a factor that was suggested by the author but not explicitly tested.
Differentiation: C colour pattern, M morphology, E ecology, E disruptive selection explicitly tested (reversed order of success across the 2 niches)
K karyotype, A fixed allozyme differences (or strongly differentiated), N fixed differences at nuclear DNA, mt fixed differences in mitochondrial DNA

Species	Prezygotic isolation				Hybrid fitness reduction			Level of distinctness		
	Assortment by encounter rate		Assortment by mate choice	Assortment before fertilisation	Intrinsic	Environment-dependent	Sexual selection	Form of differentiation	Genetic distance (Nei's D)	% hybrids in the wild (% cross-mating)
	Spatial	Temporal								
Chrysopa carnea x *downesi* (lacewings)	Y[17]	Y[17]	N[17]		N[17]			E C[17]		0[17]
Chrysopa slossonae x *quadripunctata* (lacewings)	Y[18]	Y[18]	N[18]	Y[18] (1-way)	Y[18]					
Littorina saxatilis ecotypes (marine snails)	Y[19]		Y[19]		N?[19]			E[20] M[21]	D = 0.046[19]	11-29%[21]
Heliconius himera x *erato* (butterflies)			Y[22,23]		N[22]	Y?[22]		E[24] C[25] mt[26]	D = 0.278[26]	10% (5%)[23]
Gryllus firmus x *pennsylvanicus* (field crickets)		Y[27]	Y[28]	Y?[29] (1-way)	N?[29]			E[28,30] M[31] mt N[32]	D = 0.03[33]	
Allonemobius fasciatus x *socius* (ground crickets)	N[34]	N[34]	N[35]	Y[36]	N[34]			E A[37]	D = 0.19[38]	1-7% F_1[36]
Helianthus annuus x *petiolaris* (sunflowers)		partial[39]	'N'[40]	Y[40]	Y[39]			E M K[39]	D = 0.88[38]	(0-17%)[41]
Triturus cristatus x *marmoratus* (newts)		N[42]	Y[42]		Y[42]			E M A mt K[42]	D = 0.86[42]	1.3-14.3%[42]

Table 1.1 continued

Species	Prezygotic isolation				Hybrid fitness reduction			Level of distinctness		
	Assortment by encounter rate		Assortment by mate choice	Assortment before fertilisation	Intrinsic	Environment -dependent	Sexual selection	Form of differentiation	Genetic distance (Nei's D)	% hybrids in the wild (% cross-mating)
	Spatial	Temporal								
Ficedula hypoleuca x *albicollis* (flycatchers)			Y[43]		Y[43]			C[43]		(2.6%)[43]
Mimulus cardinalis x *lewisii* (monkeyflowers)			'Y'[44]		N[44]			E C M[45]		0[44]
Lake Victoria haplochromine cichlids			Y[46]		N[46]			C[46]		
Geospiza scandens x *fortis* x *fuliginosa* (Darwin's finches)			Y[47]		N[48]	Y[49]		E[49] M[50]	D = 0.004-0.022[51]	(0-4%)[50]
Gasterosteus sp. (sticklebacks)	Y[52]		Y[53]		N[54]	Y[54]	Y[52]	E[54] M[55]	D = 0.02[55]	1%[55]
Schizocosa ocreata x *rovneri* (wolf spiders)			Y[56]		N[56]		Y[56]	M[56]		2/1500[56]

Table 1.1 continued

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Figure 1.1

Heliconius cydno chioneus

Heliconius melpomene rosina

F_1 hybrid

2

Habitat and host use as catalysts for speciation in *Heliconius* butterflies

Abstract

Divergence in habitat and host plant use have been considered as likely causes of speciation in phytophagous insects, since host adaptation and host-associated mating can lead to reproductive isolation. Here I document patterns of microhabitat and host plant use by *Heliconius cydno* and *H. melpomene*, in order to examine the role they may play in speciation. Collections of adult butterflies and host records show habitat segregation, with *melpomene* in second growth and *cydno* in closed canopy forest, although there is considerable overlap. They also differ in host plant specialisation, with *melpomene* restricted to *P. menispermifolia*, while *cydno* uses most available *Passiflora* species. Similar host plant preferences are found in insectary choice experiments, and the behaviour of hybrids suggests that specialisation is inherited as an autosomal dominant trait, although a quantitative threshold trait cannot be ruled out. The difference in preference exists despite the fact that both species can be reared successfully on most *Passiflora* hosts, and that most *Passiflora* species offer broadly similar larval resources in terms of levels of occupancy by predatory ants, and parasitoids of eggs and larvae. Community patterns of host partitioning suggest that divergence in host use was a response to the differing densities of shoots and competitors in the two microhabitats. Microhabitat divergence also seems to have provided the selection pressure for divergence in mimicry to match the local warning colour patterns, *H. melpomene* with *H. erato* and *H. cydno* with *H. sapho*. Although microhabitat use, combined with host plant specialisation, provides only a limited barrier to gene flow between the two

species, it is likely to have been the catalyst for their ecological divergence and speciation.

Introduction

Ecological divergence has generally been assumed to play two possible roles in speciation, allowing ecological coexistence of incipient species by reducing their competitive interaction, and allowing genetic coexistence through the evolution of reproductive isolation (Schluter 1998). The two effects are intimately linked, as one almost inevitable consequence of a change in niche is a reduction in the potential for gene flow between populations, through adaptive divergence in the timing or site of mating, and the production of intermediate hybrids poorly adapted to either niche. In fact, divergence might play another more subtle role as part of a feedback process during speciation (Rice & Hostert 1993). Even if the restriction of gene flow is only minor, divergence may expose the two populations to divergent selection pressures and reduce migration. Both incipient species can then more easily accumulate differences that are beneficial in one niche but deleterious in the other, further reducing gene flow.

In phytophagous insects, habitat and host use have been considered as likely niche axes along which divergence would reduce gene flow and lead to speciation (Bush 1969; Tauber & Tauber 1977; Bush 1994). If mating is associated with their habitat or host-plant, divergence in either trait will lead to assortative mating as a byproduct (Feder *et al*. 1994). This can be accompanied by selection against hybrids and individuals on the 'wrong' host, arising from adaptation to differences in host chemistry or phenology (Filchak *et al*. 2000; Via *et al*. 2000).

In *Heliconius*, most work has concerned host plant and habitat use at the community level, documenting how species partition the available *Passiflora* host species. Local diversity of *Heliconius* is directly proportional to that of their *Passiflora* hosts, with up to ten species of each plus 2-6 other heliconiines at sites across Central America (Gilbert & Smiley 1978; Thomas 1990). Species coexist due to differences in microhabitat use, host plant specialisation, and the use of new shoots or old growth on *Passiflora* vines (Benson *et al*. 1975; Benson 1978; Smiley 1978a; Waage *et al*. 1981; Mallet & Gilbert 1995). The role of host plant specialisation in speciation has been explicitly considered in only one case. No divergence in host use was found between the sister species *Heliconius erato* and *H. himera*, and this probably contributes to their restriction to parapatry with coexistence only in narrow hybrid zones (Jiggins *et al*. 1997). For several relatives of *H. erato*, including *H. himera* and *H. clysonymus*, speciation seems to have been driven by colour pattern divergence and strong habitat adaptation in the absence of host plant differentiation, preventing them from becoming sympatric (Mallet 1993; Mallet *et al*. 1998). In the *melpomene* group of species host shifts are more common between sister species, and may play a role in speciation. In particular, there is pronounced microhabitat and host plant differentiation between the closely related species, *Heliconius cydno* and *H. melpomene* in Central America (Gilbert & Smiley 1978; Smiley 1978a; Estrada & Jiggins 2001). The species have diverged in several characters, the most striking being warning coloration. Both are unpalatable Müllerian mimics of other members of the genus, *H. melpomene* of *H. erato* and *H. cydno* of *H. sapho*. They also show strong assortative mating and female hybrid sterility. However, host plant and microhabitat divergence are likely to have been an important component of their initial divergence, both as barriers to gene flow and in exposing populations to selection pressures for further divergence.

Here I present data from field collections and insectary experiments on the extent of overlap of habitat and host plant use by *H. cydno* and *H. melpomene* in Soberanía National Park in Panama. Divergence in both these traits may have been important in their speciation, and may play a role as a current barrier to gene flow between the species. I also give some evidence of the genetic basis of differences between the two species in host plant choice, and predict the patterns of oviposition by natural hybrids between the species. Finally, I consider possible selective pressures for their divergence in host plant specialisation arising from competition, predation and parasitism.

Methods

Adult butterflies, eggs and larvae were collected from Pipeline Road and nearby forest in Soberanía National Park between August 1998 and March 2000. Pipeline road is a dirt track around 4m wide that forms a transect running from open disturbed habitat into closed canopy forest. Adults were caught with a butterfly net, while eggs and larvae were taken from individually marked *Passiflora* vines along the roadside and up to 2m into the forest either side. Opportunistic collections made throughout the study period were augmented by routine plant surveys between January and July 1999. At one- or two-weekly intervals, eggs and larvae were collected during a survey of the numbered vines, whilst recording the number of shoots available and the presence of ants. Eggs and larvae were reared through to eclosion to be identified, but with experience it was also possible to identify the characteristic immature stages. Most eggs and larvae were reared individually on *Passiflora biflora*, a suitable host for the majority of the local heliconiine species (Waage *et al*. 1981). If the particular host, oviposition site, or larval morphology suggested a host specialist such as *Heliconius sara*, *Laparus doris* or *Philaethria dido*, the larva was fed using the host on which it was found. These host

records also allowed an estimate of the rate of attack by hymenopteran egg parasitoids and dipteran larval parasitoids.

A crude measure of habitat change along the road was obtained from estimates of tree height. At 500m intervals for the first 8km of the transect and 1km intervals thereafter, the heights of five randomly chosen trees were estimated. Trees were chosen by spinning a pointer on flat ground, whilst excluding scrub vegetation on the roadside. Tree height was calculated using trigonometry from a measure of the angle of sight to the tree top and distance of the observer from the tree base.

The host surveys were accompanied by experiments on the suitability of different *Passiflora* species as hosts. Larvae of *H. cydno* and *H. melpomene* were reared in the laboratory on *P. vitifolia*, *P. menispermifolia* and *P. biflora* to estimate survival under controlled conditions. First instar larvae were also put onto wild host plants to study mortality due to predation over a two day period. A paired design was used, testing each individual vine with a caterpillar of one species followed by a caterpillar of the other, to compare mortality of *cydno* and *melpomene* on host and non-host *Passiflora* species. To estimate attack rate by tachinid parasitoids, second instar larvae of *H. melpomene* were placed onto wild hosts, then recovered after four days and reared to test for attack by parasitoids.

Host choice experiments were conducted in 1m x 1m x 2m insectaries. Individual females were given a choice between *Passiflora vitifolia*, *P. menispermifolia*, and *P. biflora*, presented simultaneously either as potted vines with the same number of shoots, or as a fresh single shoot of each species in separate containers of water. Eggs were collected daily, and preference determined from the total number of eggs laid on each

Passiflora species. Experiments were carried out using inter-specific hybrids between *H. melpomene rosina* and *H. cydno chioneus*, and inter-racial crosses between *H. melpomene rosina* from Panama and *H. melpomene melpomene* from French Guiana, testing preference in the parental species, F_1 hybrids, and backcross offspring. F_1 hybrid females are sterile in crosses between *H. cydno* and *H. melpomene* and in one of the reciprocal inter-racial crosses, but these females seemed to select oviposition sites for their sterile eggs in a similar manner to the fertile females. However, females that often failed to lay eggs limited the data from certain hybrid genotypes. The natural hosts of the French Guiana race of *H. melpomene* were not available in Panama, but females were found to oviposit readily on *P. vitifolia*, a member of the same subgenus as one of its natural hosts, *P. quadriglandulosa* (Benson *et al*. 1975).

Results

Habitat use

Captures of adult butterflies show pronounced habitat segregation between *Heliconius cydno* and *H. melpomene* along Pipeline Road (figure 2.1). *H. melpomene* is generally found near the start of the road in second growth forest, with *H. cydno* dominating captures further along in the closed canopy forest. A similar pattern is seen in the wet season, from the beginning of May to late December, and the dry season, from late December to late April (Rand & Rand 1996). However, there remains considerable overlap between the two species. Excluding an extreme outlier in each species, the two species overlap in the area between 3 and 7.5km in both seasons. In the wet season, 69% of *H. cydno* were captured in this area of overlap and 56% of *H. melpomene*, while in the dry season the equivalent figures were 22% and 40%.

The pattern of segregation is repeated more strikingly in the co-mimetic species (figure 2.1c). *Heliconius erato* is found in the early part of the transect together with its co-mimic *H. melpomene*, and *H. sapho* was captured almost exclusively further along the road, in similar areas to *H. cydno*.

Distribution of eggs and larvae along the transect

The collections of eggs and larvae show a pattern similar to that of adult captures, with *H. melpomene* dominating host records from the early part of Pipeline Road, and *H. cydno* more common further along (figure 2.2). There is a similar degree of overlap in the area where adults of both species were found to occur: 29% of *H. cydno* host records and 44% of *H. melpomene* records were found between 3 and 7.5km along the road.

Habitat change along the transect

The habitat changes very gradually along the Pipeline Road transect into Soberanía National Park. There is a slight tendency for tree height to increase further along the road, and for the standard deviation of tree height to decline and become less variable (figure 2.3). There is no sharp discontinuity, although further along the transect there tends to be fewer gaps in the canopy and patches of shrubby vegetation. The pronounced segregation of *H. cydno* and *H. melpomene* may also be influenced by the surrounding habitat, with the beginning of Pipeline Road lying close to areas of open, disturbed habitat favoured by *H. melpomene*, while further along the habitat consists of closed canopy forest favoured by *H. cydno*.

Host plant use in the wild

A total of 837 eggs and larvae were collected from nine species of *Passiflora* in Panama (table 2.1). *Heliconius cydno* was the only heliconiine species that could be considered a

generalist, with records from five *Passiflora* species almost evenly distributed among the *Granadilla*, *Distephana* and *Plectostemma* subgenera. In contrast, *H. melpomene* was almost exclusively restricted to *P. menispermifolia*. The other heliconiine species were all specialists, either found predominantly on a single *Passiflora* species, or in the case of *H. erato* and *Dryas julia*, only on hosts of the *Plectostemma* subgenus.
The only other herbivores found on *Passiflora* vines were flea beetles (Coleoptera: Chrysomelidae: Alticinae), which were rarely encountered, larvae of *Josia* (Lepidoptera: Notodontidae: Dioptinae) and larvae of Riodinidae (Lepidoptera). Riodinids were of the species *Nymphidium mantus, Theope lycaenina*, and *Juditha molpe*, found on *P. vitifolia*, *P. auriculata*, *P. coriacea*, and *P. biflora*. They are defended by ants (*N. mantus* and *T. lycaenina* by *Azteca*, and *J. molpe* by *Dolichoderus*) to which they provide a nutritious secretion (DeVries 1997).

Host choice in the insectary and the genetic basis of host plant preference

The host plant preferences of *H. cydno* and *H. melpomene rosina* in the insectary are similar to those in the field (table 2.2 a and b). *H. cydno* laid eggs on all three host offered, with variation between females but an overall preference for *P. vitifolia*, while *H. melpomene* was specialised on *P. menispermifolia*. Preference for *P. menispermifolia* was also seen in F_1 hybrids between the two species (table 2.2 c), and in offspring from a backcross of an F_1 male to a female *H. melpomene* (table 2.2 e). In contrast, offspring of the backcross to *H. cydno* were more similar to *H. cydno* in using all three hosts, but with a bias towards *P. menispermifolia* (table 2.2 d).

Crosses between *H. melpomene rosina* from Panama and *H. melpomene melpomene* from French Guiana show a similar pattern. Although South American *melpomene* use a variety of hosts in the wild (Benson *et al*. 1975), in these trials *melpomene* from French

Guiana show complete specialisation on *P. vitifolia* (table 2.3 b). As in the inter-specific trials, the preference of Panama *melpomene* for *P. menispermifolia* was seen in F_1 individuals and in the offspring of backcrosses to Panama *melpomene* (table 2.3 c-f). The results from offspring of the backcross to French Guiana *melpomene* were more variable (table 2.3 g). Some females showed complete preference for *P. vitifolia*, others for *P. menispermifolia*, while a number of individuals oviposited on both. However, there were several females that would not lay eggs on either species until they were given access to plants of *P. edulis*, a cultivated species. It is not clear why they showed this latter preference, unless *P. edulis* is similar to one of their hosts in French Guiana.

It is tempting to conclude from these results that the host plant preference of *H. melpomene* in Panama, specialisation on *P. menispermifolia*, is inherited as an autosomal dominant trait in both crosses. It is the predominant behaviour in F_1 individuals of crosses of Panama *melpomene* with *H. cydno* and with *melpomene* from French Guiana, and also in the backcrosses to Panama *melpomene*. For a sex-linked trait, female offspring will carry the paternal Z chromosome, since females are the heterogametic (ZW) sex in Lepidoptera. This would predict segregation of *melpomene* and *cydno*-like behaviour with the Z chromosome in the offspring of a backcross of *cydno* by *melpomene* F_1 male to *melpomene* female (contrast table 2.2 e), and the preference of French Guiana *melpomene* for *P. vitifolia* to be seen in the F_1 offspring of French Guiana male by Panama female (contrast table 2.3 c). Neither of these results are observed, suggesting that the behaviour is autosomally inherited. Although certainly autosomal, there remains the possibility that preference is a quantitative threshold trait, with preference for *P. menispermifolia* in the F_1, but increasingly *cydno*-like behaviour with repeated backcrossing to *H. cydno*.

Selection pressures on host plant use

1) Larval feeding ability

It is possible to rear *H. cydno* and *H. melpomene* on the majority of the *Passiflora* hosts, and whilst rearing butterflies for experiments we routinely fed both species on *P. biflora*. Under our rearing conditions they showed similar but low survival to pupation on *P. vitifolia*, *P. menispermifolia*, and *P. biflora* (table 2.4). More detailed experiments using Costa Rican species have shown that larvae of *H. cydno* and *H. melpomene* achieve similar growth rates across most of the host species available (Smiley 1978a; Waage *et al*. 1981). It is therefore not immediately clear why *H. melpomene* adopts a specialist oviposition strategy on *P. menispermifolia* (or on *P. oerstedii* in north-eastern Costa Rica), in contrast to the generalist behaviour of *H. cydno*.

2) Distribution of plants

Most of the host plants were found throughout Pipeline Road, although usually with a clumped distribution (figure 2.4). Of the more common species, only *P. auriculata* showed a pronounced increase in density further along the transect. However, the transect itself, a road cut through the forest, provides an artificial sample of the surrounding habitat. *Passiflora* plants are usually more common in disturbed areas and in particular are more likely to germinate there and to form the new shoots that *Heliconius* larvae require (Smiley 1978b; Mallet 1984). Away from the roadside, plant and shoot density is likely to be lower in the forest interior further along the road than in the disturbed areas surrounding the start of Pipeline Road. Although the *cydno* habitat has a lower density of host individuals, both *cydno* and *melpomene* have access to a similar array of host plant species.

3) Distribution of competitors

Most of the species commonly found as eggs or larvae overlap to a greater extent with *melpomene* than with *cydno*. In particular, host records of both *H. erato* and *H. hecale* were more common in the area before 7km along Pipeline Road (figure 2.5).

4) Shoot occupancy

The different *Passflora* species represent broadly similar resources in a number of features. The level of shoot occupancy by eggs and larvae was fairly similar across all species, although highest in *P. menispermifolia* and *P. vitifolia* (table 2.5). Many *Heliconius* species have cannibalistic larvae, and in particular will eat eggs when encountered, so that a previously occupied shoot does not represent a favourable site for oviposition. However, groups of eggs were commonly found on new shoots (table 2.5), and although these were often clustered at the shoot tip and probably laid by the same female, in some cases larvae of several different instars were found along the same shoot.

5) Ant occupancy and larval disappearance

An important source of larval mortality is predation by ants, which are attracted to the extrafloral nectaries of *Passiflora* vines (Smiley 1985b; Smiley 1986). There are suggestive differences in survival among first instar larvae of *cydno* and *melpomene* put out onto host and non-host *Passiflora* vines in the wild (table 2.6). Mortality over two days on *P. menispermifolia* was 56.5% for *melpomene* and 69.6% for *cydno*, whilst performance was reversed on the other *Passiflora* species, 85.6% mortality for *melpomene* and 80.4% for *cydno*. However, more data would be needed to confirm the increased survival of *melpomene* on *P. menispermifolia* and test possible reasons. Much of the loss is likely to be due to ant or wasp predation (Smiley 1985b). There is some

evidence from my shoot surveys for differences between the species in the level of ant attendance (table 2.5), but the data varies greatly between surveys and between individual plants.

6) Egg parasitism

Another important source of mortality is egg parasitism by Hymenoptera. Parasitised eggs turned black after several days, and for parasitoids of the Encyrtidae and Scelionidae, a single individual emerged from each *Heliconius* egg, while for Trichogrammatidae up to 13 emerged, with extremely female-biased sex ratio. Levels of egg parasitism were fairly similar across the species for which sufficient host records were found (table 2.7).

7) Larval parasitism

Larvae are attacked by parasitoids of the Tachinidae (Diptera), which develop within apparently healthy caterpillars, to emerge and pupate when the caterpillar reaches the fifth instar. They attack larvae at an early stage, for in five cases parasitoids emerged from larvae taken from the wild in their first instar. They were found in *Dryas*, *Eueides* and *Philaethria* as well as *Heliconius*. The only other record of larval parasitism was an ichneumonid parasitoid (Hymenoptera) which emerged from a caterpillar of *Nymphidium mantus* (Riodinidae). Again, insufficient larvae were found to provide a good estimate of parasitism rates, but tachinidae did seem to be more prevalent in larvae collected from *P. vitifolia* (table 2.7). This high parasitism rate was confirmed in a small trial in which second instar larvae of *H. melpomene* were placed onto wild host plants, and recovered after four days to be reared to test for the presence of tachinid parasitoids. The majority disappeared, probably due to predation, but of five that were recovered from *P. vitifolia*, one was carrying a parasitoid.

Discussion

Habitat divergence and host use as barriers to gene flow

Ecological divergence between *H. cydno chioneus* and *H. melpomene rosina* has included the evolution of differences in habitat use and host plant preference, with *H. cydno* using the majority of host plants in the rainforest understorey, and *H. melpomene* behaving as an oviposition specialist on *P. menispermifolia* in second growth. Both traits have the potential to act as barriers to gene flow between the hybridising species. Males of *Heliconius* learn the location of *Passiflora* host plants and patrol them, and they are likely to mate with teneral females soon after they emerge from pupae on or near their host plant. Habitat and host plant differences might therefore reduce the rate at which newly emerged females are encountered by males of the other species.

The strength of this barrier to gene flow is limited by two factors, the extent to which females remate in the wild, and the degree to which resource use by the two species overlaps. Females are known to remate in the wild, with an average of 1.6 matings per female in wild-caught *H. cydno* from Costa Rica (Boggs 1979). Additional matings are not necessarily associated with the host plant, reducing the strength of the barrier due to differences in host plant use alone. Despite this, the divergence may still be important because it will reduce the encounter rate between the species when females are newly emerged and least able to resist mating by a heterospecific male. The second limitation arises because differentiation between the two species is incomplete, with overlap in habitat and host plant use. Overall, some 54% of *cydno* and 49% of *melpomene* were captured within the area in which they overlap on Pipeline Road, and the host plant of the specialist *H. melpomene* falls entirely within the host range of *H. cydno*, with 24% of *cydno* host records from the host of *H. melpomene*, *P. menispermifolia*. The effects of habitat and host use also interact, further weakening the strength of any barrier. There

are few vines of *P. auriculata* in the area between 3 and 7.5km where *cydno* and *melpomene* are found together, so that a greater percentage (38%) of *H. cydno* host records were from *P. menispermifolia* in that area. The barrier due to divergence in habitat and host plant use is therefore strong but incomplete.

Patterns of community partitioning

Although limited conclusions about community niche partitioning are possible using data from a single site, in *Heliconius* there is abundant data available from across Central and South America. In Panama as in Costa Rica, the majority of *Heliconius* species are most abundant in second growth and the forest margin (Smiley 1978b; Waage *et al*. 1981; Mallet & Gilbert 1995). The second growth community tends to partition the available *Passiflora* species, with each *Heliconius* species specialising on a single *Passiflora* or members of one subgenus. For example, in Panama you tend to find *H. melpomene* on *P. menispermifolia*, *H. hecale* on *P. vitifolia*, and *H. erato* on the subgenus *Plectostemma*. These species coexist with members of related genera including *Philaethria*, *Dryas* and *Eueides*, which use the same hosts but feed on older plant growth rather than the new shoots used by *Heliconius* (Benson *et al*. 1975; Benson 1978; Mallet 1984). In the forest interior, where new shoots of *Passflora* vines are generally rare (Smiley 1978b; Mallet 1984), *Heliconius* species tend to adopt one of two oviposition strategies: as specialists that lay clusters of eggs on rare large shoots, as does *H. sapho* on vines of subgenus *Astrophea* (for which host records were not found here), or *Heliconius* species that use the majority of available *Passiflora* species, like *H. cydno* and *H. numata* (Benson *et al*. 1975; Benson 1978; Smiley 1978a).

Selection pressures for divergence in host use

It seems likely that competition is responsible for ecological divergence in the patterns of host use by *cydno* and *melpomene*. *H. cydno* behaves as a generalist and *H. melpomene* as a host specialist, despite the fact that larvae of both species develop approximately equally well across most of the available *Passiflora* species (Smiley 1978a; Smiley 1985a). The *Passiflora* hosts also represent approximately similar resources in terms of overall levels of shoot occupancy by heliconiines and by ants, and in their levels of egg and larval parasitism. The most likely explanation is that the different host use strategies adopted by *cydno* and *melpomene* are a response to different patterns of shoot and butterfly density in their respective microhabitats. In closed canopy forest, new shoots of *Passiflora* are rare, and *cydno* females behave as generalists across the available species. In second growth where *melpomene* is found in Panama and Costa Rica, a greater abundance of *Passiflora* shoots are available but this is accompanied by a greater abundance of potential competitors. In contrast, *H. melpomene* in French Guiana is found in the absence of *cydno*, uses the forest interior to a greater extent, and is more generalist in its larval food plants (Benson *et al*. 1975). However, specialisation by *melpomene* in Central America is probably due more to competition in the second growth community, particularly among *H. erato*, *H. ismenius* and *H. hecale*, rather than character displacement with *cydno*. Competition might act through direct competition for larval resources, but also through cannibalism, apparent competition arising from increased density of shared egg and larval parasitoids, and the possibility of interference competition through pupal mortality due to the attentions of males of pupal-mating species sharing the host plant (Gilbert 1984; Gilbert 1991). There is even the possibility, although this is pure speculation in the *Heliconius* system, that sexual selection could drive host specialisation in the absence of any direct competition (Colwell 1986). Host fidelity might evolve by a runaway process if mating is associated

with the host plant, so that increasingly strict host choice creates differential mating success. A combination of these effects might have been sufficient to drive host plant specialisation in the second growth community, and lead to the differences between *H. cydno* and *H. melpomene*.

The genetic basis of host plant choice

Here I find autosomal inheritance of host preference, apparently with dominance effects although it is also possible that preference is a threshold trait controlled by a number of dominant genes. Oviposition preference has been found to have a variable genetic basis in other taxa. It can be polygenic, as in crosses between populations of the Colorado potato beetle, *Leptinotarsa decemlineata*, (Lu & Logan 1995), but is more typically controlled by one or few loci. Results have variously indicated either an additive genetic basis, as in the brown planthopper, *Nilaparvata lugens* (Homoptera) (Sezer & Butlin 1998) and *Drosophila sechellia* and *D. simulans* (Diptera) (R'Kha *et al*. 1991), or dominance effects in *Cryptomyzus* aphids (Homoptera) (Guldemond 1990) and the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera) (Lu & Logan 1995). In Lepidoptera species differences are commonly sex-linked (Prowell 1998), and two studies have demonstrated paternal inheritance of oviposition preference. There is partial sex-linkage in *Papilio* (Thompson 1988) and strong sex-linkage in the comma butterfly *Polygonia c-album* (Janz 1998), but autosomal inheritance is also known, in the moths *Heliothis virescens* and *H. subflexa* (Sheck & Gould 1995).

The host choice of hybrids will affect the extent to which they compete with the parental species, and also influence their encounter rate and so the direction in which backcrossing and gene flow is most likely. Hybrids are most readily produced by a mating between *H. cydno* female and *H. melpomene* male, so in nature F_1 hybrid larvae

are most likely to develop on the host plants of *H. cydno* and to be found in the area of overlap. Hybrids would therefore be likely to be in contact with both parental species. F_1 females are sterile, but in the early stages of their divergence before the evolution of sterility, the preference of hybrids for *P. menispermifolia* would have brought their offspring into contact with both parental species. Their (unknown) habitat preference would then have been critical in determining the pattern of further backcrossing, and will be currently for fertile F_1 males. The genetics of habitat choice and any genetic correlation with host choice would also be critical in determining how disruptive selection acts on these ecological traits.

Ecological divergence and speciation

Divergence in habitat and host plant use form strong but incomplete barriers to gene flow between *H. cydno* and *H. melpomene*. There is considerable overlap in both traits, so they would be insufficient to cause speciation alone. The role of microhabitat divergence was, however, critical in initiating a positive feedback chain of ecological divergence that led to speciation. The shift in microhabitat use has probably selected for divergence in host plant specialisation, by exposing the populations to different densities of *Passiflora* shoots and *Heliconius* competitors. It also exposed them to a different suite of potential models for Müllerian mimicry, reducing the frequency dependent advantage of the pattern of their previous microhabitat, and replacing it with selection to converge on the locally dominant pattern in the new habitat. There is good evidence that unpalatable butterflies do respond to such variation in model frequency (Beccaloni 1997; Linares 1997; Joron *et al*. 2001; Kapan 2001). Colour pattern divergence was the key step in the speciation of *cydno* and *melpomene*, for it resulted in post-mating isolation due to selection against intermediate non-mimetic hybrid phenotypes, and pre-mating isolation due to coevolution of male mating preference with

colour pattern (Jiggins *et al*. 2001). Microhabitat divergence was therefore the catalyst for the ecological speciation of *H. cydno* and *H. melpomene*.

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	P. menispermifolia	*P. vitifolia*	*P. auriculata*	*P. coriacea*	*P. biflora*	*P. ambigua*	*P. nitida*	*P. tryphostemmatoides*	*P. foetida*
Subgenus	G	D	P	P	P	G	G	T	Dy
H. cydno	14	15	19	-	-	1	9	-	-
H. melpomene	146	1	1	-	-	-	-	-	-
H. hecale	4	126	1	-	-	-	2	-	-
H. erato	-	-	3	13	12	-	-	-	-
H. ismenius	-	-	-	-	-	11	-	-	-
H. hecalesia	-	-	-	-	2	-	-	1	-
H. sara	-	-	46	-	-	-	-	-	-
Laparus doris	-	-	-	-	-	205	-	-	-
Eueides	1	11	-	-	-	-	1	-	-
Dryas julia	-	1	33	8	6	-	-	-	-
Philaethria dido	1	19	-	-	-	-	-	-	-
Dione juno	-	70	-	-	-	-	-	-	-
Agraulis vanillae	-	-	-	-	-	-	-	-	1
Riodinidae	-	3	38	5	7	-	-	-	-
Josia	3	-	-	-	-	-	-	-	-

Table 2.1 Host plant records from *Passiflora* vines

Subgenera of *Passiflora*:
G *Granadilla*, D *Distephana*, P *Plectostemma*, T *Tryphostemmatoides*, Dy *Dysosmia*.
Only three *Eueides* collections survived to eclosion and could be identified to species level (*E. aliphera* on *Passiflora vitifolia*). The Riodinidae include *Nymphidium mantus*, *Theope lycaenina*, and *Juditha molpe*. *Josia* is a notodontid moth (Notodontidae: Dioptinae).
The records for *Dione* come from a single collection, while those of *Heliconius sara* and *Laparus doris* are each from two.

Table 2.2 Results of insectary host choice experiments using *Heliconius melpomene rosina* from Panama and *H. cydno chioneus* from Panama and their hybrids. Each line shows the number of eggs laid by a single female offered a choice between *Passiflora vitifolia*, *P. menispermifolia*, and *P. biflora.* The genotype of the female parent of hybrids is given first, followed by that of the male.

(a) *H. cydno*

Female	*P. vitifolia*	*P. menisp.*	*P. biflora*
CP 151	9	26	2
Br 103	33	4	0
Br 107	40	3	0
CP 155	32	0	0
CP 104	3	0	6
CP 149	1	0	5
Br 95	4	2	0
CP 127	19	46	6
CP 185	5	0	8
Br 163	24	0	2
Br 200	22	36	2
CP 372	22	10	14
CP 360	23	0	0
CP 426	19	14	10

(b) *H. melpomene*

Female	*P. vitifolia*	*P. menisp.*	*P. biflora*
MP132	0	21	0
MP147	0	43	0
Br 65	0	35	0
MP170	0	19	0
Br 79	0	37	0
Br 81	0	46	0
Br 80	0	13	0
MP211	0	55	0
Br 35	0	60	1
MP274	0	72	0
Br 192	0	54	0

(c) F_1: *H. cydno* x *H. melpomene*

Female	*P. vitifolia*	*P. menisp.*	*P. biflora*
Br 334	0	1	0
Br 336	0	20	0
Br 338	0	17	0
Br 339	0	7	0
Br 349	0	7	0
Br 350	2	25	3
Br 369	0	3	0

(d) Backcross: *H. cydno* x F_1

Female	*P. vitifolia*	*P. menisp.*	*P. biflora*
Br 357	2	13	0
Br 360	0	1	0
Br 389	18	8	7
Br 390	0	28	0
Br 395	2	3	0
Br 402	0	25	5

(e) Backcross: *H. melpomene* x F_1

Female	*P. vitifolia*	*P. menisp.*	*P. biflora*
Br 354	0	21	0
Br 358	0	10	0
Br 365	0	13	0
Br 366	0	32	0
Br 367	0	30	1
Br 368	0	26	0
Br 372	0	33	0
Br 374	0	40	0
Br 381	0	31	0
Br 386	0	32	0
Br 396	0	32	0
Br 397	0	18	0

Table 2.2

Table 2.3 Results of insectary host choice experiments using *Heliconius melpomene rosina* from Panama and *H. melpomene melpomene* from French Guiana and their hybrids. Each line shows the number of eggs laid by a single female offered a choice between *Passiflora vitifolia*, *P. menispermifolia*, and *P. biflora.* In (g), *P. edulis* was added when the female delayed laying on any of the three species, and a hyphen in a cell indicates a trial in which one or more plant species were absent. The genotype of the female parent of hybrids is given first, followed by that of the male.

(a) *H. melpomene* Panama (MP)

Female	*P. vitifolia*	*P. menisp.*	*P. biflora*
MP132	0	21	0
MP147	0	43	0
Br 65	0	35	0
MP170	0	19	0
Br 79	0	37	0
Br 81	0	46	0
Br 80	0	13	0
MP211	0	55	0
Br 35	0	60	1
MP274	0	72	0
Br 192	0	54	0

(b) *H. melpomene* Guiana (MG)

Female	*P. vitifolia*	*P. menisp.*	*P. biflora*
MG137	34	0	0
Br 44	6	0	0
MG37	33	0	0
MG34	26	0	0
MG5	35	0	0
MG22	35	0	0
Br 147	44	0	0
MG151	25	0	0
Br 181	28	0	0
Br 182	29	0	0
MG264	36	0	0

(c) F_1: MP x MG

Female	*P. vitifolia*	*P. menisp.*	*P. biflora*
Br 46	0	29	0
Br 110	0	19	0
Br 111	0	13	0
Br 113	0	28	0
Br 123	0	16	0
Br 124	0	20	0
Br 167	0	23	0
Br 131	0	16	0
Br 158	0	25	0

(d) F_1: MG x MP

Female	*P. vitifolia*	*P. menisp.*	*P. biflora*
Br 23	37	1	0
Br 184	0	37	0
Br 189	0	37	0
Br 194	0	39	0
Br 195	1	5	0

(e) Backcross: MP x (MPxMG)

Female	*P. vitifolia*	*P. menisp.*	*P. biflora*
Br 57	0	24	0
Br 86	0	33	0
Br 91	0	20	0

(f) Backcross: (MGxMP) x MP

Female	*P. vitifolia*	*P. menisp.*	*P. biflora*
Br 52	0	39	0
Br 53	2	25	0

Table 2.3

(g) Backcross: (MGxMP) x MG

Female	*P. vitifolia*	*P. menisp.*	*P. biflora*	*P. edulis*
Br 222	11	0	-	-
Br 218	20	0	-	-
Br 216	0	26	0	-
Br 281	0	18	-	-
Br 280	30	1	-	-
Br 223	1	23	-	-
Br 254	7	13	-	-
Br 259	7	13	-	-
Br 248	5	0	-	19
Br 240	0	10	-	4
Br 225	1	6	0	19
Br 227	0	0	-	9
Br 228	0	0	-	21

Table 2.3 continued

	H. melpomene	*H. cydno*
P. menispermifolia	7/20 (35%)	5/20 (25%)
P. vitifolia	11/28 (39%)	15/28 (54%)
P. biflora	9/27 (33%)	17/27 (63%)

Table 2.4 Survival to pupation of larvae reared under controlled conditions on three different *Passiflora* host plants

	P. menispermifolia	*P. vitifolia*	*P. auriculata*	*P. coriacea*	*P. biflora*	*P. ambigua*	*P. nitida*	*P. tryphostemmatoides*
Subgenus	G	D	P	P	P	G	G	T
# plants	275	472	698	244	255	86	45	46
# eggs & larvae	144	156	100	18	16	12	20	2
# shoots	316	583	1224	167	335	61	173	25
# shoots occupied	74	102	50	16	15	4	14	2
Shoot occupancy %	23.4	17.5	4.1	9.6	4.5	6.6	8.1	8.0
Interquartile range	10.7-44.4	10.7-29.9	0-6.1	0-14.6	0-7.9	0-0	0-11.1	0-0
# plants (∃1 shoot)	170	287	554	113	161	49	45	23
# ant occupied	78	191	362	74	79	26	22	0
Ant occupancy %	45.9	66.6	65.3	65.5	49.1	53.1	48.9	0
Interquartile range	35.6-51.4	46.4-77.3	54.3-69.3	21.4-93.8	38.9-60.0	0-68.8	31.3-66.7	0-0
Eggs per shoot	1.95	1.53	2.00	1.13	1.07	3.00	1.43	1.00

Table 2.5

Host plant records from 18 surveys of *Passiflora* vines along Pipeline Road, Soberanía National Park, Panama, Jan-July 1999

"Shoot occupancy" is the percentage of shoots that bore a heliconiine immature stage

"Ant occupancy" is the percentage of plants with shoots that were occupied by ants

"Eggs per shoot" gives the average number of eggs sharing an occupied shoot

(a) *P. menispermifolia*

	H. cydno survived	*H. cydno* died
H. melpomene survived	6	4
H. melpomene died	1	12

(b) other *Passiflora*

	H. cydno survived	*H. cydno* died
H. melpomene survived	7	7
H. melpomene died	12	71

Table 2.6

Survival over a two day period of first instar larvae on *Passiflora* vines in the wild Larvae were tested in pairs, placing a caterpillar of one species on a vine, then after two days replacing it with a caterpillar of the other species.

	P. menispermifolia	*P. vitifolia*	*P. auriculata*	*P. coriacea*	*P. biflora*	*P. ambigua*	*P. nitida*	*P. tryphostemmatoides*
Subgenus	G	D	P	P	P	G	G	T
# eggs	195	215	45	16	15	10	15	2
# parasitised	17	34	7	1	1	0	5	1
% egg parasitism	8.7	15.8	15.6	6.3	6.7	0	33.3	50.0
# larvae	45	74	12	9	5	2	4	0
# parasitised	3	16	1	1	0	0	0	0
% larval parasitism	6.7	21.6	8.3	11.1	0	0	0	-

Table 2.7 Rates of egg and larval parasitism from *Passiflora* host records

Eggs are parasitised by Trichogrammatidae, Encyrtidae, and Scelionidae (Hymenoptera).

Larvae are parasitised by Tachinidae (Diptera). Tachinid larva usually emerge from the caterpillar at the fifth instar, so the larval section of the table includes only caterpillars that were reared to fifth instar or beyond. They were found in larvae taken from the wild at all developmental stages, including five examples from larvae collected as first instars.

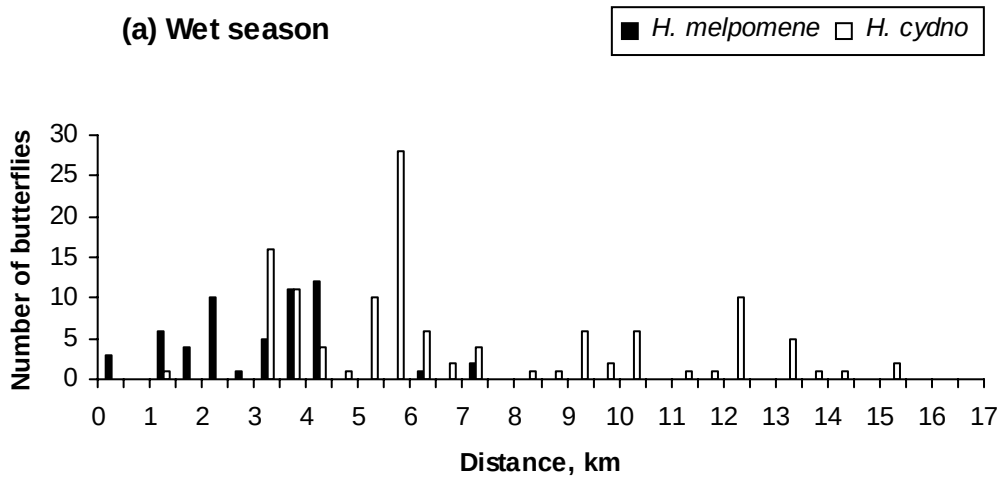
Figure 2.1 Distribution of adult butterflies along Pipeline Road in Soberanía National Park, for (a) *Heliconius melpomene* and *H. cydno* in the wet season (beginning May to late December), (b) *H. melpomene* and *H. cydno* in the dry season (late December to end April), and (c) their co-mimetic species *Heliconius erato* and *H. sapho*.

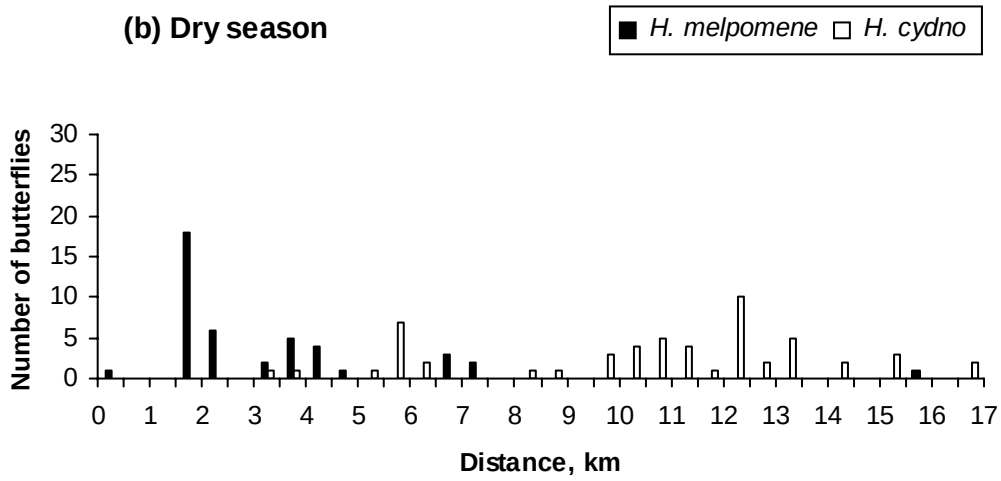
Figure 2.2 Distribution of host records along Pipeline Road, for (a) *Heliconius melpomene*, and (b) *H. cydno.*
Records from 0-0.5km include a second area of forest adjacent to Pipeline road, so the number of records should be halved to give a comparison of density along the transect.

Figure 2.3 Tree height along Pipeline Road.
Data is taken from samples of five trees along the road, showing (a) average tree height, and (b) the standard deviation of tree height in the sample.

Figure 2.4 Distribution of *Passflora* host plants along Pipeline Road, for (a) *Passiflora menispermifolia*, (b) *P. vitifolia*, (c) *P. auriculata*, (d) *P. coriacea*, (e) *P. biflora*, and (f) *P. ambigua*, *P. nitida* and *P. tryphostemmatoides*.
Records from 0-0.5km include a second area of forest adjacent to Pipeline road, so the number of plants should be halved to give a comparison of host density.

Figure 2.5 Distribution of host records along Pipeline Road, for (a) *Heliconius erato*, and (b) *H. hecale.*
Records from 0-0.5km include a second area of forest adjacent to Pipeline road, so the number of records should be halved to give a comparison of density along the transect.


(a) Wet season
H. melpomene
H. cydno
Number of butterflies
0
5
10
15
20
25
30
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Distance, km


(b) Dry season
H. melpomene
H. cydno
Number of butterflies
0
5
10
15
20
25
30
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Distance, km

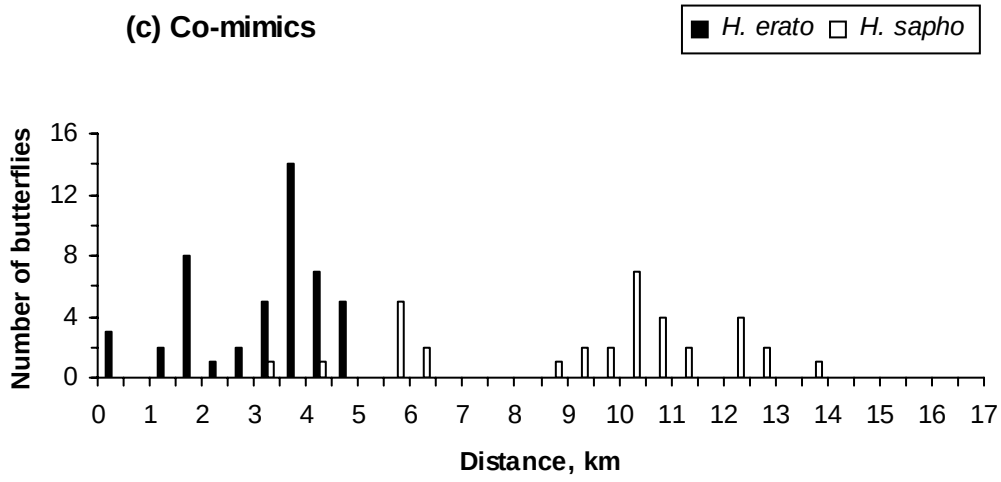

(c) Co-mimics
H. erato
H. sapho
Number of butterflies
0
4
8
12
16
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Distance, km

Figure 2.1

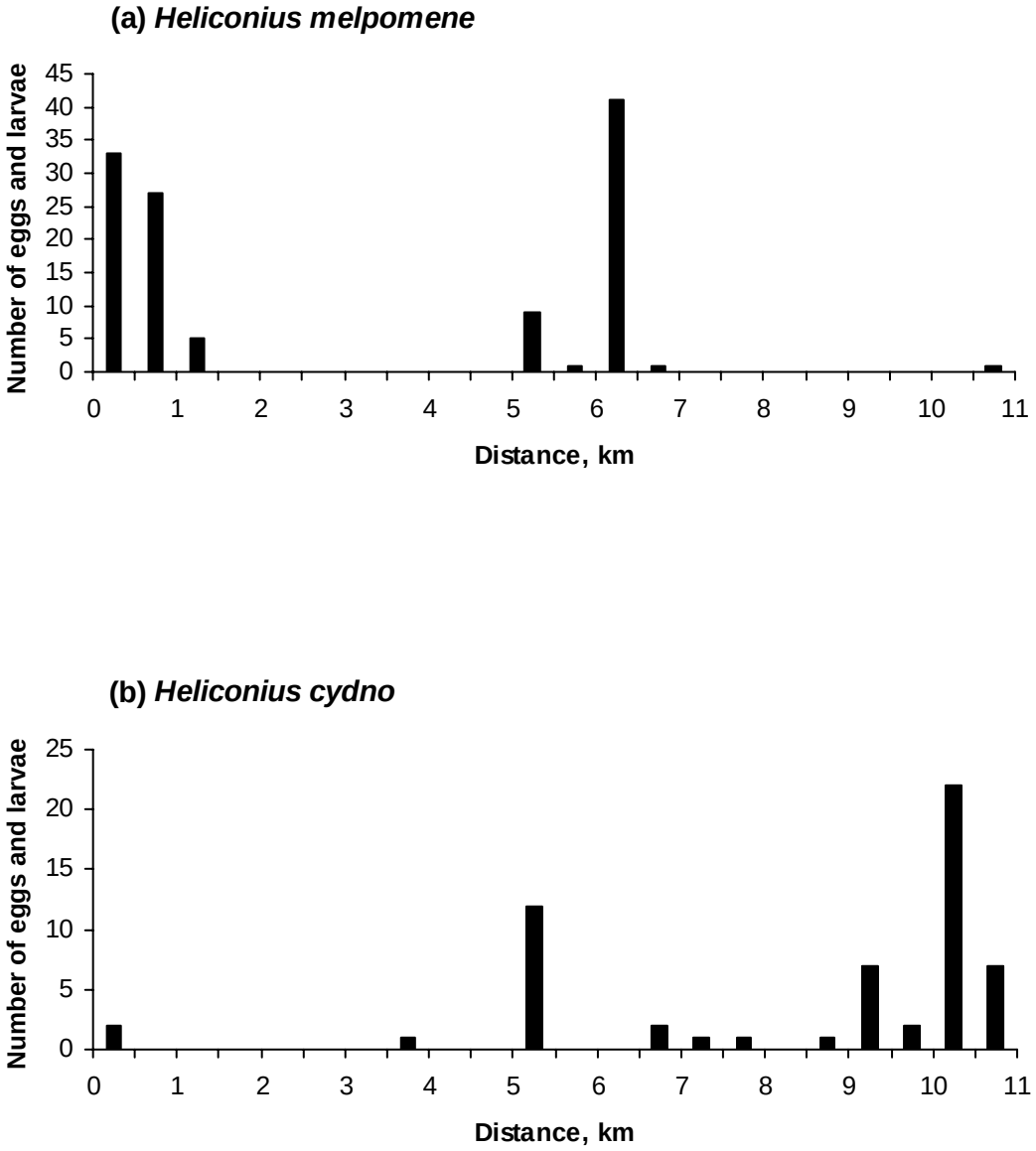
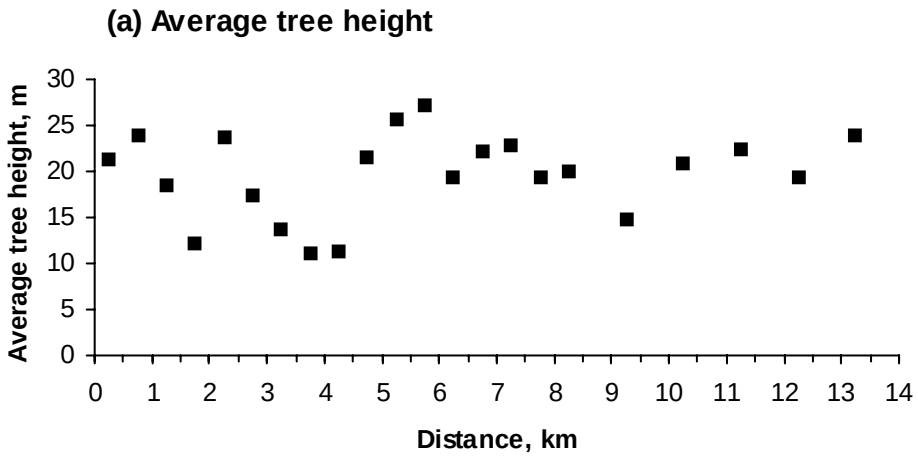

(a) Heliconius melpomene
Number of eggs and larvae
0
5
10
15
20
25
30
35
40
45
0
1
2
3
4
5
6
7
8
9
10
11
Distance, km
(b) Heliconius cydno
Number of eggs and larvae
0
5
10
15
20
25
0
1
2
3
4
5
6
7
8
9
10
11
Distance, km

Figure 2.2


(a) Average tree height
Average tree height, m
0
5
10
15
20
25
30
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Distance, km

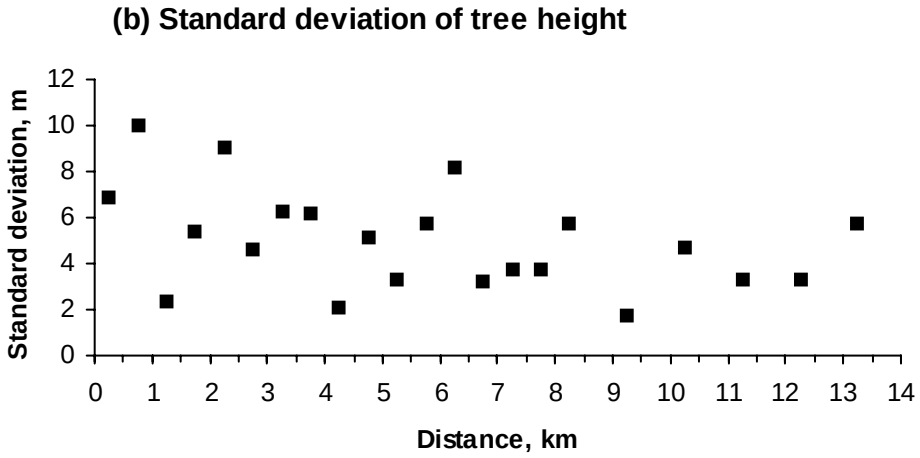
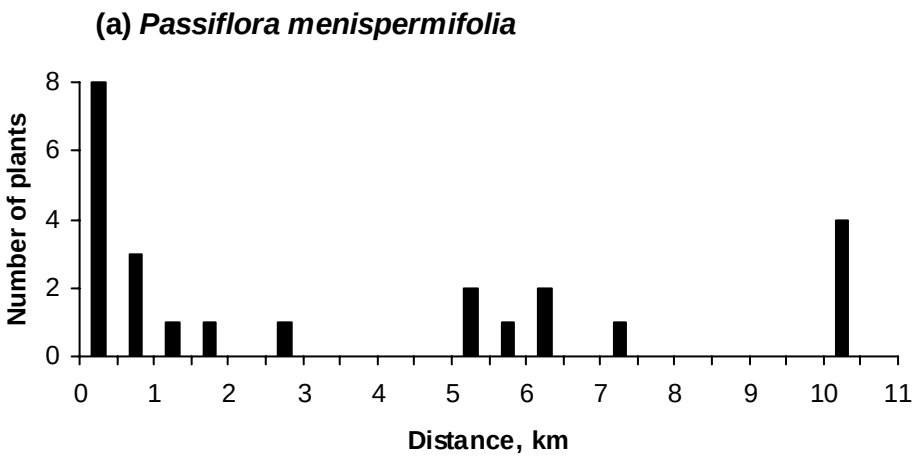
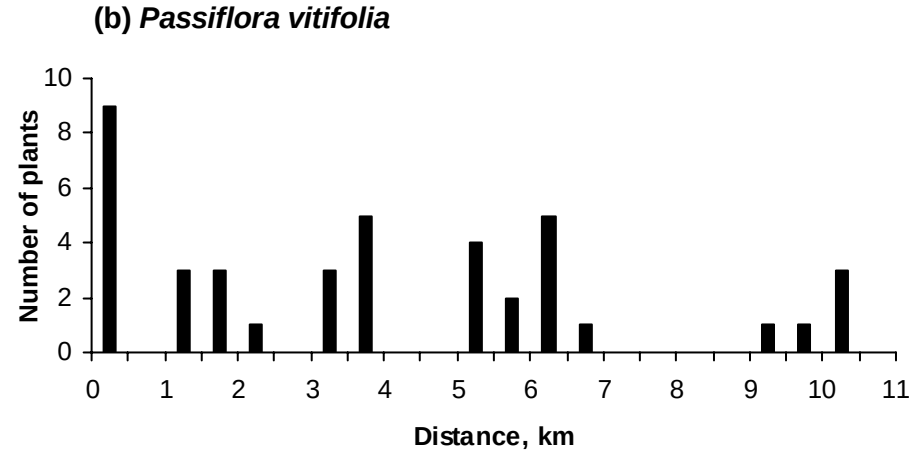

(b) Standard deviation of tree height
Standard deviation, m
0
2
4
6
8
10
12
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Distance, km

Figure 2.3


(a) Passiflora menispermifolia
Number of plants
0
2
4
6
8
0 1 2 3 4 5 6 7 8 9 10 11
Distance, km


(b) Passiflora vitifolia
Number of plants
0
2
4
6
8
10
0 1 2 3 4 5 6 7 8 9 10 11
Distance, km

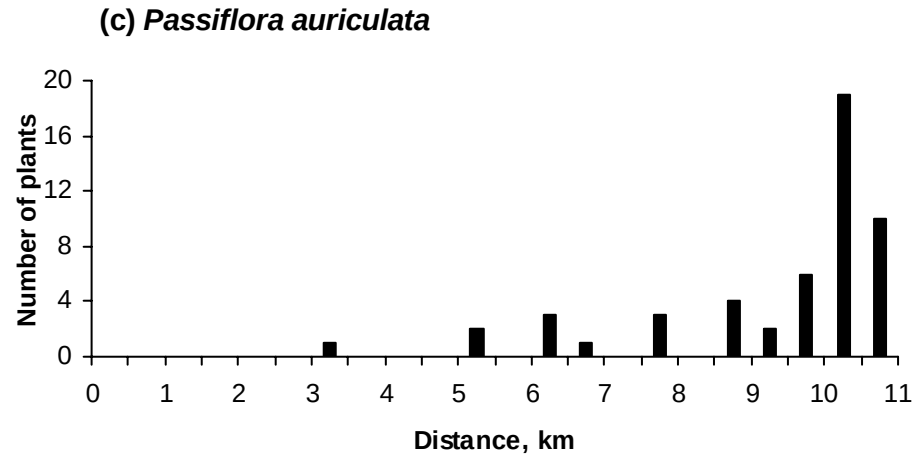
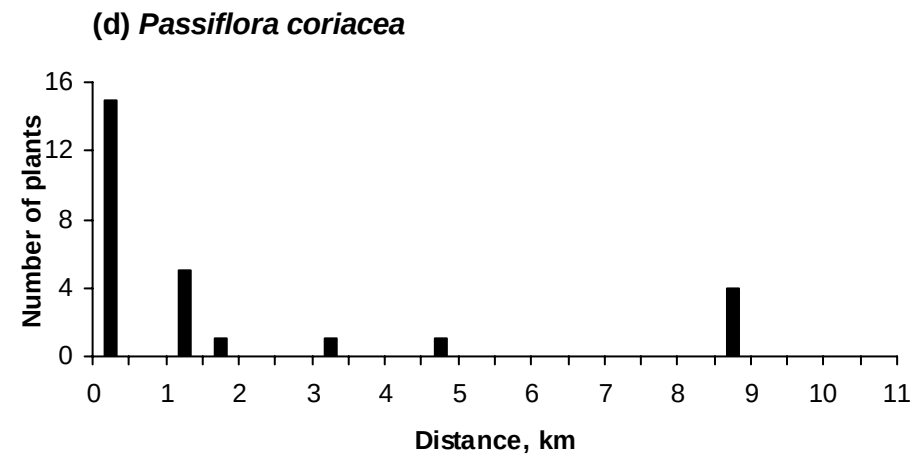
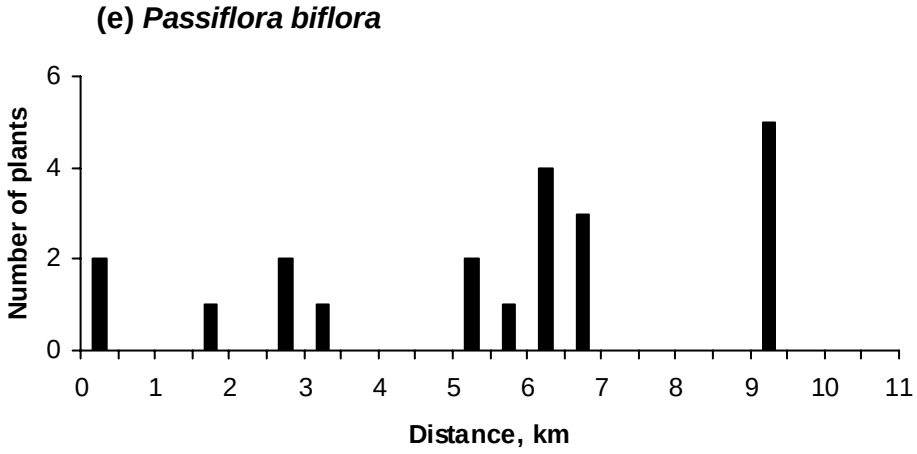

(c) Passiflora auriculata
Number of plants
0
4
8
12
16
20
0 1 2 3 4 5 6 7 8 9 10 11
Distance, km

Figure 2.4





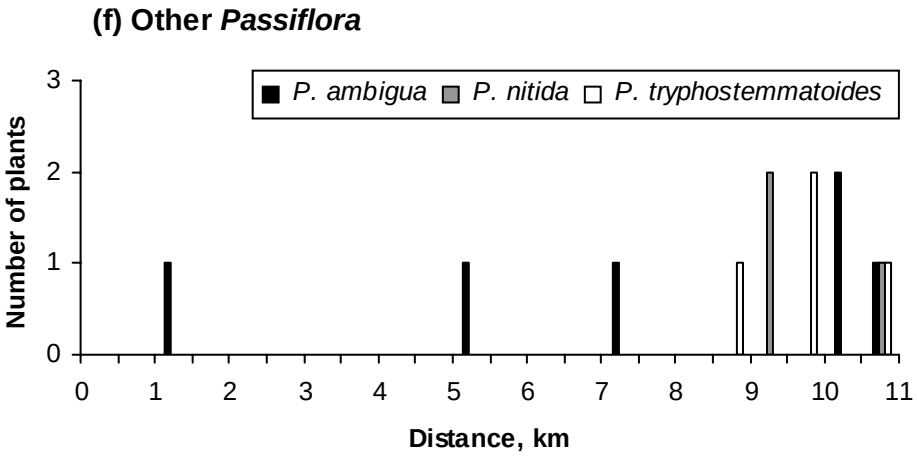


Figure 2.4 continued

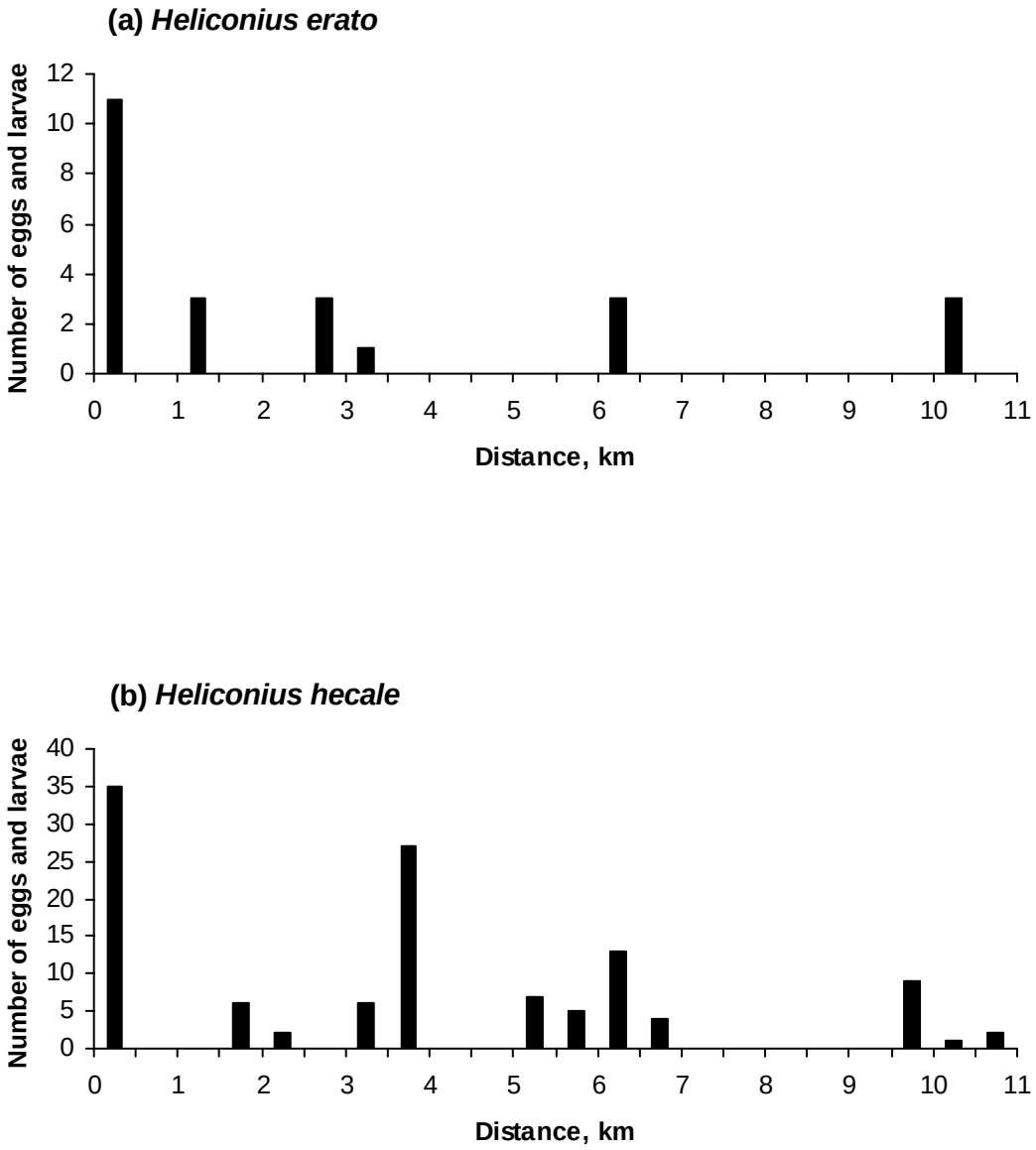

(a) Heliconius erato
Number of eggs and larvae
0
2
4
6
8
10
12
0
1
2
3
4
5
6
7
8
9
10
11
Distance, km
(b) Heliconius hecale
Number of eggs and larvae
0
5
10
15
20
25
30
35
40
0
1
2
3
4
5
6
7
8
9
10
11
Distance, km

Figure 2.5

3

The genetics of speciation: mimicry in *Heliconius cydno* and *Heliconius melpomene*

Abstract

Despite renewed interest in the role of natural selection in driving speciation, few studies have determined the genetic basis of ecologically important traits that also cause reproductive isolation. Here we describe the genetics of warning colour pattern in *Heliconius cydno* and *H. melpomene*, sister species that recently diverged to mimic different members of the genus. Divergence in mimicry has led to a degree of pre- and post-mating isolation, due to assortative mating resulting from coevolution of mate choice and colour pattern, and strong selection by avian predators against non-mimetic hybrids. Colour pattern differences have a simple genetic basis: ten loci can be identified, including genes of major effect. At least some of these loci are homologous with those controlling pattern differences between geographic races within each species. In addition, polygenic "modifiers" affect dominance and developmental canalisation within each species, causing patterns to be more variable in hybrids. Colour pattern elements interact epistatically, which influences ecological selection against hybrid phenotypes. Linkage is tighter between major loci than expected from independent distribution across chromosomes, suggesting that colour pattern evolution uses pre-existing linked elements, which may exist because of tandem duplication. Linkage, modifiers and epistasis strongly affect the strength of warning colour selection as a current barrier to gene flow between these naturally hybridising species, leaving open the possibility of introgression.

Introduction

Recent work suggests that speciation is often driven by divergent natural selection, which can lead to both pre- and post-mating isolation (Filchak *et al*. 2000; Rundle *et al*. 2000; Jiggins *et al*. 2001; Podos 2001). However, few studies have identified both the key traits initiating reproductive isolation and their genetic basis (for notable exceptions see Bradshaw *et al*. 1995; Schemske & Bradshaw 1999; Hawthorne & Via 2001). Despite over 70 years of study (Haldane 1929), several questions remain largely unanswered both for speciation (Coyne & Orr 1998) and for adaptation in general (Orr & Coyne 1992). Firstly, does divergence typically proceed by the substitution of many genes of minor effect (Fisher 1930), or can genetic changes of large effect contribute to adaptation? Recent theory considering the entire approach to the optimum phenotype suggests that adaptation will actually fix an exponential distribution of gene effects, with many factors of small effect and a few of large effect (Orr 1998; 1999). Secondly, is the mechanism of species divergence fundamentally distinct from that of normal adaptive evolution, or are the changes involved in speciation simply an extension of adaptive differentiation between populations? It has been suggested that analyses of sterility and inviability reveal a distinction between interspecific and intraspecific differences (Wu & Davis 1993; Wu 2001), but this comparison has yet to be made for traits involved in adaptive speciation. Thirdly, what are the roles of linkage, dominance and epistasis in adaptive speciation, and are they incidental properties of newly evolved genes or do they themselves evolve? Tight linkage between functionally related loci would suggest that evolution proceeds after tandem duplication, but may also have an adaptive explanation, since clustering of loci into linked blocks will facilitate the build-up of correlations between coadapted alleles by selection in the face of gene flow (Rieseberg 2001). Epistasis in particular is fundamental to speciation, since it is the

production of maladaptive gene combinations in hybrids that causes reproductive isolation (Whitlock *et al*. 1995).

Mimicry in butterflies is generally recognised as a visually appealing, intuitive example of adaptive evolution, involving readily understood strong selection (Bates 1862; Müller 1879; Poulton 1890; Mallet & Barton 1989; Kapan 2001). The evolution of mimicry is also a useful model system for studying speciation: diversification into distinct mimicry rings provides an analogue of adaptive radiation into distinct species. Divergence in mimicry can also drive speciation directly, because reproductive isolation can arise as an incidental by-product of differentiation (Bates 1862; Darwin 1863; Vane-Wright 1978; Turner 1981; Mallet *et al*. 1998). Recently we have demonstrated that a shift in mimicry in the butterflies *Heliconius cydno* and *H. melpomene* (Lepidoptera: Nymphalidae), has led to both pre- and post-mating isolation (Jiggins *et al*. 2001, appendix 1). The sister species coexist in Central and Andean South America despite hybridising at a low frequency (Mallet *et al*. 1998). Both species are unpalatable and warningly coloured, and have diverged to mimic distantly related *Heliconius* species, *H. melpomene* as a Müllerian mimic of *H. erato*, and *H. cydno* mostly of *Heliconius sapho* or *H. eleuchia* (figure 3.1) (Linares 1997b; Jiggins *et al*. 2001). Colour pattern is important in mate choice, so the shift in mimicry ring led to assortative mating (Jiggins *et al*. 2001). The mimicry shift will also result in selection against intermediate non-mimetic hybrids, since they will not be recognised as unpalatable by predators. The species differ in other respects, but traits such as female hybrid sterility (Naisbit *et al*. 2002) and reduced hybrid mating success (Naisbit *et al*. 2001) are likely to have evolved after their initial divergence, whilst others such as differences in microhabitat (Mallet & Gilbert 1995) and host plant use (Smiley 1978) provide only very weak

barriers to gene flow. The genes controlling colour pattern differences between the two species therefore represent 'speciation genes'.

Several authors have found that a number of major genes control colour pattern variation of geographic races within each species (Sheppard *et al*. 1985; Mallet 1989; Linares 1996; Linares 1997b). The fact that *H. cydno* and *H. melpomene* retain the ability to hybridise has also been used to transfer colour pattern genes between the species (Linares 1989; Gilbert 2001). Here we present the first systematic genetic analysis of their colour pattern differences, in order to answer three questions. Have major genes played a role in the colour pattern divergence of *H. cydno* and *H. melpomene*? Do the colour pattern genes involved in speciation differ from those controlling inter-racial variation within each species? What role do epistasis, linkage and dominance play in the evolution of colour pattern in *Heliconius*?

Methods

Crosses were performed in Gamboa, Republic of Panama between August 1998 and March 2000. *Heliconius cydno chioneus* and *H. melpomene rosina* were collected continuously from nearby forest in Soberanía National Park. To obtain the required crosses, we isolated reared virgin females together with wild caught males of the pure species, or with laboratory hybrids. After mating, females were kept individually in 1x1x2m outdoor insectaries and supplied daily with pollen sources (*Lantana* and *Psiguria*), artificial nectar (10% sugar solution), and potted *Passiflora* vines for oviposition. Eggs were collected daily and caterpillars fed on new growth of *P. biflora*. After eclosion, wings were removed and stored in glassine envelopes, and the bodies frozen in liquid nitrogen for genetic analysis.

We were only able to obtain offspring from crosses between male *H. melpomene* and female *H. cydno*; the reverse cross was rare due to strong asymmetrical mate choice. Sterility of F_1 females prevented F_2 crosses, but colour pattern was examined in F_1 individuals and offspring from backcrosses of fertile F_1 males to both parental species. Individuals were scored for the presence or absence of each of the colour pattern elements seen in the parental species. Single gene control was assumed when a 1:1 ratio of distinct phenotypes segregated in the backcross to the parental species bearing the recessive form of the trait, and unless indicated in the results, all G tests are for deviation from this 1:1 ratio. Where a continuous distribution of intermediate phenotypes was produced for any single pattern element, control was assumed to be polygenic. Homology was inferred where the patterns of gene action or linkage were identical with loci previously described from inter-racial crosses within *H. cydno* and *H. melpomene*. Summaries of individual genotypes are given in tables 3.1 to 3.3. Ratios of alleles segregating and recombination frequencies include data from 19 additional individuals (14 from backcrosses to *H. cydno* and 5 from backcrosses to *H. melpomene*) that could not be scored at all loci due to wing damage or failure to eclose fully.

Clustering of colour pattern loci into tight linkage groups is not expected in monomorphic Müllerian mimics, and we compared the extent of linkage among the ten colour pattern loci found here with a null model assuming random distribution of genes across chromosomes, to perform a test similar to that in Turner (1984: p.158). The null distribution was obtained from simulations run 1 million times placing ten loci across the 21 chromosomes, using as a test statistic the G statistic with expected 10/21 genes per chromosome.

Results

Crosses between *Heliconius cydno chioneus* and *H. melpomene rosina* reveal a number of genes with major effect on colour pattern.

B locus: controls the presence (*BB*, *Bb*) or absence (*bb*) of the red forewing band of *H. melpomene* (backcross to *H. cydno Bb*:*bb* 94:80 G_1=1.13, p>0.05). There is epistatic interaction with the unlinked *N* locus, so that in *B*- N^N- individuals the red is moved distally in comparison with the normal position in *H. melpomene* (figure 3.2). The pattern of gene action, epistasis with *N*, and linkage (see below) are very similar to those of the *B* locus in inter-racial crosses of *H. melpomene* (Turner 1972; Sheppard *et al*. 1985).

N locus: controls the presence (N^NN^N, N^NN^B) or absence (N^BN^B) of an area of white or yellow in the forewing band seen in *H. cydno* (backcross to *H. melpomene* N^BN^B:N^NN^B 54:65 G_1=1.02, p>0.05). The mode of gene action and its linkage (see below) are identical to that of the *N* locus segregating in inter-racial crosses of *H. melpomene* (Sheppard *et al*. 1985). The locus is apparently distinct from the *L* locus which controls the forewing band in crosses between several Colombian races of *H. cydno* (Linares 1996; Linares 1997b). *L* lacks the linkage seen here of *N* with *Yb* and *Sb*, and differs in gene action as there is almost complete dominance of the melanic allele.

Yb locus: controls the presence (*ybyb*) or absence (Yb_cYb_c, Yb_cyb) of the hindwing yellow bar of *H. melpomene* (figure 3.3). In heterozygous individuals the hindwing bar is usually visible as a shadow of melanic scales with altered reflectance, but occasionally very sparse yellow scales are present (backcross to *H. cydno* Yb_cYb_c:Yb_cyb 80:86 G_1=0.22, p>0.05, backcross to *H. melpomene* Yb_cyb:*ybyb* 61:57 G_1=0.14, p>0.05). On the basis of identical gene action and linkage, these crosses confirm the

homology of this locus in the two species, previously described from inter-racial crosses within *H. melpomene* (Sheppard *et al*. 1985), and *H. cydno* (Linares 1997b).

Sb locus: controls the presence (Sb_3) or absence (Sb_1) of the white submarginal band on the hindwing of *H. cydno* (figure3.4). The strength of expression in heterozygotes depends on at least one unlinked modifier (*J* – see below). The pattern of linkage and gene action is identical to that of *Sb* in inter-racial crosses of *H. cydno* (Linares 1996; Linares 1997b).

K locus: controls forewing band colour (figure 3.5), expressed as white (K^wK^w, K^wK^y) or yellow (K^yK^y) (backcross to *H. melpomene* in N^NN^B individuals, K^wK^y:K^yK^y 35:30 G_1=0.38, p>0.05). This is probably homologous with the *K* locus segregating in inter-racial crosses of *H. cydno* (Linares 1997b). There is no evidence from these crosses for any influence on the colour of the yellow hindwing bar, but the locus can cause the inclusion of yellow scales in the normally white hindwing submarginal band. Fore-and hindwing band colour is jointly controlled in polymorphic populations of *H. cydno* in Ecuador (Kapan 1998), but in some Colombian races a yellow forewing band is found with a white hindwing margin (Linares 1997b).

In addition, there are several genes with less dramatic effects on the mimetic colour pattern.

Vf locus: controls scale colour on the ventral surface of the red forewing band (figure 3.6), either dark (Vf_1Vf_1, Vf_1Vf_2) or white/yellow (Vf_2Vf_2) as in *H. melpomene* (backcross to *H. melpomene* Vf_1Vf_2:Vf_2Vf_2 62:56 G_1=0.31, p>0.05). This locus has not been described in either species, although its action has been noted in inter-specific crosses (Gilbert 2001).

Ac locus: controls the presence ($ac^c ac^c$) or absence (*AcAc*, $Acac^c$) of the anterior triangle of a white hourglass shape in the main forewing cell of *H. cydno* (figure 3.7) (backcross to *H. cydno* $Acac^c$:$ac^c ac^c$ 97:79 G_1=1.84, p>0.05). On the basis of gene action, this is thought to be homologous with the *Ac* locus that segregates in crosses between a race of *H. melpomene* from Trinidad with the red forewing band, and Amazonian races in which the hourglass is present (Sheppard *et al*. 1985).

Br locus: controls the presence (*BrBr*, *Brbr*) or absence (*brbr*) of a pincer-shaped brown marking on the ventral surface of the hindwing seen in *H. cydno* (figure 3.8) (backcross to *H. melpomene Brbr*:*brbr* 56:63 G_1=0.41, p>0.05). The extent of expression is variable in heterozygotes, lacking most of the distal part of either or both arms of the pincer. This is complicated by an epistatic interaction with the yellow bar which occupies a similar position on the hindwing. The colour is also variable, brown in *H. cydno*, but typically more orange in hybrids (compare figure 3.1 with 3.8). It is probable that this locus is homologous to the *D* locus described in *H. melpomene*, controlling the 'dennis' pattern of red on fore- and hind-wing (Sheppard *et al*. 1985). There may in fact be separable loci controlling the anterior and posterior components of the pincer (M. Linares, pers. comm.).

G locus: controls the presence (G_2) or absence (G_1) of a short red line at the base of the costal vein on the ventral surface of the forewing, seen in *H. melpomene* (figure 3.1). Expression is intermediate and variable in heterozygotes (backcross to *H. cydno* G_1G_1:G_1G_2 82:95 G_1=0.96, p>0.05, backcross to *H. melpomene* G_1G_2:G_2G_2 61:57 G_1=0.14, p>0.05). This locus was first described from inter-racial crosses of Colombian *H. cydno* (Linares 1996).

Several of these loci are involved in epistatic interactions of varying strength, in addition to the interaction between *N* and *B* controlling forewing bar shape and colour. Some traits are only expressed in certain genotypes, for instance *K* only in N^N- individuals, and *Vf* only in the *B*- genotype. There are also a number of modifier loci that adjust the strength and position of expression of other loci.

J locus: this could be described as a co-dominant modifier of dominance of the *Sb* locus controlling the hindwing submarginal band (figure 3.4). In Sb_1Sb_3 heterozygotes, individuals of J_1J_1 genotype express the submarginal band in the form of melanic scales with altered reflectance on the ventral surface only, J_1J_2 individuals show a mixture of white and melanic scales giving the impression of a blue-grey band expressed most strongly on the dorsal surface, whilst J_2J_2 individuals are indistinguishable from the Sb_3Sb_3 phenotype with a white submarginal band. In the backcross to *H. melpomene*, this gives an expected 2:1:1 ratio of absent (Sb_1Sb_1 J_1J_1 and Sb_1Sb_1 J_1J_2) to altered reflectance (Sb_1Sb_3 J_1J_1) to scattered white scales (Sb_1Sb_3 J_1J_2) (57:28:32, G_2=0.34, p>0.05), whilst in the backcross to *H. cydno* we expect a 3:1 ratio of full expression (Sb_3Sb_3 J_1J_2, Sb_3Sb_3 J_2J_2 and Sb_1Sb_3 J_2J_2) to scattered white scales (Sb_1Sb_3 J_1J_2) (137:40, G_1=0.56, p>0.05).

Forewing band width: in both backcrosses there is continuous variation in the position of the distal edge of the white or yellow part of the forewing band of N^N- individuals, suggesting additive polygenic control of expression of the *N* locus (figure 3.9). However, there are likely to be relatively few modifier loci controlling this variation, as individuals of the most extreme phenotypes are relatively common in both backcrosses. For instance, around 8% of individuals in the backcross to *H. melpomene* show a band

width similar to that in the F_1, and around 6% in the backcross to *H. cydno*. In the background of these modifiers, there is evidence of a slight effect of the *N* and *B* loci on the width of the white or yellow part of the forewing band. On average, the band is slightly wider in $N^N N^N$ than $N^N N^B$ individuals (Turner 1972) (using linkage with *Yb* to distinguish heterozygotes from homozygotes for *N*), and the band is increasingly wide in *BB*, *Bb*, and *bb* individuals (using evidence from linkage to *G* to distinguish *Bb* heterozygotes from *BB* homozygotes). The distal edge of the red part of the forewing band is also variable in position. Its boundary is often moved distally and is much less sharply defined in hybrids than in *H. melpomene* (figure 3.10). In the offspring of the backcross to *H. melpomene*, the distal boundary varies between the position in *H. melpomene* and that in F_1 individuals, independently of the effect of the *N* locus in moving the proximal edge (figure 3.10). In offspring of the backcross to *H. cydno* it generally falls at a point similar or slightly distal to that in F_1 individuals.

Forewing band colour: there is also apparently continuous variation in the colour of the forewing red. In the backcross to *H. melpomene* this ranges from the scarlet of *melpomene* through to an orange-red, whilst in the backcross to *H. cydno* the colour varies from orange-red to brownish. This continuous variation suggests that control is probably not homologous with the *Or* locus controlling red versus orange coloration in *H. melpomene* (Sheppard *et al*. 1985).

Red spots: *H. melpomene* has a variable number of red spots at the base of the hindwing on the ventral surface (figure 3.1). There is often a single spot, in the angle where the first anal vein meets the discal cell, but may be up to three more, in the angle of the second anal vein and the wing margin, within the discal cell, and in the angle where the subcosta meets the discal cell. Penetrance of this character is variable in hybrids. It is

absent from many F_1 offspring, but seen in almost all offspring from backcrosses to *H. melpomene*, and absent from almost all offspring of one backcross to *H. cydno,* but over-represented in another (brood 342 present:absent 46:26, G_1=5.63, p<0.05, compared with a 1:1 expectation).

Iridescence: the black areas of the wing are iridescent blue in *H. cydno*, and matt black in *H. melpomene* (figure 3.1). The trait is difficult to score consistently, and appears to be under polygenic control; strongly expressed in the backcross to *H. cydno*, intermediate in F_1 individuals and many of the backcross to *H. melpomene*, and absent in others from that backcross.

Linkage: the ten loci fall into two linkage groups of *Br-B-G* and *N-Sb-Vf-Yb*, with three further unlinked loci, *K*, *Ac*, and *J*. There is a recombination frequency of 23/118 (19.5% with support limits 12.9%, 27.4%) between *Br* and *G* in the backcross to *H. melpomene* (table 3.2). *B* and *G* are very tightly linked or may be pleiotropic effects of the same gene, as no recombinants segregate amongst 174 individuals in the backcrosses to *H. cydno*. The loci in the other linkage group can be placed in order by assuming that double recombinants are very rare. In the backcross to *H. melpomene*, heterozygotes can be distinguished from homozygotes at all four loci so that crossing-over between any of the genes could be detected. Gene order is most likely to be *N*, *Sb*, *Vf*, *Yb*, with 4.3% recombination between *N* and *Sb-Vf-Yb* (5/115, support limits 1.5%, 9.2%), and 0.9% between *Yb* and *Vf-Sb-N* (1/115, support limits 0.05%, 3.9%). For pictures of recombinant phenotypes see figures 3.11 and 3.4 bottom right. *Sb* and *Vf* are tightly linked or pleiotropic effects of the same gene, with no recombinants amongst 115 individuals. Since there are 21 chromosomes in *H. cydno* and *H. melpomene* (Brown *et al*. 1992), the clustering of genes into linkage groups exceeds that expected if

the colour pattern genes were distributed randomly across chromosomes (number of genes per chromosome, variance/mean = 2.44, Poisson expected variance/mean = 1, by simulation, $p<0.001$).

Presumed genotypes for the two species are $bbN^{N}N^{N}Yb_{c}Yb_{c}Sb_{3}Sb_{3}K^{w}K^{w}Vf_{1}Vf_{1}ac^{c}ac^{c}BrBrG_{1}G_{1}J_{2}J_{2}$ for *Heliconius cydno chioneus* and $BBN^{B}N^{B}ybybSb_{1}Sb_{1}K^{y}K^{y}Vf_{2}Vf_{2}AcAcbrbrG_{2}G_{2}J_{1}J_{1}$ for *H. melpomene rosina*. These loci are involved in producing almost perfect resemblance of the co-mimetic species, *H. erato* and *H. sapho* (figure 3.1). This extends to such minor details as the lightening of the ventral forewing produced by Vf_2, the red spots at the base of the hindwing, and the red line in the costal vein at the forewing base produced by G_2, all of which are seen in *H. erato* and replicated in *H. melpomene*. The only exceptions are the yellowing of pale forewing produced by K^{y}, which cannot be expressed on the normal red forewing band of *H. melpomene rosina*, and the brown pincer-shaped mark on the hindwing of *H. cydno* produced by *Br*. *H. sapho* differs from *H. cydno* in that it has large red patches near the base of the hindwing, and has the red line in the costal vein that is seen in *H. erato* and *H. melpomene*.

Discussion

The role of major genes in mimicry

Ten genes can be identified that act together with several additional polygenic traits to control the differences in the mimetic colour patterns of *H. cydno* and *H. melpomene*. Among the ten, many are genes of major effect on the colour pattern. There is evidence for epistatic interactions and linkage of several of the loci.

The gene effects are major in the sense that individual genes control a large proportion of the difference between the two species and affect large areas of the wing surface, and also in that they cause changes far beyond the normal variation seen within populations (True *et al*. 1997; Orr 2001). Most populations of *Heliconius* outside of zones of inter-racial hybridisation have relatively uniform wing patterns, although there are polymorphisms in several species, including populations of *H. cydno* from Colombia and Ecuador (Linares 1996; Joron *et al*. 2001; Kapan 2001; Mallet 2001). These genes cause changes in scale pigmentation and morphology in specific areas of the wing (Gilbert *et al*. 1988), and whilst not dramatic developmentally, they are under very strong selection arising from mate choice (Jiggins *et al*. 2001) and mimicry (Mallet & Barton 1989; Kapan 2001). Adaptation has not proceeded by the fixation of genes of minor effect only.

The general acceptance in the modern synthesis that adaptation would typically involve the fixation of very many mutations of small effect arose from Fisher's multidimensional geometric model of adaptation (Fisher 1930). He showed that the probability that a random mutation is advantageous approaches 0.5 as mutation size approaches zero, but that this probability rapidly declines with increasing mutation size. Large mutations are therefore unlikely to be favourable and so play a role in adaptation. However, this ignores the effect of selective advantage on the stochastic probability of fixation of a new mutation (Kimura 1983), and the fact that adaptation is a sequential process involving substitution of numerous alleles as the optimum is approached. When the entire process is considered, an exponential distribution of gene effects fixed during adaptation is expected (Orr 1998; 1999). This is largely independent of the distribution of mutation sizes, and is supported by the results of QTL analyses (Orr 1998).

However, mimicry may not fit the Fisher/Kimura/Orr model of adaptation. Mimicry is likely to evolve in two stages, as originally proposed by Nicholson, involving both major and minor mutations (Turner 1977). An initial major mutation yielding approximate resemblance is necessary for a population to overcome strong stabilising selection and cross the adaptive valley between two very distinct protected colour patterns. This is followed by improvement of resemblance through the fixation of modifiers and genes of more minor effect. In the evolution of mimicry we are therefore concerned with a rugged adaptive landscape, where warning colour phenotypes are separated by adaptive valleys of decreased protection (Sheppard *et al*. 1985). In contrast, both the models of Fisher and Orr consider a smooth adaptive surface, with gradual evolution towards a single optimum. Despite this difference, our empirical results with mimicry suggest that a distribution of few large and several small mutations are involved in adaptation, similar to that expected under Orr's model (Orr 1998). It may therefore be difficult to distinguish between the two types of adaptive landscape on the basis of the distribution of gene effects.

Divergence of *H. cydno* and *H. melpomene* seems to have occurred in the presence of target warning patterns as required for Nicholson's model. Mitochondrial DNA divergence at genes COI and COII between their two models, *H. erato* and *H. sapho* (average divergence 8.69%) is almost three times that between *H. cydno* and *H. melpomene* (3.07%) (Brower & Egan 1997; Mallet *et al*. 2001), suggesting that *H. cydno* and *H. melpomene* diverged to mimic the pre-existing warning patterns of their co-mimics. Colour pattern differentiation between *H. cydno* and *H. melpomene* probably followed a shift in habitat use, for their current distribution matches that of their comimetic species, *H. melpomene* in second growth and *H. cydno* in the forest understorey, but with considerable overlap (Mallet & Gilbert 1995). This habitat shift

would have brought the species into contact with a different suite of potential models, relaxing the purifying selection on their original pattern and replacing it with selection to converge on the local dominant warning pattern. There is good evidence that populations of *Heliconius* do respond to temporal (Linares 1997a) and spatial (Kapan 2001) variation in model frequency. This adaptive divergence in colour pattern incidentally led to a degree of reproductive isolation, through coevolution of mate choice and disruptive selection against non-mimetic hybrids (Jiggins *et al*. 2001).

Similar genetic architecture in intra- and inter-specific divergence

Colour pattern is a striking component of adaptive radiation within *Heliconius,* involving convergence between the major clades of the genus, racial differentiation, and speciation (Turner 1976; Jiggins & McMillan 1997; Mallet *et al*. 1998; Gilbert 2001). Both *H. cydno* and *H. melpomene* have diversified into an array of geographic colour pattern races across Central and South America, matching those of their co-mimics *H. sapho* and *H. erato* (Brown 1979). Most of the loci encountered here have been previously described from inter-racial crosses within *H. melpomene* (*N*, *B*, *Yb*, *Ac*) (Sheppard *et al*. 1985; Mallet 1989) or *H. cydno* (*Sb*, *Yb*, *K*, *G*) (Linares 1996; Linares 1997b). Linkage relationships are also similar to those previously described: *N* with *Yb* in *H. melpomene* (Sheppard *et al*. 1985), and *Sb* with *Yb* in *H. cydno* (Linares 1997b). The linkage of *B* and *Br* suggests there is homology of *Br*, which here controls the brown pincer-shaped mark on the hindwing of *H. cydno*, with the *D* locus segregating in crosses between *H. melpomene* races, which has similar linkage with *B* and controls the 'Dennis' pattern of red on the proximal part of the fore- and hind-wings in Amazonian races (Sheppard *et al*. 1985). Similar homology is seen between the two major loci responsible for most of the colour pattern differences between another pair of sister species, *Heliconius erato* and *H. himera,* and those controlling pattern variation within

H. erato (Jiggins & McMillan 1997). There is therefore no clear distinction between inter- and intra-specific differentiation in colour pattern. Both are driven by identical strong selection at many of the same loci.

Sister species in *Heliconius* typically belong to different mimicry rings (Mallet *et al*. 1998), but speciation is not an inevitable consequence of mimetic divergence. There is geographic variation in colour pattern within most species of *Heliconius*, in particular within *H. erato*, *H. melpomene* and *H. cydno*, and racial differences are often as dramatic as those between *H. cydno* and *H. melpomene* (Brown 1979). Contact zones between these geographic races are characterised by rampant hybridisation, so that their divergence has not resulted in speciation (Mallet 1993). This is despite the fact that colour pattern change carries the same potential for strong selection against non-mimetic forms within the hybrid zones (Mallet & Barton 1989). The initiation of speciation seems to depend on the mimetic shift involving a change in the dominant colour of the species, a colour that may normally act as a courtship releaser (Crane 1955). In most races of *H. melpomene* red is the dominant colour, in comparison with white or yellow in *H. cydno*, and such a colour pattern shift may be sufficient for male preference to coevolve and so reduce male courtship interest towards females of the other incipient species (Jiggins *et al*. 2001). Any initial reduction in gene flow due to pleiotropy with mate choice and selection against non-mimetic hybrids will facilitate further adaptive divergence and completion of speciation (Rice & Hostert 1993).

Linkage and the evolution of Müllerian mimicry

Seven of the ten loci described here fall into two linked groups. This linkage of colour pattern loci would be expected in a polymorphic Batesian mimic such as *Papilio memnon*, where a palatable species includes several morphs that mimic unpalatable

species (Clarke *et al*. 1968; Clarke & Sheppard 1971). In a Batesian mimic, each morph is under negative frequency dependent selection since the more common it becomes the lesser the selective advantage of mimicry. This can lead to a polymorphism of several mimetic forms, but only if new colour pattern genes emerge at linked loci. New alleles at unlinked loci will be removed by selection when recombination produces non-mimetic patterns. This forms what has been called an evolutionary 'sieve', whereby polymorphism can only become established in species that possess suitable "supergenes", tightly clustered groups of genes affecting mimicry (Turner 1984). This contrasts with the situation in Müllerian mimicry systems like those in which *Heliconius cydno* and *H. melpomene* participate, where both model and mimic are unpalatable. Purifying frequency dependent selection is expected to lead to monomorphic populations and so no selection for linkage (Turner 1984; Joron & Mallet 1998). Analysis of the mimicry genes known within the species *H. erato* and *H. melpomene* did not show significant clustering of colour pattern genes (Turner 1984). The significant clustering of colour pattern loci on some chromosomes in these *Heliconius cydno* and *H. melpomene* crosses is therefore unexpected, and has two possible explanations. On the one hand, linkage in *Heliconius* might suggest that divergence of colour pattern occurred in sympatry. Hybridising incipient species would in effect form a polymorphic population, into which new mutations must pass through a linkage 'sieve' like that in Batesian mimicry in order to become established. Although sympatric divergence seems unlikely, some form of parapatric divergence, perhaps on a microhabitat or altitudinal scale, is probable in *Heliconius cydno* and *H. melpomene.* The adaptive explanation of linkage is therefore possible, although not likely as strong as for Batesian sympatry. Linkage might instead result from limited sites at which mutations can produce changes in colour pattern (Mallet 1989). Clustering of loci would therefore represent a constraint on the mutable sites that can produce the relevant colour pattern, rather than a constraint

on the substitutable sites that can become established. This genetic architecture is likely to have arisen by duplication of regulatory genes, so that subsequent evolution of colour pattern will tend to proceed by mutation within linked blocks (Mallet 1989). Duplicated loci seem likely in the *Heliconius* system, where the linked genes affect similar pattern elements; the inclusion of red for the *B* linkage group, and white/yellow effects for the *N* group. Some of these linkage groups may show homology at a deep level within *Heliconius*, such as the similar linkage of yellow hindwing bar and white hindwing margin in the distantly related *H. erato* group (Turner & Sheppard 1975; Jiggins & McMillan 1997). In *Papilio*, supergenes include tightly linked loci controlling wing shape (the presence of tails on the hindwing), and body colour as well as wing colour (Clarke & Sheppard 1971). However, in butterflies a similar cascade of signalling molecules controls colour pattern and wing shape produced by cell death at the margin of the wing imaginal disc (Carroll *et al*. 1994), so that both might be under similar genetic control. A complete explanation of linkage in both systems must await a better understanding of the homologies of loci involved in wing development, but it is tempting to conclude that our finding of "supergene" inheritance in a Müllerian mimic is due to the underlying genomic organisation of colour pattern control.

The evolution of dominance

In our crosses there is evidence that dominance evolves rather than simply being an intrinsic property of an allele, for dominance is at least in part influenced by genetic background. In fact, a specific modifier of dominance can be identified. The two alleles of the *Sb* locus show variable dominance, controlled to a large extent by the *J* locus. When heterozygous for *Sb*, the *J* locus controls the strength of expression, from complete dominance of the melanistic allele, through to strong expression of white in the hindwing margin. Such variation casts doubt on the previous use of the 'dominance

sieve' to infer ancestral colour patterns in *H. melpomene* and *H. erato* (Sheppard *et al*. 1985). This relies on the fact that an advantageous allele that is dominant will be expressed when heterozygous and so be more likely to respond to selection during initial establishment (Haldane 1924), so that the recessive allele at each locus is most likely ancestral. However, if dominance can be altered to the extent seen here, a strong phylogenetic signal is unlikely to be preserved (Mallet 1989). It is also of little use in determining the direction of evolution in *H. melpomene* and *H. cydno*, as dominant colour pattern alleles are possessed by both species.

The role of epistasis in adaptive speciation

Epistasis plays a profound role in all models of speciation, as the source of hybrid dysfunction through genomic or ecological incompatibilities. In these crosses there is evidence for epistasis at several levels. There are strong epistatic effects of some loci over others, for example the failure to express the *K* genotype on a $N^B N^B$ background, the pronounced interaction between *N* and *B* in positioning elements of the forewing band, and that between *Sb* and *J* in the strength of expression of the hindwing submarginal band. General epistasis is apparent in the influence of genetic background, with colour pattern elements being less sharply defined in hybrids. This suggests that pattern development is normally canalised but this breaks down in the absence of coadapted genes (Mallet 1989). There is also epistasis for fitness between colour pattern loci, identical to that between the loci that cause hybrid inviability and sterility (contrast Orr 2001). Quantitative genetic analyses of morphological differences between species have typically found little evidence of epistasis in phenotypic measures (Orr 2001), but epistasis for fitness arising from niche divergence is likely to be almost universal even where the underlying genetic basis of the ecologically important trait is additive (Whitlock *et al*. 1995). Epistasis for fitness here arises from increased predation on non-

mimetic combinations of colour pattern elements, since hybrids fall between the protected patterns of the two species. For hybrid sterility and inviability, epistasis involves negative interactions between heterospecific genes on a hybrid genetic background. Both illustrate the coadaptation of the genome that binds loci together, so that an incidental by-product of divergence is hybrid dysfunction due to epistasis.

Genetic architecture and the possibility of introgression

The genetic properties of linkage, dominance, and epistasis affect how colour pattern acts as a barrier to gene flow between these two hybridising species. As with the loci involved in hybrid sterility (Naisbit *et al*. 2002), colour pattern loci are not distributed homogeneously across the genome. The main differences are controlled by genes in just three regions of the genome, close to the *B*, *N* and *K* loci, which may constrain or prohibit introgression between species around these loci. This leaves other regions relatively free to introgress. Also, due to linkage of *N*, *Sb*, *Vf* and *Yb*, and modifiers of dominance such as *J*, almost half of the offspring from backcross broods have colour patterns similar to those of *H. cydno* and *H. melpomene* at the forewing band, hindwing yellow bar and submarginal band. Natural hybrids between the two species may often remain undetected and so be more common than inferred from museum 'hybrids' (Mallet *et al*. 2001). These cryptic hybrids should also escape the strong selection due to predation on non-mimetic patterns. The genetic architecture of colour pattern therefore directly influences its efficiency as a barrier to gene flow, creating a semipermeable species boundary.

Mimicry and adaptive radiation

Mimicry is the most striking feature of an adaptive radiation in which *Heliconius* has diversified into a bewildering array of geographic races and species across the

Neotropics. Yet at first glance, mimicry is a conservative force, resisting divergence (Mallet & Joron 1999). The genetics of colour pattern in *H. cydno* and *H. melpomene* illustrate the coadaptation of the genome through epistatic interaction, both at the level of gene interaction in producing sharply defined pattern elements, and at the level of fitness in producing mimetic pattern combinations. Strong purifying selection by predators acting against novel patterns will resist diversification. Yet selection can be relaxed, for example by ecological shifts in microhabitat use which expose populations to a new suite of protected models. When such populations respond, evolution appears to involve changes at relatively few genes of major effect arranged in tightly linked blocks. The same selection by predators now acts to preserve diversity, by removing intermediate hybrids. Where shifts in colour pattern are sufficiently bold, mate choice may coevolve with colour pattern, so that adaptive divergence results in speciation.

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Genotype	Brood 345	Brood 341	?
[BG_2br][-G_2br][$ybSb_1Vf_2N^B$][$ybSb_1Vf_2N^B$](K^yK^y)(J_1J_1)*Ac*- [BG_2br][-G_2br][$ybSb_1Vf_2N^B$][$ybSb_1Vf_2N^B$](K^yK^y)(J_1J_2)*Ac*- [BG_2br][-G_2br][$ybSb_1Vf_2N^B$][$ybSb_1Vf_2N^B$](K^yK^w)(J_1J_1)*Ac*- [BG_2br][-G_2br][$ybSb_1Vf_2N^B$][$ybSb_1Vf_2N^B$](K^yK^w)(J_1J_2)*Ac*-	8/9	5/4	
[BG_2br][-G_2br][$ybSb_1Vf_2N^B$][$Yb_cSb_3Vf_1N^N$]$K^yK^yJ_1J_1$*Ac*-	3/2	0/1	
[BG_2br][-G_2br][$ybSb_1Vf_2N^B$][$Yb_cSb_3Vf_1N^N$]$K^yK^yJ_1J_2$*Ac*-	1/4	0/0	
[BG_2br][-G_2br][$ybSb_1Vf_2N^B$][$Yb_cSb_3Vf_1N^N$]$K^yK^wJ_1J_1$*Ac*-	2/1	0/2	
[BG_2br][-G_2br][$ybSb_1Vf_2N^B$][$Yb_cSb_3Vf_1N^N$]$K^yK^wJ_1J_2$*Ac*-	3/1	0/1	
[BG_2br][-G_1Br][$ybSb_1Vf_2N^B$][$ybSb_1Vf_2N^B$](K^yK^y)(J_1J_1)*Ac*- [BG_2br][-G_1Br][$ybSb_1Vf_2N^B$][$ybSb_1Vf_2N^B$](K^yK^y)(J_1J_2)*Ac*- [BG_2br][-G_1Br][$ybSb_1Vf_2N^B$][$ybSb_1Vf_2N^B$](K^yK^w)(J_1J_1)*Ac*- [BG_2br][-G_1Br][$ybSb_1Vf_2N^B$][$ybSb_1Vf_2N^B$](K^yK^w)(J_1J_2)*Ac*-	2/6	2/6	
[BG_2br][-G_1Br][$ybSb_1Vf_2N^B$][$Yb_cSb_3Vf_1N^N$]$K^yK^yJ_1J_1$*Ac*-	1/1	1/0	
[BG_2br][-G_1Br][$ybSb_1Vf_2N^B$][$Yb_cSb_3Vf_1N^N$]$K^yK^yJ_1J_2$*Ac*-	3/1	0/2	0/1
[BG_2br][-G_1Br][$ybSb_1Vf_2N^B$][$Yb_cSb_3Vf_1N^N$]$K^yK^wJ_1J_1$*Ac*-	3/3	0/1	
[BG_2br][-G_1Br][$ybSb_1Vf_2N^B$][$Yb_cSb_3Vf_1N^N$]$K^yK^wJ_1J_2$*Ac*-	2/0	3/3	

Table 3.1 Genotypes produced without crossing-over in the backcross to *H. melpomene* (female *H. melpomene* x male F_1)

Genes within square brackets are linked, with maternal *H. melpomene* alleles given first. Genes in curved brackets are not expressed on that genetic background (*K* on N^BN^B, and *J* on Sb_1Sb_1). A dash indicates an allele that cannot be determined due to dominance of the alternative allele. Counts are given as females/males.

Genotype	Brood 345	Brood 341	?
[BG_2br][-G_2br][$ybSb_1Vf_2N^B$][$\mathbf{Yb_c}Sb_1Vf_2N^B$](K^y-)(J_1-)Ac-	0/1		
[BG_2br][-G_1Br][$ybSb_1Vf_2N^B$][$ybSb_1Vf_2\mathbf{N^N}$]K^yK^w(J_1-)Ac-	0/1		
[BG_2br][-G_1Br][$ybSb_1Vf_2N^B$][$ybSb_1Vf_2\mathbf{N^N}$]K^yK^y(J_1-)Ac-	1/1		
[BG_2br][-$\mathbf{G_2Br}$][$ybSb_1Vf_2N^B$][$ybSb_1Vf_2\mathbf{N^N}$]K^yK^y(J_1-)Ac-	1/0		
[BG_2br][-$\mathbf{G_1br}$][$ybSb_1Vf_2N^B$][$Yb_cSb_3Vf_1\mathbf{N^B}$](K^y-)J_1J_1Ac-		0/1	
[BG_2br][-$\mathbf{G_1br}$][$ybSb_1Vf_2N^B$][$Yb_cSb_3Vf_1N^N$]$K^yK^wJ_1J_1Ac$-	1/0	0/1	
[BG_2br][-$\mathbf{G_1br}$][$ybSb_1Vf_2N^B$][$Yb_cSb_3Vf_1N^N$]$K^yK^wJ_1J_2Ac$-	1/0	1/0	1/0
[BG_2br][-$\mathbf{G_1br}$][$ybSb_1Vf_2N^B$][$Yb_cSb_3Vf_1N^N$]$K^yK^yJ_1J_2Ac$-		0/1	
[BG_2br][-$\mathbf{G_1br}$][$ybSb_1Vf_2N^B$][$Yb_cSb_3Vf_1N^N$]$K^yK^yJ_1J_1Ac$-	1/1		
[BG_2br][-$\mathbf{G_1br}$][$ybSb_1Vf_2N^B$][$ybSb_1Vf_2N^B$](K^y-)(J_1-)Ac-	2/0	2/1	
[BG_2br][-$\mathbf{G_2Br}$][$ybSb_1Vf_2N^B$][$Yb_cSb_3Vf_1N^N$]$K^yK^wJ_1J_2Ac$-	0/2		
[BG_2br][-$\mathbf{G_2Br}$][$ybSb_1Vf_2N^B$][$Yb_cSb_3Vf_1N^N$]$K^yK^yJ_1J_1Ac$-	1/1		
[BG_2br][-$\mathbf{G_2Br}$][$ybSb_1Vf_2N^B$][$Yb_cSb_3Vf_1N^N$]$K^yK^yJ_1J_2Ac$-	0/1		
[BG_2br][-$\mathbf{G_2Br}$][$ybSb_1Vf_2N^B$][$ybSb_1Vf_2N^B$](K^y-)(J_1-)Ac-	2/1		

Table 3.2 Genotypes produced by crossing-over in the backcross to *H. melpomene* (female *H. melpomene* mated to male F_1)

Conventions as in table 3.1. Loci affected by crossing-over are shown in bold.

Genotype	Brood 304	Brood 342	Brood 347	Brood 351
[bG_1Br][bG_1-][$Yb_cSb_3(Vf_1)N^N$][Yb_cSb_3--]K^w-J_2J_2acac [bG_1Br][bG_1-][$Yb_cSb_3(Vf_1)N^N$][Yb_cSb_3--]K^w-J_1J_2acac [bG_1Br][bG_1-][$Yb_cSb_3(Vf_1)N^N$][Yb_cSb_1--]K^w-J_2J_2acac	1/3	3/1	4/0	0/3
[bG_1Br][bG_1-][$Yb_cSb_3(Vf_1)N^N$][Yb_cSb_3--]K^w-J_2J_2Acac [bG_1Br][bG_1-][$Yb_cSb_3(Vf_1)N^N$][Yb_cSb_3--]K^w-J_1J_2Acac [bG_1Br][bG_1-][$Yb_cSb_3(Vf_1)N^N$][Yb_cSb_1--]K^w-J_2J_2Acac	1/2	1/5	1/6	3/0
[bG_1Br][bG_1-][$Yb_cSb_3(Vf_1)N^N$][$ybSb_3$--]K^w-J_2J_2acac [bG_1Br][bG_1-][$Yb_cSb_3(Vf_1)N^N$][$ybSb_3$--]K^w-J_1J_2acac [bG_1Br][bG_1-][$Yb_cSb_3(Vf_1)N^N$][$ybSb_1$--]K^w-J_2J_2acac	3/0	1/1	2/3	
[bG_1Br][bG_1-][$Yb_cSb_3(Vf_1)N^N$][$ybSb_3$--]K^w-J_2J_2Acac [bG_1Br][bG_1-][$Yb_cSb_3(Vf_1)N^N$][$ybSb_3$--]K^w-J_1J_2Acac [bG_1Br][bG_1-][$Yb_cSb_3(Vf_1)N^N$][$ybSb_1$--]K^w-J_2J_2Acac	0/0	4/3	1/1	
[bG_1Br][bG_1-][$Yb_cSb_3(Vf_1)N^N$][$ybSb_1$--]K^w-J_1J_2acac	3/1	1/3	1/1	
[bG_1Br][bG_1-][$Yb_cSb_3(Vf_1)N^N$][$ybSb_1$--]K^w-J_1J_2Acac	0/1	3/1	4/2	
[bG_1Br][BG_2-][$Yb_cSb_3Vf_1N^N$][Yb_cSb_3--]K^w-J_2J_2acac [bG_1Br][BG_2-][$Yb_cSb_3Vf_1N^N$][Yb_cSb_3--]K^w-J_1J_2acac [bG_1Br][BG_2-][$Yb_cSb_3Vf_1N^N$][Yb_cSb_1--]K^w-J_2J_2acac	1/3	4/4	4/2	0/1
[bG_1Br][BG_2-][$Yb_cSb_3Vf_1N^N$][Yb_cSb_3--]K^w-J_2J_2Acac [bG_1Br][BG_2-][$Yb_cSb_3Vf_1N^N$][Yb_cSb_3--]K^w-J_1J_2Acac [bG_1Br][BG_2-][$Yb_cSb_3Vf_1N^N$][Yb_cSb_1--]K^w-J_2J_2Acac	1/3	9/6	2/3	
[bG_1Br][BG_2-][$Yb_cSb_3Vf_1N^N$][$ybSb_3$--]K^w-J_2J_2acac [bG_1Br][BG_2-][$Yb_cSb_3Vf_1N^N$][$ybSb_3$--]K^w-J_1J_2acac [bG_1Br][BG_2-][$Yb_cSb_3Vf_1N^N$][$ybSb_1$--]K^w-J_2J_2acac	2/3	1/4	3/2	

Genotype	Brood 304	Brood 342	Brood 347	Brood 351
[bG_1Br][BG_2-][$Yb_cSb_3Vf_1N^N$][$ybSb_3$--]K^w-J_2J_2Acac [bG_1Br][BG_2-][$Yb_cSb_3Vf_1N^N$][$ybSb_3$--]K^w-J_1J_2Acac [bG_1Br][BG_2-][$Yb_cSb_3Vf_1N^N$][$ybSb_1$--]K^w-J_2J_2Acac	3/1	2/4	0/1	
[bG_1Br][BG_2-][$Yb_cSb_3Vf_1N^N$][$ybSb_1$--]K^w-J_1J_2acac	1/1	3/0	1/0	
[bG_1Br][BG_2-][$Yb_cSb_3Vf_1N^N$][$ybSb_1$--]K^w-J_1J_2Acac	2/1	2/2	2/2	

Table 3.3 Genotypes in the backcross to *H. cydno* (female *H. cydno* mated to male F_1)

Genes within square brackets are linked, with the maternal *H. cydno* alleles given first. Genes in curved brackets are not expressed on that genetic background (*Vf* on *bb*). Certain genotypes cannot be distinguished due to epistasis involving *Sb* and *J*, so that full expression of white hindwing margin could be produced by Sb_3Sb_3 or Sb_1Sb_3 J_2J_2. Counts are given as females/males.

Crossing over was not observed in this backcross, and would have been detectable between only two pairs of loci; *B* and *G*, and *Yb* and *Sb* (in the latter case only in J_1J_2 individuals, and only for the cross-over producing *YbYb* Sb_1Sb_3, giving a butterfly with no yellow hindwing bar shadow and medium expression of the hindwing margin, because the phenotype of the reciprocal cross-over *Ybyb* Sb_3Sb_3 is also produced by a J_2J_2 genotype in heterozygotes at *Yb* and *Sb*).

Figure 3.1 Müllerian mimicry of distantly related *Heliconius* species by *H. melpomene* and *H. cydno* in Panama.

H. melpomene with its co-mimic *H. erato*, and *H. cydno* with its co-mimic *H. sapho*. The F_1 hybrid between *H. cydno* and *H. melpomene* is intermediate and non-mimetic. In these and all following pairs of wings, the upper surface is shown on the right, and the lower surface on the left. Wings are reproduced at 60% of life size.

Figure 3.2 Interaction between the *N* and *B* loci in the forewing band. A dash indicates an allele that cannot be determined due to dominance of the alternative allele.

Figure 3.3 Control of the yellow hindwing bar of *H. melpomene* by the *Yb* locus. The effect of the *H. melpomene* allele is shown to the left, and that of the *H. cydno* allele to the right. The presence of the white submarginal band in the wings on the right is controlled by the *Sb* locus.

H. erato

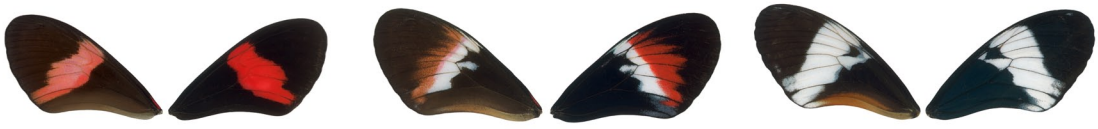
H. sapho

H. melpomene

F_1

H. cydno

Figure 3.1



B- N^BN^B

B- N^N-

bb N^N-

Figure 3.2

ybyb

Yb_c yb

Yb_c Yb_c

Figure 3.3

Figure 3.4 Control of the white hindwing submarginal band of *H. cydno* by the *Sb* locus, and modification of dominance by *J*. The *H. cydno* submarginal band phenotype is shown top left, and that of *H. melpomene* bottom right. The centre row shows modification of dominance in *Sb* heterozygotes by the *J* locus.

Figure 3.5 Control of forewing band colour by the *K* locus. In this and the following two figures, the effect of the *H. melpomene* allele is shown in the top row, above that of the *H. cydno* allele.

Figure 3.6 Control of the colour of the underside of the red forewing band by the *Vf* locus.

Figure 3.7 Control of the anterior half of the forewing hourglass by the *Ac* locus.

$Sb_3\ Sb_3\ J\text{-}$

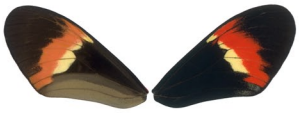
$Sb_1\ Sb_3\ J_2\ J_2$

$Sb_1\ Sb_3\ J_1\ J_2$

$Sb_1\ Sb_3\ J_1\ J_1$

$Sb_1\ Sb_1\ J\text{-}$

Figure 3.4



K^yK^y

$Vf_2\ Vf_2$

$Ac\ \text{-}$

$K^w\text{-}$

$Vf_1\text{-}$

$ac\ ac$

Figure 3.5

Figure 3.6

Figure 3.7

Figure 3.8 Control of the brown pincer-shaped marking of *H. cydno* by the *Br* locus. The effect of the *H. cydno* allele is shown to the left, and that of the *H. melpomene* allele to the right.

Figure 3.9 Variation in the width of the white portion of the forewing band. The wing in the centre shows the phenotype of an F_1 hybrid.

Figure 3.10 Variation in the width of the red portion of the forewing band.

Figure 3.11 Two hybrid phenotypes produced by recombination within the *N-Sb-Vf-Yb* linkage group. Both are the result of a cross-over between *N* and *Sb-Vf-Yb* in a backcross of F_1 male to *H. melpomene* female. Left, genotype $N^{N}N^{B}Sb_{1}Sb_{1}Vf_{2}Vf_{2}ybyb$, right, genotype $N^{B}N^{B}Sb_{1}Sb_{3}Vf_{1}Vf_{2}Yb_{c}yb$. Shown at 80% life-size. The only other crossover seen within this linkage group was between *Yb* and *Vf-Sb-N*, producing the genotype $N^{B}N^{B}Sb_{1}Sb_{1}Vf_{2}Vf_{2}Ybyb$. The hindwing is shown in figure 3.4, lower right, and the forewing was like that of *H. melpomene*.

Br Br *Br br* *br br*

Figure 3.8

Figure 3.9

Figure 3.10

Figure 3.11

4

Disruptive sexual selection against hybrids contributes to speciation between *Heliconius cydno* and *Heliconius melpomene*

Abstract

Understanding the fate of hybrids in wild populations is fundamental to understanding speciation. Here we provide evidence for disruptive sexual selection against hybrids between *Heliconius cydno* and *H. melpomene*. The two species are sympatric across most of Central and Andean South America, and coexist despite a low level of hybridisation. No-choice mating experiments show strong assortative mating between the species. Hybrids mate readily with one another, but both sexes show a reduction in mating success with the parental species, by over 50% on average. Mating preference is associated with a shift in adult colour pattern, which is involved in predator defence through Müllerian mimicry but also strongly affects male courtship probability. The hybrids, which lie outside the curve of protection afforded by mimetic resemblance to the parental species, are also largely outside the curves of parental mating preference. Disruptive sexual selection against F_1 hybrids therefore forms an additional post-mating barrier to gene flow, blurring the distinction between pre-mating and post-mating isolation, and helping to maintain the distinctness of these hybridising species.

Introduction

Studies of recently diverged species are increasingly producing examples of sympatric species that hybridise in the wild yet remain distinct (Grant & Grant 1992; Mallet *et al*. 1998). Understanding the fate of hybrids is therefore critical to our understanding of the nature of the species boundary, and of the forces that drive speciation.

Given incomplete assortative mating, the fitness of hybrids will determine the extent to which gene flow occurs between species. Hybrid fitness can be reduced by (1) sterility or inviability ("genomic" incompatibility), (2) disruptive ecological selection, or (3) disruptive sexual selection. Most attention has been given to the first effect, hybrid sterility and inviability, with the majority of studies using *Drosophila*. That work has yielded a number of consistent generalisations about speciation, for instance Haldane's rule, which states that where one sex of F_1 hybrid is absent, rare, or sterile, it is the heterogametic sex (Haldane 1922; Coyne 1992; Sperling 1993). However, these studies of genomic incompatibility arguably tell us little about the early stages of speciation: a growing body of work demonstrates that speciation can proceed in the absence of this form of post-mating isolation (Feder *et al*. 1994; Bradshaw *et al*. 1995; Grant & Grant 1997; McMillan *et al*. 1997; Seehausen *et al*. 1997; Hatfield & Schluter 1999). More recently, there has been an upsurge of theoretical and empirical interest in the second source of selection against hybrids: ecological forms of disruptive selection (McMillan *et al*. 1997; Schluter 1998; Dieckmann & Doebeli 1999; Kondrashov & Kondrashov 1999).

Rather less experimental work has investigated mate choice during speciation, and the possibility of the third type of selection against hybrids: disruptive sexual selection. Recent theory suggests that disruptive sexual selection could be extremely important in

speciation (Payne & Krakauer 1997; Higashi et al. 1999). Several recent examples have shown reduced mating probability of F_1 hybrids between a pair of butterfly species (Davies *et al*. 1997), preference for conspecific over F_1 hybrid males in sticklebacks (Vamosi & Schluter 1999), strong mating discrimination against hybrids in lacewings (Wells & Henry 1998), and almost complete behavioural sterility of both sexes of F_1 hybrid in wolf spiders (Stratton & Uetz 1986). This form of selection against hybrids provides an additional element of speciation for which Haldane's rule might hold, as in the effects on vigour or choosiness of female hybrids seen in *Anartia* butterflies (Davies *et al*. 1997). Sexual selection will limit the extent to which introgression is possible following hybridisation, and if mating asymmetries exist they will influence the direction in which gene flow might proceed.

Three processes are likely to contribute to divergence in mating preference: (1) pleiotropic or otherwise genetically correlated effects of ecological selection, (2) disruptive sexual selection and (3) reinforcement. First, assortative mating can arise as a by-product of disruptive natural selection (Schluter 1998). Assortment will result if a trait under ecological selection also forms the basis of mate choice, or through the recruitment of other traits involved in mate choice by the build-up of linkage disequilibrium with the ecological character (Dieckmann & Doebeli 1999; Kondrashov & Kondrashov 1999). This will generate populations with divergent ecology which are also reproductively isolated, and therefore that are able to coexist. Second, recent theory suggests that the genetic correlations between preferences and traits generated purely by disruptive sexual selection can also drive sympatric speciation (Turner & Burrows 1995; Payne & Krakauer 1997, 2000; Higashi *et al*. 1999). However, the daughter populations from such a process may be able to coexist genetically, but without adaptive differentiation they will not be able to coexist ecologically (but see Lande & Kirkpatrick

1988; van Doorn *et al*. 1998). Reinforcement provides a third process that can strengthen pre-mating isolation, however produced, between differentiated populations through selection for assortative mating, if hybridisation produces offspring with low fitness (Liou & Price 1994). Interestingly, once mating divergence occurs, hybrids are potentially left in an adaptive valley between the ranges of mating preference of the two parental species, which itself can promote additional divergence by further reinforcement.

Heliconius cydno and *Heliconius melpomene* (Lepidoptera: Nymphalidae) are sister species which are sympatric across much of Central and Andean South America below 1500m (Brown 1979; Brower 1996). Both are unpalatable and warningly coloured, and speciation has accompanied a shift in Müllerian mimicry. *H. melpomene* is black with red and yellow markings and mimics *Heliconius erato*, while *H. cydno* is black with yellow or white markings and usually mimics members of the *Heliconius sapho*/*eleuchia* clade (Linares 1997; Jiggins *et al*. 2001). The two species have diverged in habitat use, with *H. melpomene* in second growth and *H. cydno* in forest understorey, matching the distribution of their co-mimetic species (Smiley 1978*a*; Waage *et al*. 1981; Mallet & Gilbert 1995). However, there is considerable overlap and the two species can be found flying together. They also differ in their degree of host plant specialisation within the *Passiflora* (Smiley 1978*a,b*) and female F_1 hybrids are sterile (Linares 1989). Despite these differences the two species do hybridise in the wild, and F_1 and backcross hybrids are known from across their range, although they probably form less than 0.1% of natural populations (Mallet *et al*. 1998).

In this chapter, I investigate mating interactions between these two species and estimate the extent of sexual selection against hybrids.

Methods

The work was carried out with *H. melpomene rosina*, *H. cydno chioneus* and their F_1 hybrids. Crosses and mating trials were performed in outdoor insectaries in Gamboa, Republic of Panamá, between September 1999 and March 2000. This area lies close to Pipeline Road in Soberanía National Park where stocks of both species were collected. The two species will cross in the insectary although rarely, and one direction of cross, *H. melpomene* female x *H. cydno* male, could not be obtained to supply hybrids for these experiments: males of *H. cydno* are consistently more reluctant to mate with heterospecifics than are *H. melpomene* males. Although the *H. melpomene* female x *H. cydno* male cross has been produced on two earlier occasions, in both cases the female died before laying eggs.

Mating behaviour was analysed using no-choice trials. Each female was left to eclose in a 1x1x2m insectary in the presence of a single male, and the pair observed at half hourly intervals during the daylight hours for a minimum of two days. Males were allowed to mature beforehand for at least five days with access to pollen sources (*Psiguria* and *Lantana*) and artificial nectar (10% sugar solution) (McMillan *et al*. 1997). All individuals were reared without access to the opposite sex and were used once only. Pairs stay coupled for at least an hour so all matings were observed. In addition females were dissected to check for the presence of a spermatophore. At each observation period the pair were disturbed if perching, and once they had come into contact, male behaviour such as chasing the female, fluttering courtship, or attempted mating was recorded. This no-choice experimental design was adopted in order to mimic the natural situation, where males patrol larval host plants and mate teneral females soon after eclosion (Mallet 1986). Male choice is almost certainly the primary determinant of mating probability at this stage. Females use their wings to fend off unwanted males,

but the wings of teneral females are too soft to be used in this way. A female can use sperm from a single spermatophore to fertilise several eggs each day over her six month lifespan, although a fraction of females do remate in the wild (Boggs 1979).

Mating probabilities were estimated using likelihood in order to test between hypotheses differing in complexity, as well as to obtain measures of reliability (McMillan *et al*. 1997). For each combination of *i*-type female and *j*-type male, a binomial mating probability P_{ixj} was obtained, maximising the expression for $\log_e$ likelihood given by

$$m\log_e P_{ixj} + n\log_e(1-P_{ixj})$$

where *m* and *n* are the number of trials in which the pair mated or remained unmated respectively. The $\log_e$ likelihoods for P_{ixj} values were maximised using the 'Solver' algorithm supplied with Microsoft Excel. Support limits for P_{ixj} were obtained at the parameter values that led to a decrease in $\log_e$ likelihood of two units, asymptotically equivalent to 95% confidence intervals (Edwards 1972). In the case of parameters *a*, *b*, *c*, and *e*, which are multiplicatively combined in the final model (see below), support limits were obtained while maximising likelihoods for the other parameters. Fitting models with different numbers of parameters allowed a test for differences in mating probability across trials, using a likelihood ratio test with $G = 2\Delta\log_e L$, which asymptotically follows a χ^2 distribution (Edwards 1972).

The likelihood model was fitted in a stepwise manner, adding parameters to an initial null model with a single mating probability (*a*=*b*=*c*=*d* in table 4.2) across all trials. Estimating mating probability separately for interspecific trials (*c*, versus *a*=*b*=*d* for the rest) gave a significant improvement (G=30.12, df=1, $p < 0.01$). Further improvement was achieved in a three-parameter model estimating separate probabilities for trials

among individuals of like genotype (*a* in table 4.2), hybrid x parental (*b*=*d*), and interspecific mating (*c*) (G=10.14, df=1, p<0.01). Throughout the period of the experiment, males of *H. cydno* displayed consistently poor performance, with reduced courtship effort and mating success in all trials including those with a conspecific female. This was not the case in a different set of experiments performed the year before (Jiggins *et al*. 2001). It was apparent in offspring of several wild caught females and seemed to reflect poor adaptation of *H. cydno* to the cage environment. Here we account for the poor performance of *H. cydno* males by estimating an extra parameter (*e*) (G=14.25, df=1, p<0.01). Separate mating probabilities (*b*, *d*) were estimated for the reciprocal trials of hybrid with parental individual, since although this did not significantly improve the model (G=1.29, df=1, p>0.05), it allowed the mating success of the three genotypes to be compared for each sex. The adoption of a full nine-parameter model with separate probabilities estimated for each genotype by genotype combination gave no significant improvement over this five-parameter model (G=2.39, df=4, p>0.05).

Results

There was very strong assortative mating between the species, with no interspecific mating occurring in 30 trials (table 4.1). Hybrid males had a high mating success with hybrid females, but both sexes of hybrids had considerably reduced mating probabilities with the parental species (table 4.1). Male courtship in the first two hours after female eclosion was a good predictor of male mating success in the trials (figure 4.1): male mating probability is highly correlated with male courtship probability (r^2=0.65, p<0.01).

Interpretation of the likelihood model is complicated by the poor performance of the *H. cydno* males in both courtship effort and mating success (figure 4.1; parameter *e*, table 4.2). Since courtship appears to determine male mating performance, it is reasonable to treat the performance parameter (*e*) as an adjustment for the effect of poor performance by male *H. cydno*. We believe this to be an artefact of this experiment, since a different study with the same pair of species showed similar conspecific mating probabilities within *H. cydno* and within *H. melpomene* (Jiggins *et al*. 2001). This variation between experiments places doubt on the precision of some parameter estimates, but the relationships between parameters holds up for the males of each pure species (for example $a>b>c$), regardless of whether the *H. cydno* performance correction is used. We can then compare the four mating parameters (*a-d* in table 4.2). There is very strong assortative mating of the parental species (test of $a=c$, $G=44.33$, $df=1$, $p<0.01$), and there is good evidence of discrimination against female hybrids (test of $a=b$, $G=6.41$, $df=1$, $p<0.05$). Males of the parental species are therefore most likely to mate with conspecific females, less so with hybrids, and are extremely unlikely to pair with heterospecifics. For hybrid males, pairing with hybrid females is more likely than with either parental species (test of $a=d$, $G=15.79$, $df=1$, $p<0.01$): the probability of mating between hybrid males and parental females is estimated to be less than half that of parental by parental or hybrid by hybrid (table 4.2). As noted previously, the mating success of male and female hybrids with parental partners does not differ significantly (test of $b=d$, $G=1.29$, $df=1$, $p>0.05$).

Discussion

There is extremely strong assortative mating between *Heliconius cydno* and *H. melpomene*, and reduced mating success of F_1 hybrids of both sexes with the parental species. Probability of mating is strongly correlated with probability of courtship,

suggesting that while female receptivity may play a role, male choice is the primary determinant of mating probability. Although hybrids were not tested in earlier work, a similar correlation was obtained in a study of male courtship and mate choice in *H. cydno* from Panama and *H. melpomene* from Panama and French Guiana: in both cases, mating success seems mainly a result of male courtship interest due largely to the males' colour pattern preference (Jiggins *et al*. 2001). Male choosiness is not unexpected since the spermatophore of *Heliconius* represents a considerable nutrient investment, providing the female with amino acids used in egg production (Boggs 1979). In the experiments described here, mating probabilities for male and female parentals are intermediate when tested with hybrid individuals (conspecific>hybrid>heterospecific) and hybrid success is greatest with hybrids (hybrid>parental). Although the precise nature of mating cues are uncertain, from our data hybrid male preferences and hybrid female signals seem to be intermediate between those of the two parental species, suggesting an approximately additive genetic basis of both mating cue and response. Similar cases of intermediate signals and preferences of hybrids exist in other species, for example in tree frogs (Doherty & Gerhardt 1983) and lacewings (Wells & Henry 1998), but there are also instances where the mating success of hybrids is similar to that of the parentals (Davies *et al*. 1997; McMillan *et al*. 1997). In only one published case we could find, a spider, was hybrid mating success non-additive, being very low with all genotypes (Stratton & Uetz 1986).

The concept of the "shape" of mating preference provides a unifying theme in the study of sexual selection and species recognition (Ryan & Rand 1993; Ritchie 1996). The shape of mating preference is the relationship between values of a trait and their probability of acceptance by a mating partner (Ritchie 1996). It determines how sexual selection acts within a population, as well as the probability of hybridisation between

populations. The distribution of trait values within a population and the shape of preference are expected to coevolve closely, although recognition may be elicited by trait values beyond the normal range of the population, as found for syllable number in the song of the cricket, *Ephippiger ephippiger* (Ritchie 1996). In the initial stages of divergence the preference distributions of two incipient species will overlap. The trait values found in hybrids will depend on the genetic architecture of those traits, but if a number of cues combine additively to form the basis for recognition, hybrids will lie at some intermediate point. Hybrids may therefore be recognised as potential mates by both species, and could even have superior mating success to parentals in mixed populations (dashed line in figure 4.2*a*). Another pair of sister species, *Heliconius himera* and *H. erato,* illustrate a possible intermediate stage of divergence as in figure 4.2*a*, with strong assortative mating but little discrimination against hybrids (McMillan *et al*. 1997). At this point, mate choice on its own merely provides stabilising selection, opposing further divergence. However, if hybrids are selected against, reinforcement can lead to a narrowing of the preference function of the parental species, which might reduce the extent to which hybrids are accepted as mates (figure 4.2*b*). Alternatively, discrimination may result from further trait divergence of the parental species and coevolution of their mating preference (figure 4.2*c*). In either case, disruptive sexual selection against hybrids is generated. In *H. melpomene* and *H. cydno* this is exactly what we see, with an additive pattern of hybrid mating, very strong assortative mating, and a reduction in mating success of hybrids with the parental species of over 50%. This sexual selection may only delay mating for hybrid females, but should result in a strong reduction in lifetime reproductive success for male hybrids. The strength of this sexual selection depends on an Allee effect (Allee *et al*. 1949) for hybrids: male mating opportunities are only scarce because female hybrids are rare. Hybrids between *H. melpomene* and *H. cydno* are always at extremely low density (less than 1 in 1000

individuals (Mallet *et al*. 1998)), so they are unlikely to encounter other hybrids as compatible mates. This creates what is in effect a novel form of post-mating isolation due to poor hybrid mating success.

In this pair of species, ecological adaptation initiated a chain of divergence that led to speciation. Changes in habitat use exposed populations to different suites of potential Müllerian co-mimics (Waage *et al*. 1981; Mallet & Gilbert 1995), selecting for a shift in mimetic allegiance. This switch in mimicry then led to pleiotropic changes in mate choice, as assortative mating coevolved with colour pattern (Jiggins *et al*. 2001). Reduced production of hybrids created further disruptive predator selection leading to a low fitness of non-mimetic hybrids, probably in the order of 50% as seen in *H. erato* hybrid zones (Mallet & Barton 1989). Selection within each nascent species after separation almost certainly led to the acquisition of female hybrid sterility (Linares 1989), presumably via Muller's classical pleiotropic route for the evolution of genomic incompatibility between populations (Muller 1940). These ecological and genomic post-mating barriers created the conditions necessary for reinforcement, which appears to have strengthened the assortative mating in sympatric populations: allopatric *H. melpomene* court and mate with *H. cydno* more readily than the same species pair in sympatry (Jiggins *et al*. 2001). This greater choosiness in sympatry very likely resulted in still stronger disruptive sexual selection against hybrids. Thus, it is possible to see how simple ecological divergence can trigger a cascade of further changes that lead to full speciation. Each step in divergence we have documented leads to conditions that promote further divergence, by reducing gene flow and creating additional disruptive selection pressures, in a series of examples of positive feedback in the speciation process. Of course not every species is mimetic, or has such clear selective forces at work, but mimicry is a particularly good example where an ecological change that has

pleiotropic effects both on pre-mating isolation and on post-mating isolation may ultimately cause speciation. Many other examples of similar pleiotropy probably exist.

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Table 4.1 Results from no-choice mating trials

Each cell contains the number of trials on which mating occurred, the total number of trials, the expected number of matings estimated from the five-parameter model, and the parameters (table 4.2) from which this expected value was obtained.

		male		
		H. melpomene	F_1	*H. cydno*
female	*H. melpomene*	15/17 13.4 *a*	5/14 4.2 *d*	0/10 0 *ce*
	F_1	8/18 8.2 *b*	11/16 12.6 *a*	3/16 2.7 *be*
	H. cydno	0/20 0 *c*	4/16 4.8 *d*	5/18 5.3 *ae*

Table 4.2 Multiplicative mating probability parameters from the five-parameter model, with support limits

type of parameter	parameter	max likelihood value	support limits
pure x pure and hybrid x hybrid mating probability	*a*	0.786	(0.625, 0.903)
pure male x hybrid female mating probability	*b*	0.455	(0.257, 0.672)
interspecific mating probability	*c*	0	(0, 0.081)
pure female x hybrid male mating probability	*d*	0.300	(0.155, 0.479)
H. cydno male mating performance parameter	*e*	0.371	(0.176, 0.648)

Figure 4.1. Courtship and mating in the no-choice trials. Each cell shows the percentage of males displaying courtship interest towards the female during four observations in the first two hours after eclosion, and the percentage mating over the whole trial period. Courtship was taken to include chasing the female, fluttering courtship above her, and attempted or actual mating.

Figure 4.2. Hypothetical example of variation in mating cue in two species and their F_1 hybrids (shaded) and the shape of mating preference in each species (solid lines)
(a) after initial divergence, with strong assortative mating but little discrimination against hybrids, (b) following reinforcement of assortative mating, (c) an alternative source of discrimination, after further divergence but without reinforcement.
The dashed line in each figure shows the mating probability for both parental species combined, assuming equal population sizes.

Figure 4.1

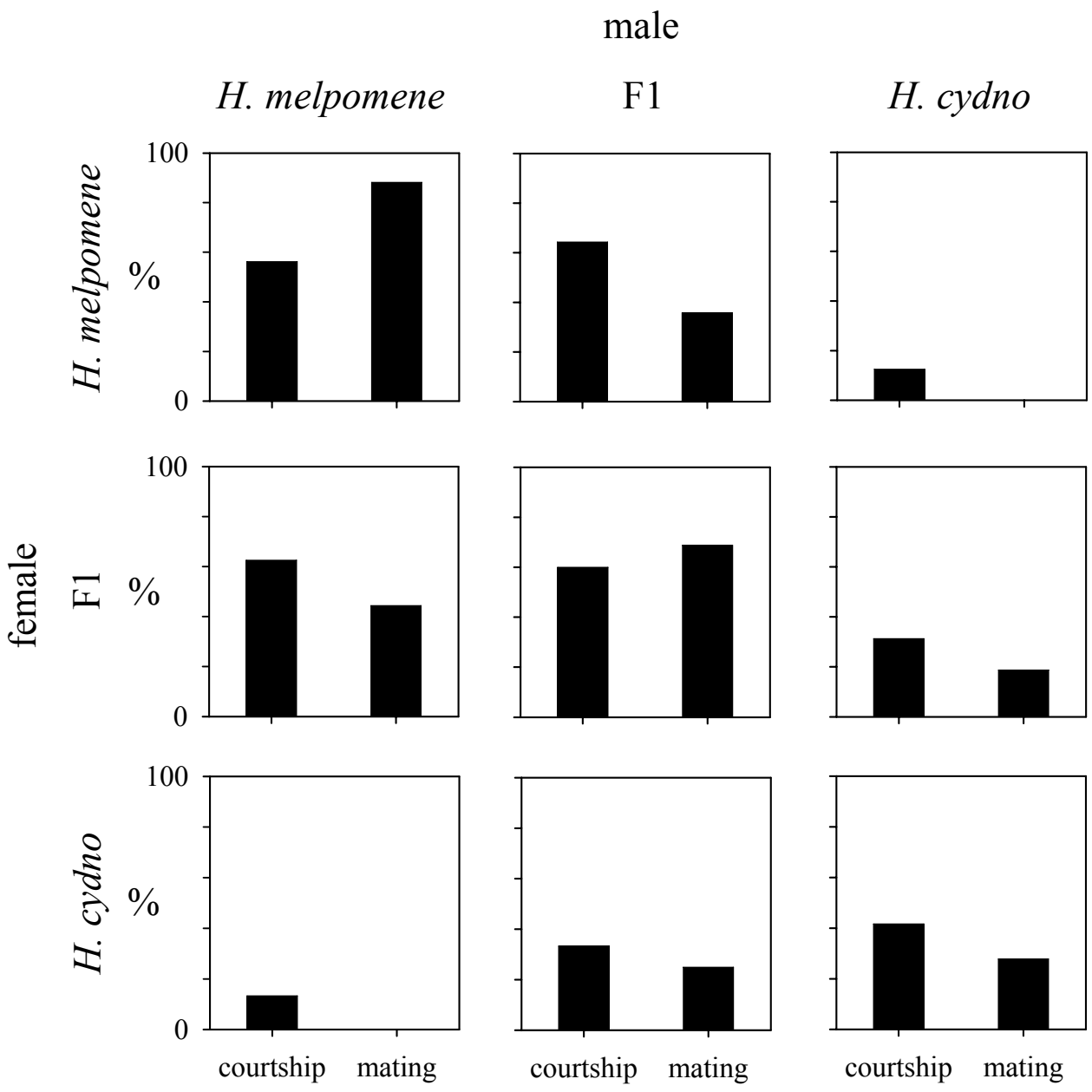
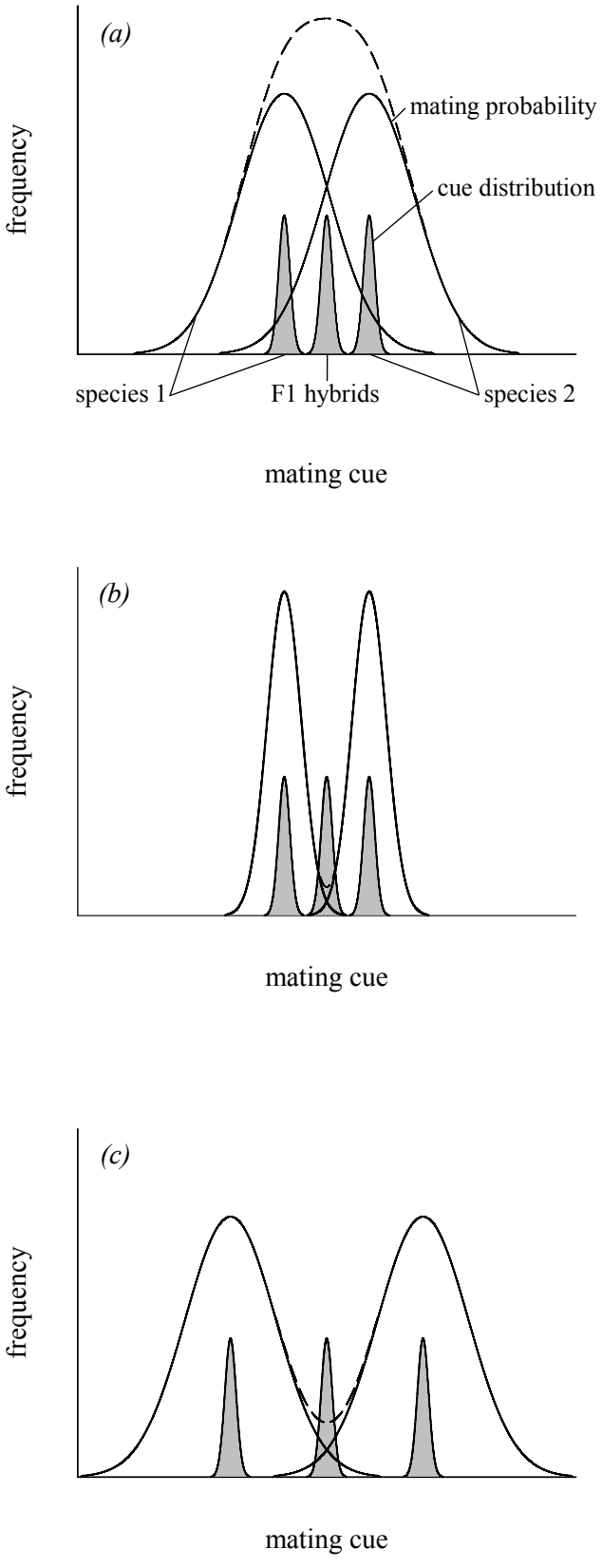

male
H. melpomene
F1
H. cydno
female
H. melpomene
F1
H. cydno
100
%
0
courtship
mating

Figure 4.2


(a)
mating probability
cue distribution
frequency
species 1
F1 hybrids
species 2
mating cue
(b)
frequency
mating cue
(c)
frequency
mating cue

5

Hybrid sterility, Haldane's rule, and speciation in *Heliconius cydno* and *Heliconius melpomene*

Abstract

Most genetic studies of Haldane's rule, the tendency for sterility or inviability to affect the heterogametic sex of hybrids preferentially, have focussed on *Drosophila*. It therefore remains unclear to what extent their conclusions apply more generally. Here we present a genetic analysis of Haldane's rule in *Heliconius* butterflies. Female F_1 hybrids between *Heliconius melpomene* and *H. cydno* are completely sterile, while males have normal to mildly reduced fertility. In backcrosses of male F_1 hybrids, female offspring range from completely sterile to fully fertile. Linkage analysis using the Z-linked *triose-phosphate isomerase* locus demonstrates a "large X" (Z) effect on sterility. Phenotypic expression of female sterility varies among different crosses between *Heliconius melpomene* and *H. cydno,* from the production of normal but infertile eggs to a complete failure to develop ovarioles, and production of small infertile eggs in previous crosses between races of *H. melpomene*. These results conform to the expectations of "dominance" rather than "faster male" theories of Haldane's rule, and suggest that several different loci with major effects are responsible. The two species are broadly sympatric and hybridise in the wild, so that female hybrid sterility forms one of several strong but incomplete barriers to gene flow in nature. Female sterility has an effect comparable to that of selection against non-mimetic hybrids, whilst mate choice forms a much stronger barrier to gene transfer.

Introduction

Haldane's rule has proved an enduring generalisation. It states that when one sex is absent, rare, or sterile in the F_1 offspring of a cross between two races or species, that sex is most commonly the heterogametic sex (Haldane 1922). It holds for over 95% of 324 crosses from 6 classes and several insect orders (Laurie 1997), and seems to be a standard feature of the evolution of complete sterility and inviability (Coyne & Orr 1989a; 1997). However, a universal explanation has proved elusive.

Recently a degree of consensus has finally been reached that Haldane's rule is largely explained by "dominance theory", with a contribution in *Drosophila* from "faster male evolution", and possibly "faster X evolution" (Wu & Davis 1993; Turelli & Orr 1995; Laurie 1997; Orr 1997; Turelli 1998; Turelli & Orr 2000). All of these theories rely on the existence of complementary genes (Bateson 1909; Dobzhansky 1936; Muller 1940), that can produce hybrid inviability or sterility between two populations without either having passed through an adaptive valley. Divergent alleles become fixed at different loci between populations, and cause incompatibility only when brought together in novel hybrid genotypes. "Dominance theory" stems directly from this epistatic model. If members of complementary sets of genes are found on the sex chromosomes, and act as recessive "loss of function" alleles in their effect on hybrid sterility, the heterogametic sex will suffer incompatibilities before the homogametic sex. This will produce Haldane's rule, and may be sufficient to explain it (Turelli & Orr 1995). However, several observations from studies of Diptera have motivated other theories. In that group, cases of Haldane's rule for sterility vastly outnumber those for inviability (Wu & Davis 1993). Attached-X hybrid females, with the same degree of X-autosome imbalance as F_1 males, fail to show effects on fertility when their male siblings are sterile (Coyne 1985), but do accompany them in cases of inviability (Orr 1993). Sterility

factors seem to accumulate more rapidly in males and are sex-specific, unlike inviability loci. Male sterility is also found in *Aedes* mosquito hybrids, which lack a hemizygous X and therefore in which dominance theory could not act (Presgraves & Orr 1998). These observations may be explained by "faster male" evolution, the faster evolution of male sterility alleles, either due to greater sensitivity of spermatogenesis to disruption, or rapid divergence of male reproductive characters driven by sexual selection (Wu & Davis 1993; Wu *et al*. 1996). The incidence of Haldane's rule may also be promoted by "faster X" evolution, where hemizygosity enhances selection of favorable recessive alleles on the X chromosome (Haldane 1924; Charlesworth *et al*. 1987). If these are to produce Haldane's rule effects the theory must act in conjunction with dominance theory or sex-specific expression of alleles (Orr 1997). The only two sufficiently detailed introgression studies provide little support for a greater density of sterility factors on the X than on autosomes (Hollocher & Wu 1996; True *et al*. 1996). They do however provide evidence of a contribution of both dominance and faster male evolution, with many recessive autosomal sterility factors that are only effective when made homozygous, and a greater density of male than female sterility factors. In sum, dominance theory seems to provide a fundamental explanation of Haldane's rule across all taxa for sterility and inviability, while faster male evolution contributes to the disproportionate representation of hybrid male sterility among Dipteran examples.

Birds and Lepidoptera may play an important role in distinguishing the contribution of the three theories. Their females are heterogametic yet both groups still display Haldane's rule. Therefore, heterogamy rather than sex is critical. Faster male theories are insufficient, and models of dominance theory suggest that it can produce Haldane's rule effects in females even when opposed by faster evolution of male hybrid sterility (Turelli & Orr 1995). In Lepidoptera the X (Z) chromosome represents a relatively

small proportion of the genome which would slow the origin of Haldane's rule effects (Turelli & Begun 1997), and yet they show a bias towards Z-linkage of species differences (Prowell 1998). Unfortunately, detailed genetic analyses have yet to be performed within the Lepidoptera to look at the rates of accumulation of male relative to female sterility factors and X relative to autosomal effects (Hollocher & Wu 1996; True *et al*. 1996).

Here we study the genetic basis of sterility between *Heliconius cydno* and *H. melpomene* (Lepidoptera: Nymphalidae). These sister species are sympatric across much of Central and Andean South America below 1500m (Brown 1979; Brower 1996). Both are unpalatable and warningly coloured, and their speciation has been accompanied by a shift in Müllerian mimicry. *H. melpomene* is black with red and yellow markings and mimics *Heliconius erato*, while *H. cydno* is black with yellow or white markings and usually mimics *Heliconius sapho* or *H. eleuchia* (Linares 1997; Jiggins *et al*. 2001b). This shift in mimetic allegiance appears to have driven speciation, resulting in both selection against hybrids due to their intermediate non-mimetic colour pattern, and assortative mating through a pleiotropic effect on mate recognition (Jiggins *et al*. 2001b). Colour pattern divergence probably followed a change in habitat use, bringing the two into contact with different suites of potential comimics. *H. melpomene* is found in second growth and *H. cydno* in forest understorey, but there is considerable overlap and the two species commonly fly together (Benson 1978; Smiley 1978b; Waage *et al*. 1981; Mallet & Gilbert 1995). They also differ in their degree of specialisation among *Passiflora* host plants (Smiley 1978a). Despite these differences the species do hybridise in the wild, albeit rarely: F_1 and backcross hybrids are known from across their range, although they probably form less than 0.1% of natural populations (Mallet *et al*. 1998b).

A previous study has shown female hybrid sterility between *Heliconius melpomene* from French Guiana and allopatric races from Panama and Colombia (Jiggins *et al*. 2001a, appendix 2). Here we investigate hybrid incompatibility between the genetically more divergent sister taxa, *H. cydno* and *H. melpomene*, allowing the first comparative phenotypic and genetic study of hybrid sterility in Lepidoptera.

Methods

Details of collection localities are given in table 5.1. Crosses involving *Heliconius melpomene rosina* and *H. cydno chioneus* from Panama and *H. melpomene melpomene* from French Guiana were performed in Gamboa, Republic of Panama between August 1998 and March 2000. Crosses involving Colombian butterflies were made in La Vega, 50km northwest of Santafé de Bogotá, Colombia. *H. melpomene vulcanus* and *H. cydno* were collected in the west of the Cauca valley, in the Dagua Pass. In this region three races of *Heliconius cydno* form a hybrid zone, between *H. cydno zelinde*, *H. cydno cydnides*, and *H. cydno weymeri* (Linares 1997), but with no detectable reduction in hybrid fertility or viability. Stocks of *H. melpomene melpomene* and *H. cydno cordula* were collected from the foothills of the eastern slopes of the Andes.

Females were kept individually in 1x1x2m outdoor insectaries and supplied daily with pollen sources (*Lantana* and *Psiguria*) and artificial nectar (10% sugar solution). Potted *Passiflora* vines were provided for oviposition, mainly *P. menispermifolia* and *P. edulis*. Eggs were collected daily and kept individually in small plastic pots with moist cotton wool to maintain humidity. Caterpillars were fed on new growth of *P. biflora* in Panama and *P. edulis* in Colombia, reared individually until the third instar to avoid cannibalism and then in groups of 2-8. After pupation they were transferred to baskets

until eclosion. The number of eggs laid, hatch rates, larval survival and eclosing butterflies were recorded. F_1 males and females as well as female offspring of backcrosses were tested for fertility.

Crosses were attempted in all possible combinations, but very strong asymmetrical mate choice prevented one direction of interspecific cross (*H. melpomene* female x *H. cydno* male) from Panama populations, and this direction of cross was obtained only once each using Colombia and French Guiana females (table 5.2). Crosses between the two species in Colombia were largely restricted to stocks from within a geographic region. Results from inter-racial *H. melpomene* crosses are described separately (Jiggins *et al*. 2001a, appendix 2).

Statistical analysis: Counts of egg hatch rates were analysed using maximum likelihood based on a beta-binomial model (Ziheng Yang, in Jiggins *et al*. 2001a). This method takes account of brood-to-brood variation in counts within each type of cross, when the real interest is in variation between cross types. Rather than estimating a single binomial parameter for each data class, for example hatch rate within one cross type, the model allows genetic and environmental variation between broods of a given class (e.g. cross type) to be taken into account. A binomial parameter is estimated for each brood, and these are assumed to vary across broods within each class according to a beta distribution, for which values of mean, variance and their standard errors are estimated. Classes can then be compared on the basis of these beta distribution parameters of mean and variance. This provides five alternative models for a data set containing replicate broods of several classes: 1) a classical binomial parameter for each class, which assumes a zero brood-to-brood variance; 2) a single beta mean and variance for the entire data set; 3) different means for each class but a single variance;

4) a single mean but different variances; and 5) a different mean and variance for each class. Further comparisons can be made by fitting models after combining parts of the dataset. For example, to compare the fertility of different male genotypes whilst controlling for female genotype, models are fitted before and after combining brood classes of the same maternal genotype but different paternal genotype. These models are then compared using likelihood (L) ratio tests, where $G = 2\Delta\log_e L$, which asymptotically follows a χ^2 distribution (Edwards 1972). Data from crosses performed in Colombia was analysed separately as hatch rates of control broods were lower than those in Panama, presumably for environmental reasons. For sex ratio, where a simple binomial was the best fitting model, support limits were obtained at the parameter values that led to a decrease in $\log_e$ likelihood of two units, asymptotically equivalent to 95% confidence intervals (Edwards 1972).

Linkage analysis: Intron 4 of the sex-linked *triose-phosphate isomerase* (*Tpi*) gene was amplified using primers situated in the surrounding exons. Evidence for sex linkage, primer sequences and PCR conditions are described by Jiggins *et al*. (2001a). This intron contains a 39 base pair insertion which is common (but not fixed) in the Panama *H. cydno* population, hereafter the '*cydno* insertion', as compared to *H. melpomene*. This size variation was used to follow segregation of *Tpi* in Panama backcross broods. All broods proved informative with regard to the segregation of alleles in female offspring, having F_1 fathers heterozygous for the insertion. Alleles were separated on 6% acrylamide gels run for 4 hours at 125V and stained using ethidium bromide.

Results

Crosses between the sister species in Panama: Female hybrids between *H. cydno* and *H. melpomene* were completely sterile (table 5.2). In the sympatric Panamanian cross 25

F_1 females were tested, producing 209 apparently normal eggs, not one of which hatched. Dissections show that they develop normal ovaries (table 5.3). Fifteen of those females failed to lay eggs despite surviving over 15 days, by which time fertile control broods had invariably begun laying. Male hybrids were fully fertile, with no significant difference in hatch rate between their offspring and those of control broods (G=2.90, df=4, p>0.05).

Sex ratio across all broods was best described by a simple binomial model with an average proportion of females of 0.516 (support limits 0.491, 0.542), across 1553 adults from 48 broods. There was no evidence for differences in the sex ratio of adults emerging from nine Panama and French Guiana control, F_1 and Panama backcross brood classes (G=9.15, df=8, p>0.05), suggesting no Haldane's rule effect of differential female inviability.

When fertile F_1 males were backcrossed to females of the two parental species, female offspring were recovered showing the full range of fertility but with pronounced bimodality at complete sterility and apparently normal fertility (table 5.4 parts A and B and figures 5.1 and 5.2). In the backcross to *H. cydno*, complete sterility was usually manifested as a failure to lay eggs, while from the backcross to *H. melpomene*, sterile females typically laid eggs which did not hatch. However, F_1 females were variable and often did not lay eggs.

There was strong linkage between sterility and Z-linked *Tpi* in female offspring from the backcross to *H. melpomene* (table 5.4A). All of the six females that were completely sterile had the insertion characteristic of *H. cydno*, while all of five females that showed at least some fertility lacked the insertion as in *H. melpomene* (G=15.16, df=1,

$p<0.001$). In contrast, in the backcross to *H. cydno* there was no association between *Tpi* genotype and sterility (table 5.4B, $G=1.98$, $df=1$, $p>0.05$).

Crosses between *H. melpomene* from French Guiana and *H. cydno* from Panama: Inter-specific hybrid females of the allopatric cross were also sterile (table 5.2). Female offspring of *H. cydno* female x *H. melpomene* male failed to lay eggs in all 10 cases examined. Dissections showed that the failure to lay eggs in these females was due to a complete failure to develop ovaries (table 5.3). Only two females could be produced from the reciprocal cross and although both were sterile, one actually laid eggs. There was also a reduction in F_1 male fertility (table 5.2). Broods fathered by F_1 males had hatch rates of 0.615±0.041 with a *H. cydno* female and 0.611±0.124 with *H. melpomene*, significantly lower than the rates of 0.859±0.039 and 0.901±0.027 for control broods of *H. cydno* and Guiana *H. melpomene* respectively ($G=16.94$, $df=4$, $p<0.01$). This is hybrid male sterility, rather than incompatibility when crossing parents of different genotypes, since there was no evidence of a reduction in hatch rate among eggs fertilised by a heterospecific male when F_1 and control broods are compared ($G=3.58$, $df=1$, $p>0.05$).

Crosses between Colombian sister species: In both the Eastern Andean foothills and Cauca valley crosses, female F_1 hybrids of *H. cydno* female by *H. melpomene* male were completely sterile, either failing to lay eggs or laying eggs that never hatched (table 5.2). Hybrid males were fully fertile: hatch rates of their offspring were higher than those of control broods, but not significantly so ($G=6.235$, $df=4$, $p>0.05$). Some partially fertile females were produced in the reciprocal cross, of a *H. melpomene* female from the Eastern Andes and a *H. cydno* male from the Cauca valley (table 5.2).

Females were tested from a single brood, with three laying few eggs that never hatched, and the remaining four producing eggs with hatch rates of around 20%.

Fertile F_1 males backcrossed to *H. cydno* produce female offspring with the full range of fertility (table 5.4 parts C and D, figure 5.2). The ratio of completely sterile to fertile females in backcrosses does not differ significantly between the eastern foothills, Cauca valley or Panama backcrosses to *H. cydno* (G test of heterogeneity, G=0.880, df=2, p>0.05).

Discussion

Female F_1 hybrids between *Heliconius cydno* and *H. melpomene* are completely sterile in five of the crosses described here, and their fertility is dramatically reduced in the sixth. In contrast, males have normal fertility in all but one of the crosses, with fertility only reduced in hybrids between *H. cydno* from Panama and *H. melpomene* from French Guiana. The lack of variation in sex ratio across control and F_1 broods suggests that there is no reduction in female viability. This brings the total to 20 cases of sex-limited sterility in Lepidoptera, all but one conforming to Haldane's rule (Laurie 1997; Jiggins *et al*. 2001a), compared to 68 examples of unisexual hybrid inviability (Laurie 1997). This is the reverse of the pattern seen in *Drosophila* where sterility predominates (Wu & Davis 1993; Laurie 1997). These results are expected under dominance theory, which explains why both taxa conform to Haldane's rule for sterility and inviability regardless of which sex is heterogametic (Turelli & Orr 1995), while faster male evolution may explain the strong excess of sterility in *Drosophila* (Wu & Davis 1993; Turelli 1998).

The association between sterility and *Tpi* genotype shown here provides evidence of a large X (Z) effect on sterility, as is common in *Drosophila* (Coyne & Orr 1989b) and very similar to that shown in inter-racial crosses in *H. melpomene* (Jiggins *et al*. 2001a). The locus forms a terminal marker in linkage maps of the Z chromosome in *Heliconius erato* and *Helicoverpa armigera* (N. Flanagan, *pers. comm*.; D. Heckel, *pers. comm*.) so that linkage suggests factor(s) in that region able to produce complete sterility. The effect of introgression of this region of the Z chromosome is asymmetric. Introgression of the *H. cydno* region into a predominantly *H. melpomene* genotype produces complete sterility, whilst there is no detectable linkage between sterility and the *Tpi* locus of *H. melpomene* when introgressed into a predominantly *H. cydno* genetic background. Such asymmetric effects are predicted by Muller's model of complementary gene evolution (Muller 1942). In the early stages of differentiation when few substitutions have occurred, if alleles in the *Tpi* region of the sex chromosome of one species interact with autosomal alleles in the other species to produce sterility or inviability, the reciprocal cross may remain compatible. Similar effects have been seen in *Drosophila* crosses (Wu & Beckenbach 1983). Sterility in the backcross to *H. cydno* must be produced by different factors at least some of which must be less tightly linked to *Tpi* if they are on the *H. melpomene* Z chromosome. However, dominant-dominant and dominant-recessive interactions between autosomal loci cannot be ruled out for this direction of cross.

Here we find sterility in females with the *H. cydno Tpi* allele on a Panama *H. melpomene* autosomal background. Interestingly, in hybrids within *H. melpomene* between geographic races from Panama and French Guiana, sterility is again associated with the same Z-linked *Tpi* marker; in this case it is the French Guiana *Tpi* that is associated with sterility on a Panama autosomal background (Jiggins *et al*. 2001a).

According to recent molecular evidence, the two *H. melpomene* races are most closely related, with *H. cydno* as an outgroup (Beltrán *et al*. 2002; Bull *et al*. 2002), in spite of previous evidence for paraphyly of *H. melpomene* relative to *H. cydno* (Brower 1996). Two possible explanations exist for the observed pattern of sterility in the three taxa. First, sterility is due to a Z-linked region involved in female fertility and ovary development, that has diverged rapidly between populations. Independent substitutions in the *Tpi* regions of both *H. cydno* and Guianan *H. melpomene* must then have occurred to cause sterility on a Panamanian *H. melpomene* autosomal background. Secondly, and more parsimoniously, sterility in both cases is due to the same derived Z-linked and autosomal alleles substituted within *H. melpomene* in Panama. In both crosses, sterility is found in females with an autosomal background predominantly derived from Panamanian *H. melpomene*, but lacking the Z-linked *Tpi* region of that population. Sterility arises when an individual lacks the epistatic sex-linked genes of Panama *H. melpomene* needed to complement the autosomes of that population, providing good evidence of complementary genes (Muller 1940 p. 203). Absence of this Z-linked region could therefore be acting as a recessive "loss of function" allele, as predicted under dominance theory. However, loci causing sterility are likely to be sex-specific, as in *Drosophila*. Ideally, therefore, we would need fertility data from attached-Z males to test Haldane's rule more effectively. However, no such strains exist and it is not even clear that they would be phenotypically male under Lepidopteran sex-determination.

Three different sterility phenotypes appear in female hybrids involving *H. melpomene*. (1) In the cross described here between female Panamanian *H. melpomene* and male *H. cydno*, F_1 females lay normal-sized eggs which never hatch. (2) The sterile eggs of hybrids are much smaller in inter-racial crosses between female *H. melpomene* from Panama and male *H. melpomene* from French Guiana (Jiggins *et al*. 2001a). (In the

reciprocal cross egg size and fertility is normal). (3) The third sterility phenotype is more extreme: hybrid females between French Guiana *H. melpomene* and Panama *H. cydno* fail to develop ovaries at all. These distinct sterility phenotypes suggest that at least some different loci are involved in each case, even though a parsimonious explanation of the results involving Panamanian *H. melpomene* (see previous paragraph) imply some genes in common.

At least five features of these crosses suggest that a small number of loci each of major effect are important in producing sterility. (1) The asymmetry seen in crosses between French Guiana and Panama or Colombia *H. melpomene* implies that few loci are involved, so that, by chance, incompatibilities have arisen in one but not both reciprocal crosses (Muller 1942). It has been asserted on the basis of an unpublished analysis that such asymmetry might arise even if large numbers of loci produce sterility (Turelli & Orr 1995, p. 395). However, many other examples of heterogeneity between the crosses studied here add to an impression that a few different major-effect loci are important in each cross, rather than many loci of individually small phenotypic effect. For instance, (2) there are strong effects of loci linked to *Tpi* in some crosses but not others, and (3) the sterility phenotypes are very different between the different crosses. Neither would be expected if sterility were a polygenic effect of steadily accumulating genetic differences. (4) In the backcross to *H. melpomene*, sterility is associated with *cydno Tpi*, on a Z chromosome which forms about 5% of the genome. Of those with the *H. melpomene Tpi* allele, interaction with *H. cydno* autosomal alleles would still be possible, since about 50% of autosomal loci are heterozygous for *cydno* and *melpomene* alleles. These autosomal loci can have a dominant effect, indeed alleles at such *cydno* loci produce sterility in the F_1. In the backcross to *H. melpomene*, therefore, there could be additional interactions between recessive *H. melpomene* autosomal factors (on the

50% of the genome that is homozygous) and many dominant *H. cydno* factors (on the remaining heterozygous 50%). If autosomal sterility factors were scattered throughout the genome, most offspring should have low fertility given that about ten times as much of the genome could be producing recessive autosomal sterility than hemizygous sterility in this backcross. As some individuals with the *melpomene Tpi* are fertile, this implies that the *cydno Tpi*-linked sterility is unusual in comparison with the rest of the genome. (5) Alternatively, one might expect to see a range of intermediate fertility levels if many loci combine to produce sterility. Instead, the distribution of sterility in the backcrosses is strongly bimodal. All of these considerations suggest that several, rather than many genomic regions are important, at least some of which have major effects on sterility.

Whilst female sterility is complete in F_1 hybrids between a *H. cydno* female and *H. melpomene* male, it remains uncertain if sterility is normally present in hybrids from the reciprocal cross as few could be produced due to strong behavioural isolation. There is evidence for sterility in both directions of cross between *H. cydno* from Panama and *H. melpomene* from French Guiana. In the Colombian crosses there is variation among females from the one brood of *H. melpomene* female by *H. cydno* male, with some showing complete sterility and others having low fertility with around 20% of eggs hatching. This variation within F_1 females is difficult to explain, but both parents come from regions where interspecific hybridisation occurs, *H. melpomene melpomene* with *Heliconius heurippa* in the Eastern Andean foothills, and *H. cydno* with *H. melpomene vulcanus* in the Cauca valley and Pacific slopes (Linares 1989; Mallet *et al*. 1998b). Populations may therefore carry introgressed alleles and not be fixed for whatever loci cause the interspecific sterility so that there is segregation in our F_1 cross. On the basis of the linkage between sterility and *Tpi,* we predict that female offspring from the

unobtainable *H. melpomene* female x *H. cydno* male Panama cross should also be sterile. These would have a *H. cydno* Z chromosome and heterozygous *H. melpomene* autosomes. This is similar to the interaction that causes sterility in the backcross to *H. melpomene* shown by *Tpi* linkage, but which consists of the *H. cydno* Z together with on average 50% homozygous and 50% heterozygous *H. melpomene* autosomes. These were all female-sterile.

Closely related species of *Heliconius* typically have multiple, incomplete barriers to gene flow that can include assortative mating, hybrid sterility, selection against hybrids due to increased predation on their non-mimetic colour patterns, and the divergent ecology of the parental species (Mallet *et al*. 1998b). Speciation is probably most closely associated with the origin of assortative mating and ecological divergence in *Heliconius* and other taxa (Mallet *et al*. 1998b; Jiggins & Mallet 2000; Kirkpatrick & Ravigné 2001). Hybrid sterility is neither necessary nor sufficient for speciation. For example, *Heliconius erato* and *H. himera* show no evidence for a reduction in viability or fertility of interspecific hybrids, and yet the two remain distinct in hybrid zones characterised by assortative mating and probably strong ecological selection against hybrids (Jiggins *et al*. 1997; McMillan *et al*. 1997; Mallet *et al*. 1998a). In contrast, unidirectional female hybrid sterility has been found between the French Guiana and Panama races of *H. melpomene* (Jiggins *et al*. 2001a), although these are traditionally considered part of the same species, and thought to be connected via continuous populations (Mallet 1986). Hybrid sterility may arise more as a consequence than a direct cause of speciation. If spatial isolation or assortative mating limit gene flow, even weak divergent selection in the two populations may lead to the accumulation of epistatic incompatibilities in hybrids. Sterility can of course promote further divergence by reducing gene flow still further and providing a selection pressure for increased

assortative mating through reinforcement. In *H. cydno* and *H. melpomene* sterility currently forms one of three strong barriers reducing the fitness of F_1 and later generation hybrids. Genomic incompatibility resulting in female sterility produces strong selection against F_1 hybrids, of 50% when averaged across both sexes, and backcrosses produce females with an average sterility of about 80%, so that the total barrier due to F_1 and backcross sterility is around 0.7 (0.5+0.5x0.5x0.8). Ecological incompatibility causing selection by predators against hybrid colour patterns is of a similar strength, around 50% estimated from a *H. erato* hybrid zone (Mallet & Barton 1989). Nearly half of backcross offspring should escape this increased predation on hybrids, because their colour patterns are similar to those of the parentals due to modifier loci and linkage of several major colour pattern loci (chapter 3), so that the overall barrier due to mimicry is around 64%. Mate choice between the two species forms the strongest barrier to gene transfer, since they hybridise extremely rarely in nature, only with great difficulty in no choice tests, and never in laboratory choice tests (Naisbit *et al*. 2001, chapter 4). This is accompanied by disruptive sexual selection against hybrids causing reduced mating success, forming an overall mating barrier of more than 99.9% (Mallet *et al*. 1998b; Naisbit *et al*. 2001).

A consequence of these multiple incomplete barriers to gene flow is that certain hybrid genotypes will suffer disproportionately while others escape, creating a selectively permeable species boundary. As in hybrid zones, different regions of the genome will cross the species boundary and introgress with varying ease, with some halted by selective barriers and others relatively unrestricted (Barton 1979; 1983; Rieseberg *et al*. 1999). In this case, the barrier due to sterility is incomplete, as males are fertile and after only one generation of backcrossing a fraction of females are produced with normal fertility and nearly normal mimicry phenotype. Introgression should therefore be

possible in both directions, and museum specimens confirm that backcrossing occurs in nature (Mallet *et al*. 1998b; Gilbert 2001). Transfer of some genes will be opposed by selection, such as colour pattern genes or sterility factors. Strongly selected loci will also affect associated regions, for example the *Tpi* locus or mitochondrial DNA, the introgression of which is prevented by female sterility (Sperling 1993). However, universally advantageous alleles will be barely affected if loosely linked. Even the strong Haldane's rule sterility found here between a pair of *Heliconius* species may not result in complete evolutionary independence in nature.

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Table 5.1 Collection localities for the *Heliconius* races used in these crosses.
Each race has been given a two-letter code used in later tables.
Heliconius cydno in the Cauca Valley is from a three-way inter-racial hybrid zone (see methods for details).

Race	Locality and altitude	Latitude and longitude
	Panama	
H. cydno chioneus (CP)	Pipeline road, Soberanía National Park	9° 08' N, 79° 42' W
H. melpomene rosina (MP)	Pipeline road, Soberanía National Park	9° 08' N, 79° 42' W
	French Guiana	
H. melpomene melpomene (MG)	Pointe Macouria, near Cayenne	4° 54.8' N, 52° 21.6' W
H. melpomene melpomene (MG)	Sablance, near Cayenne	4° 57.8' N, 52° 25.2' W
	Colombia, Eastern Andean foothills	
H. cydno cordula (CE)	Barro Negro, Casanare 1050 m	6º 01' 6"N, 72º 05' 47"W
H. melpomene melpomene (ME)	Chirajara, Cundinamarca 1150-1450m	4º 12'48"N, 73º 47'70"W
H. melpomene melpomene (ME)	Dele B (Río Charte), Casanare 1150m	5º 25'5"N, 72º 31' 20"W
H. melpomene melpomene (ME)	Pajarito, Casanare 940m	5º 17'30"N, 72º 42'30"W
	Colombia, Cauca valley	
H. cydno (CV)	Atuncela, Valle del Cauca 1400m	3º 44' 3"N, 76º 41' 53"W
H. cydno (CV)	Río Bravo, Valle del Cauca 1000m	3º 54'13"N, 76º 38'18"W
H. melpomene vulcanus (MV)	Queremal, Valle del Cauca 1200m	3º 31'28"N, 76º 45'25"W
H. melpomene vulcanus (MV)	Río Bravo, Valle del Cauca 1000m	3º 54'13"N, 76º 38'18"W

Table 5.2 Hatch rate of control, F_1 and backcross broods

In this and later tables, individuals of hybrid genotype are coded as maternal x paternal genotype.

CP = *H. cydno* from Panama

MP = *H. melpomene* from Panama

MG = *H. melpomene* from French Guiana

CE = *H. cydno* from the Eastern Andean foothills, Colombia

ME = *H. melpomene* from the Eastern Andean foothills, Colombia

CV = *H. cydno* from the Cauca Valley, Colombia

MV = *H. melpomene* from the Cauca Valley, Colombia

Mean, variance and standard error estimates are from a beta-binomial model (for details see methods). The data includes *H. melpomene* broods from Jiggins *et al.* 2001a.

Maternal genotype	Paternal genotype	# broods	# eggs	Mean hatch rate	s.e.	Variance	s.e.
Panama and French Guiana crosses							
Pure							
CP	CP	16	820	0.859	0.039	0.021	0.011
MP	MP	22	943	0.945	0.013	0.002	0.002
MG	MG	18	881	0.901	0.027	0.011	0.007
F_1 intraspecific							
MP	MG	7	316	0.915	0.022	0.001	0.002
MG	MP	7	200	0.946	0.022	0.001	0.002
F_1 interspecific							
CP	MP	5	516	0.955	0.007	0.000	0.000
CP	MG	5	250	0.932	0.019	0.000	0.002
MG	CP	1	15	1.000	-	-	-
Fertile backcrosses							
CP	CPxMP	5	594	0.887	0.046	0.010	0.009
MP	CPxMP	5	451	0.977	0.011	0.000	0.000
CP	CPxMG	2	143	0.615	0.041	0.000	0.000
MG	CPxMG	4	178	0.611	0.124	0.059	0.033

Table 5.2 continued

Maternal genotype	Paternal genotype	# broods	# eggs	Mean hatch rate	s.e.	Variance	s.e.
Sterile backcrosses							
CPxMP	CP/MP/MG/F_1	25	209	0	-	-	-
CPxMG	CP/MG	10	0	0	-	-	-
MGxCP	MG	2	23	0	-	-	-
Colombia crosses							
Pure							
CE	CE	8	386	0.588	0.099	0.078	0.028
ME	ME	11	416	0.520	0.065	0.038	0.017
CV	CV	14	791	0.665	0.073	0.073	0.023
F_1 interspecific							
CE	ME	3	81	0.574	0.105	0.025	0.024
CV	MV	2	68	0.925	0.085	0.013	0.028
ME	CV	1	82	0.6	-	-	-
Fertile backcrosses							
CE	CExME	3	241	0.629	0.101	0.025	0.021
CV	CVxMV	5	83	0.894	0.041	0.003	0.008
MExCV	CV/F_1	7	257	0.172	0.036	0.003	0.004
Sterile backcrosses							
CExME	CE/ME/MV/F_1	15	51	0	-	-	-
CVxMV	CV/MV	8	0	0	-	-	-

Table 5.3 Sterility phenotypes of female F_1 hybrids from inter-specific and inter-racial crosses

Cross	Phenotype of ovary	Egg phenotype	Fertility
CPxMG	Undeveloped	-	Sterile
MGxCP		Normal	Sterile
CPxMP	Normal	Normal	Sterile
MPxCP	unknown	unknown	Sterile[1]
MPxMG	Normal	Small	Sterile[2]
MGxMP	Normal	Normal	Fertile[2]

[1]Inferred (see discussion)

[2]Jiggins *et al.* 2001a (appendix 2)

Table 5.4 Hatch rate of eggs laid by the female offspring of backcross broods
A hyphen in the *Tpi* genotype column indicates cases where the individual was not preserved for genetic analysis, while data in brackets was excluded from the analysis of linkage and sterile:fertile ratios as the female died before sterility could confidently be assigned. Colombian material was not genotyped for *Tpi*.

Female #	Offspring of brood #	# days in cage	# eggs laid	Hatch rate %	*Tpi* genotype
A. Offspring of backcross to Panama *H. melpomene* MP x (CP x MP)					
354	341	6	27	0	C
355	341	(10)	(3)	(100)	-
358	341	6	12	0	C
365	345	14	13	0	-
366	345	14	32	97	M
367	341	12	31	0	C
368	345	18	26	81	M
372	345	16	33	0	C
374	341	7	52	83	M
381	345	10	31	0	C
385	345	(11)	(0)	(-)	(M)
386	345	6	31	19	M
396	341	12	32	13	M
397	345	13	18	0	C

Table 5.4 continued

Female #	Offspring of brood #	# days in cage	# eggs laid	Hatch rate %	*Tpi* genotype
B. Offspring of backcross to Panama *H. cydno* CP x (CP x MP)					
308	304	57	0	-	M
310	304	53	0	-	M
321	304	41	0	-	C
323	304	18	3	0	C
326	304	23	58	64	M
357	342	16	15	40	C
359	342	26	0	-	-
360	342	(9)	(1)	(0)	-
363	347	20	0	-	M
383	342	34	0	-	M
388	347	(14)	(0)	(-)	(M)
389	347	6	31	81	M
390	347	5	31	84	C
391	347	(14)	(0)	(-)	(M)
393	342	20	0	-	M
394	347	21	0	-	M
395	347	21	5	0	M
399	347	(5)	(0)	(-)	-
402	347	9	28	11	C

Table 5.4 continued

Female #	Offspring of brood #	# days in cage	# eggs laid	Hatch rate %	*Tpi* genotype
C. Offspring of backcross to Colombia eastern *H. cydno* CE x (CE x ME)					
65	C-630	(12)	(0)	(-)	
63	C-630	22	0	-	
10	C-630	15	13	0	
1	C-630	12	6	0	
30	C-630	22	7	0	
66	C-630	5	13	15	
16	C-630	51	108	10	
21	C-637	16	0	-	
3	C-637	20	0	-	
2	C-637	25	26	0	
1	C-637	20	6	66	
12	C-637	65	15	26	
20	C-637	34	7	42	
30	C-637	56	31	92	
10	C-634	88	41	17	
15	C-634	19	12	0	

Table 5.4 continued

Female #	Offspring of brood #	# days in cage	# eggs laid	Hatch rate %	*Tpi* genotype
D. Offspring of backcross to Colombia Cauca valley *H. cydno* CV x (CV x MV)					
1	C-656	31	0	-	
13	C-656	17	0	-	
3	C-656	(5)	(1)	(0)	
11	C-656	61	23	100	
25	C-629	21	0	-	
1	C-626	13	7	100	
3	C-624	(12)	(0)	(-)	
18	C-624	(12)	(0)	(-)	
8	C-624	17	0	-	
1	C-619	18	0	-	
2	C-619	16	0	-	
2	C-618	19	0	-	
4	C-618	48	35	60	

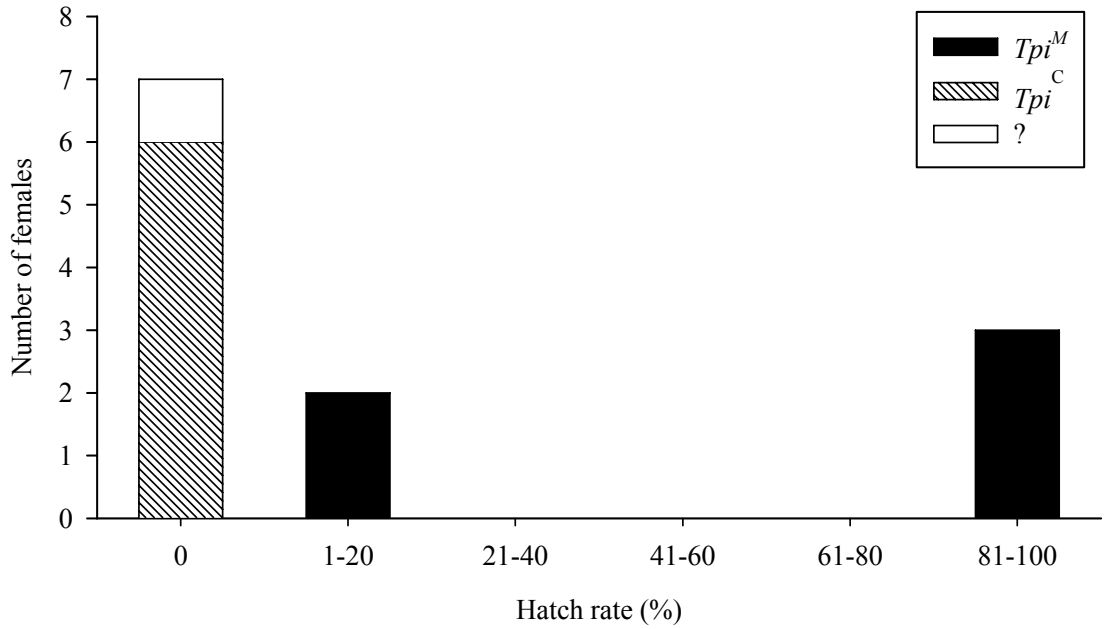

Figure 5.1 Hatch rate of eggs laid by female offspring of the backcross to *Heliconius melpomene* MPx(CPxMP)

Shading indicates genotype at the *triose-phosphate isomerase* locus, with superscripts for the alleles of *H. melpomene* and *H. cydno*, and ? for individuals that were not analysed.

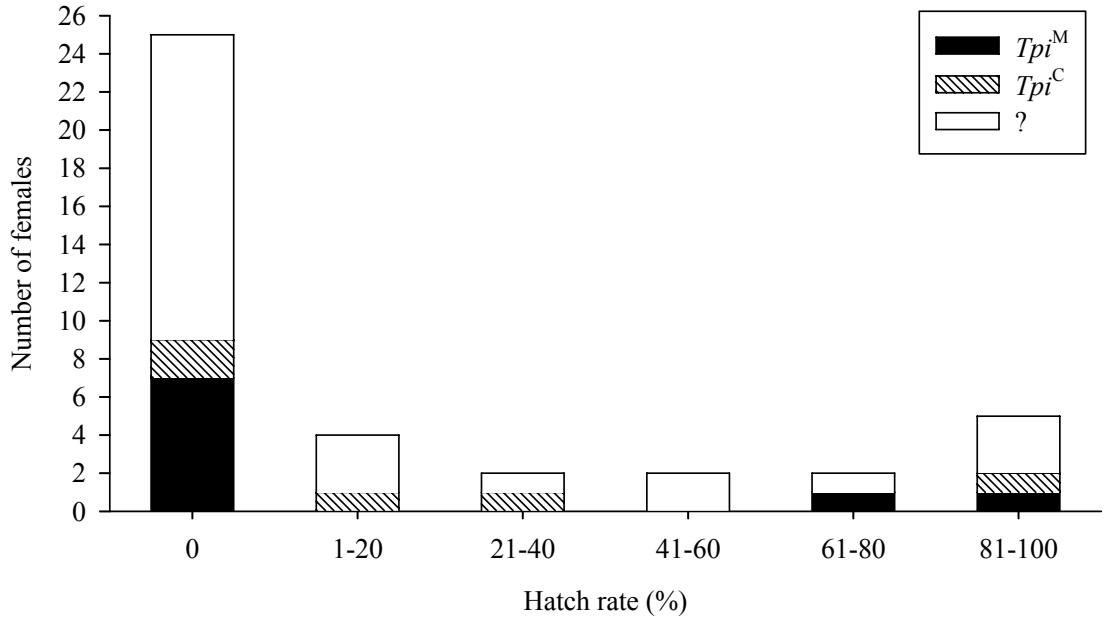

Figure 5.2 Hatch rate of eggs laid by female offspring from all backcrosses to *Heliconius cydno* Cx(CxM)

Shading as in figure 5.1

6

What causes speciation in *Heliconius*?

Heliconius cydno and *H. melpomene* are sister species that differ in many aspects of behaviour and ecology. They have diverged in Müllerian mimicry, *melpomene* as a mimic of *Heliconius erato*, and *cydno* as a mimic of *Heliconius sapho*. They also differ in microhabitat and host plant use, with *cydno* using most *Passiflora* species in the forest interior, and *melpomene* specialised on *P. menispermifolia* in second growth. They also display most of the barriers to gene flow known from other systems, despite having diverged relatively recently (probably within the last 1.5 million years (Beltrán *et al*. 2002)). There is premating isolation due to host plant use, habitat divergence, and strong mate choice, and three forms of strong selection against hybrids arising from disruptive sexual selection, increased predation on their intermediate non-mimetic colour patterns, and female hybrid sterility.

So what drove speciation in these *Heliconius*? With so many incomplete barriers to gene flow it is unclear which were critical in the divergence of *cydno* and *melpomene*, and which might have accumulated as a result of further divergence after speciation. In *Heliconius* we are fortunate in being able to adopt a comparative approach using populations at a variety of levels of differentiation, from broadly overlapping polymorphic forms through to distinct sympatric species. Many of the selection pressures acting on populations are also understood so it is possible to reconstruct the probable stages of divergence.

It is likely that a change in microhabitat use initiated divergence in *cydno* and *melpomene*, triggering a cascade of further changes that led to full speciation (figure 6.1). *H. cydno* is found in the forest interior, whilst *H. melpomene* dominates open, disturbed habitats, although there is considerable overlap (chapter 2). This shift would have brought an immediate degree of premating isolation by reducing the encounter rate between the incipient species, but more importantly, exposed them to selection pressures for further divergence. The abundance of host plants and potential competitors among other *Heliconius* species differs in the two habitats, leading to selection on their host plant preferences. Their microhabitats also represent different thermal regimes. *H. cydno* is likely to be adapted to cooler conditions, being found in deep forest and at higher altitudes and latitudes (Brown 1979), whilst *H. melpomene* is restricted to the lowlands in open areas and savannah. Habitat divergence is also likely to have led to the key stage of speciation in *melpomene* and *cydno*, divergence in mimicry. Their co-mimetic species, *Heliconius erato* and *H. sapho*, are not sister species and show almost three times the mitochondrial DNA sequence divergence of *cydno* and *melpomene*, which implies that *melpomene* and *cydno* diverged to mimic the pre-existing warning colour patterns of *erato* and *sapho,* rather than vice versa (Brower & Egan 1997; Mallet *et al*. 2001). They also show strong habitat divergence, *erato* in second growth where *melpomene* is found, and *sapho* in the forest interior with *cydno* (Waage *et al*. 1981; Mallet & Gilbert 1995). The different light conditions in the two microhabitats may have played a role in mimetic divergence: as in the sexual signals of dewlaps of male *Anolis* lizards (Losos 1994), bright red and yellow markings like those of *erato* and *melpomene* may be more effective warning signals in sunlit open habitats, whilst the lighter white or yellow patterns of *sapho* and *cydno* may be more apparent in the darker forest interior. Habitat divergence by *cydno* and *melpomene* would have brought them

into a new mimetic environment, relaxing the selection pressure on their original pattern, and replacing it with strong selection to converge on the local colour pattern.

Differences in habitat use would have generated selection for divergence in host plant specialisation. The density of *Passiflora* shoots is lower in the forest interior, and as the larvae of both *cydno* and *melpomene* are able to feed on most species of *Passiflora*, it is not surprising to find that *cydno* adopts a generalist oviposition strategy (chapter 2). Different *Passiflora* species offer broadly similar resources in terms of levels of occupancy by predatory ants and parasitoids of eggs and larvae. In second growth there is a much greater density of *Passiflora* vines, but also of *Heliconius* species. In Central America, the second growth heliconiine community tends to partition the available *Passiflora*, by species and age of vegetation used, and in Panama *melpomene* is a specialist on *P. menispermifolia, P. oerstedii,* and a few related species. Brazilian races of *melpomene*, which are found in the absence of *cydno* and inhabit the forest interior as well as second growth, display a generalist oviposition strategy (Benson 1978). Interestingly, the difference between *melpomene* populations is likely to be a result of competition among the *Heliconius* community in second growth, rather than character displacement between *melpomene* and *cydno* as a result of competition with its sister species.

Microhabitat adaptation and host plant divergence allows broad sympatry between *cydno* and *melpomene*, with overlap along the boundary of the two types of forest (chapter 2). In contrast, another pair of sister species, *Heliconius erato* and *H. himera,* share host-plants in forest edge habitats and only meet in narrow hybrid zones at the boundary of wet and dry forest in Ecuador and Peru (Mallet 1993; Jiggins *et al*. 1997a). Strong habitat adaptation to different macro-ecological conditions forms an additional

barrier to gene flow, but together with a lack of microhabitat and host plant divergence is probably the reason for their restriction to parapatric distributions (Jiggins *et al*. 1997b; McMillan *et al*. 1997; Mallet *et al*. 1998; Davison *et al*. 1999).

Mimetic divergence was the crucial step in speciation, for it had two important effects on gene flow, generating selection against hybrids and assortative mating (chapter 4 & appendix 1). Divergence in colour pattern leads to selection against hybrids because their intermediate non-mimetic patterns are not recognised as being unpalatable by predators. This selection is likely to be strong, for instance selection coefficients are around 50% against foreign colour patterns transported across a *H. erato* hybrid zone (Mallet & Barton 1989), and 60% against *H. cydno* colour forms transported to an area where theirs is not the locally dominant warning pattern (Kapan 2001). Colour pattern differences between *cydno* and *melpomene* have a simple genetic basis: ten loci can be identified, including genes of major effect (chapter 3). Many of these loci are homologous with those controlling pattern differences between geographic races within each species. Epistatic interaction between loci will affect the way ecological selection acts against non-mimetic hybrid genotypes. Several of the major loci are clustered into linked blocks, suggesting that colour pattern evolution uses pre-existing linked elements, which may arise by tandem duplication.

Mimetic divergence is the most striking aspect of the adaptive radiation within the genus (Turner 1976; Brown 1979). Almost all forms are involved in Müllerian mimicry, where unpalatable species share a warning colour pattern and hence the costs of predator education. Mimicry rings typically include two or more species of *Heliconius*, often with other Lepidoptera in particular from the Ithomiinae. Yet, on the whole, divergence in colour pattern rarely leads to speciation in *Heliconius*. Most species have

diversified into geographic colour pattern races (Brown 1979), with rampant hybridisation between these parapatric races so that contact zones are usually dominated by hybrids. Most divergence is thus parapatric but populations of *Heliconius* may also respond to variation on a finer spatial scale, for example forms of *H. numata* and *H. cydno* track the frequency of their comimics in the Ithomiinae over a scale of tens to hundreds of km (Joron *et al*. 2001; Kapan 2001). The balance between local adaptation and migration can then lead to polymorphism, as in some *melpomene*-group species, such as within *H. cydno* in Western Ecuador and *H. numata* in the Amazon basin (Joron *et al*. 2001; Kapan 2001). These forms have clearly not speciated, despite hybrids being under strong selection due to predation, and with colour pattern variation determined by many of the same loci as the interspecific differences found here (chapter 3). However, there is an association between colour pattern divergence and speciation: eight out of nine pairs of sister species in *Heliconius* belong to distinct mimicry rings (Turner 1976; Brower 1994; Mallet *et al*. 1998). Divergence seems to lead to speciation only when dramatic changes in colour pattern have pleiotropic effects on mate choice. Colour may perhaps seem an unlikely mating cue in a genus where mimicry is so common, but it is important in the initial attraction of males. Males in the wild can often be observed approaching and inspecting butterflies with similar coloration, and then at close range mate recognition presumably relies on pheromone differences in addition to colour. The dominant colour pattern of a species can thus form an important mating cue, as does red coloration in *H. erato* (Crane 1955). The dramatic change in the predominant colour between *cydno* and *melpomene* caused male mating preference to coevolve with the change in colour pattern, leading to assortative mating (appendix 1).

Once assortative mating had evolved, together with selection against hybrids it created partially isolated populations that could more easily diverge further and accumulate

adaptations beneficial in only one population. It also created the conditions most favourable for the action of reinforcement, the increase in assortative mating driven by natural selection when hybrids have low fitness (Liou & Price 1994). This seems to have strengthened male mating preference, for males of *H. melpomene melpomene* from French Guiana where *cydno* is not found, are more likely to court and mate female *cydno*, than are males of *H. melpomene rosina* from Panama where this selection for reinforcement has had the opportunity to act (Jiggins *et al*. 2001).

H. cydno and *H. melpomene* show very strong assortative mating even in no-choice trials, and there is evidence for disruptive sexual selection against F_1 hybrids (chapter 4). Hybrids mate readily with one another, but both sexes have reduced mating success with the parental species. This discrimination at mating against hybrids is not an inevitable product of assortative mating between species. Another pair of sister species, *H. erato* and *H. himera*, show strong assortative mating but lack any evidence for disruptive sexual selection against most hybrid genotypes (McMillan *et al*. 1997). Tightening of male mating preference under reinforcement and/or greater divergence of mating cues may be responsible for the origin of the discrimination against F_1 hybrids seen in *melpomene* and *cydno*.

Female hybrid sterility is likely to have evolved after assortative mating and mimicry differences led to *cydno* and *melpomene* becoming strongly isolated. Female F_1 hybrids are completely sterile in crosses between *cydno* from Panama or Colombia, and *melpomene* from Panama, Colombia or French Guiana (chapter 5). However, male hybrids are fertile, and some fertile female hybrids are recovered in backcrosses. Similar sterility is found in crosses between geographic races of *melpomene* from French Guiana and Panama or Colombia (appendix 2). Partial isolation due to intrinsic or

geographic barriers would allow the accumulation of differences in each species, leading to dysfunction when combined in hybrid genotypes. The normal within-species functions of genes that cause hybrid sterility are not understood, but divergent natural or sexual selection is presumably responsible for their differences. In *cydno* and *melpomene,* sterility might be a by-product of thermal adaptation or egg size differences (Brown 1981), the larger eggs of *H. cydno* perhaps adapted to less frequent and more dispersed but safer oviposition sites in the forest interior.

Ecological adaptation is becoming a common theme among studies of speciation. Recent theory suggests that even where mate choice and ecological adaptation are governed by distinct loci, disruptive selection in sympatry can build up linkage disequilibrium between the two traits leading to assortative mating (Dieckmann & Doebeli 1999; Kondrashov & Kondrashov 1999). However, speciation is facilitated when there is pleiotropy between ecological selection and reproductive isolation, so that adaptation reduces the rate of interbreeding and the survival of hybrids (Rice & Hostert 1993; Dieckmann & Doebeli 1999; Kondrashov & Kondrashov 1999; Kirkpatrick 2000). Partial parapatry caused by microhabitat divergence would make speciation still more likely, reducing gene flow and exposing populations to divergent selection pressures. Pleiotropy between ecology and mate choice is commonly seen in phytophagous insects, where host-associated mating and host-specialisation lead to disruptive selection and assortative mating (Wood 1980; Feder & Bush 1989; Katakura *et al*. 1989; Craig *et al*. 1993; Feder *et al*. 1994; Via 1999), even in taxa where pheromones are involved in long-range mate attraction (Emelianov *et al*. 2001). Similar pleiotropy can occur when mate choice is based on the trait under disruptive selection, as in body size in sticklebacks (Rundle *et al*. 2000), and morphology in Darwin's finches (Podos 2001). Reproductive isolation in *cydno* and *melpomene* arose largely as a

response to disruptive natural selection on habitat use and the resulting differences in mimetic environment. Disruptive selection on mimicry then led to premating and postmating isolation, through coevolution of colour and mate choice, and selection against non-mimetic hybrids (appendix 1). Increasingly Darwin's insight is vindicated: natural selection is not only the mechanism of adaptation, but also the means by which species originate.

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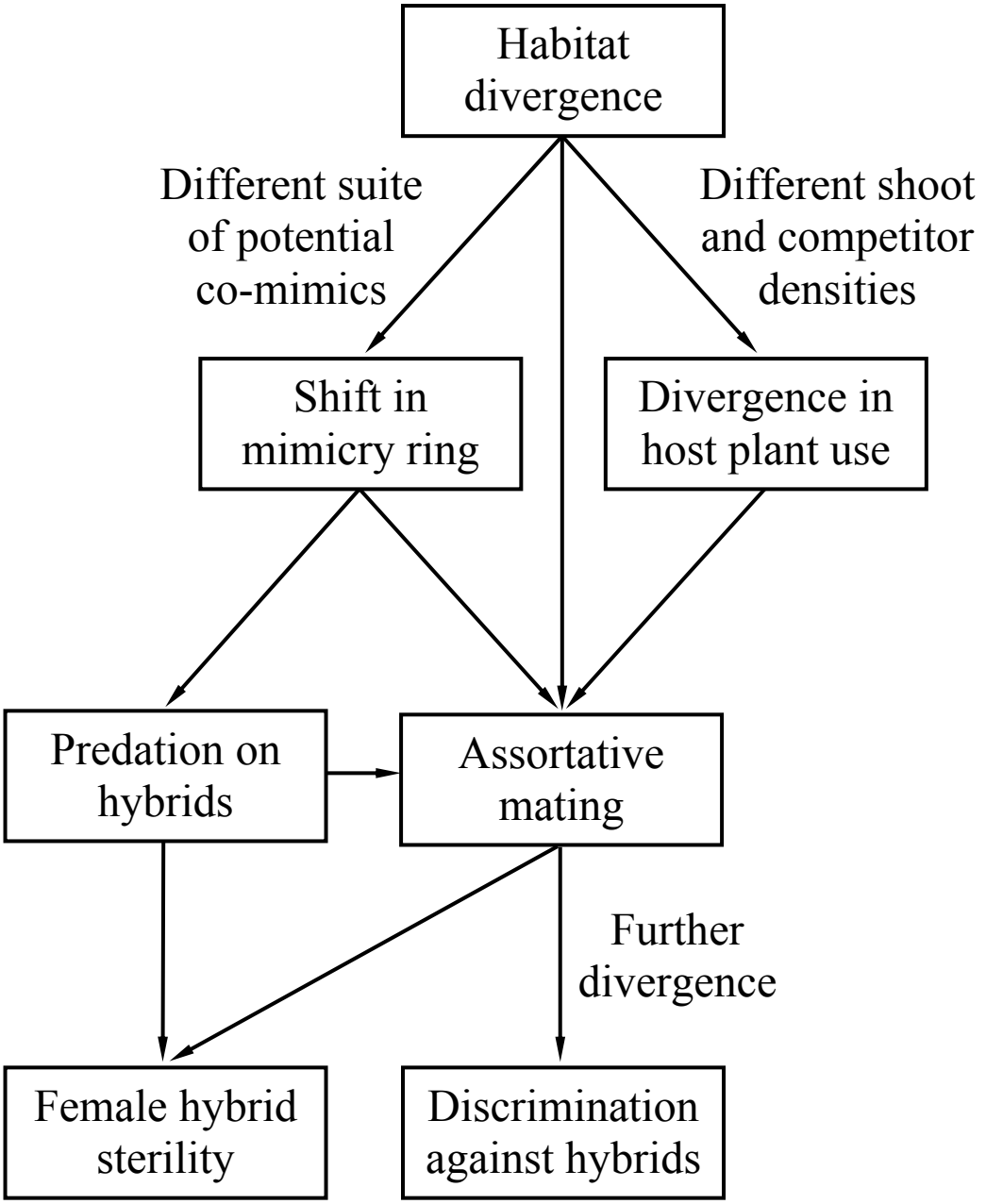

Figure 6.1 Probable course of events during speciation of *Heliconius cydno* and *Heliconius melpomene*

Appendix 1

Reproductive isolation caused by colour pattern mimicry

(d) of the worm is 200 μm (see Supplementary Information). All assumptions are conservative and result in an overestimation of sulphide flux from the sediment (see Supplementary Information). Internal sulphide production from the symbionts is based on SRRs measured in worms incubated in sand (Table 1), assuming that all sulphide produced is consumed by the sulphide-oxidizing symbionts. SRRs in the worms are assumed to be underestimated, given that no external electron donor was used and experimental conditions are suboptimal in comparison to the natural environment.

Received 25 September 2000; accepted 26 February 2001.

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

Acknowledgements

We thank F. Widdel and B. Barker Jørgensen for reviewing this manuscript; E. Llobet-Brossa and A. Boetius for discussions; the Hydra Institute for Marine Sciences on Elba for logistical support during collection of the worms; and J. Wulf, D. Lange, G. Eickert and A. Eggers for technical support. The Max-Planck Society and the German Research Foundation (DFG) provided financial support.

Correspondence and requests for materials should be addressed to N.D. (e-mail: ndubilie@mpi-bremen.de). GenBank accession numbers: 16S rRNA: γ-Proteobacteria symbiont AF328856, δ-Proteobacteria symbiont AF328857; DSR: δ-Proteobacteria symbiont AF244995, *D. variabilis* AF191907.

Reproductive isolation caused by colour pattern mimicry

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Speciation is facilitated if ecological adaptation directly causes assortative mating[1], but few natural examples are known. Here we show that a shift in colour pattern mimicry was crucial in the origin of two butterfly species. The sister species *Heliconius melpomene* and *Heliconius cydno* recently diverged to mimic different model taxa, and our experiments show that their mimetic coloration is also important in choosing mates. Assortative mating between the sister species means that hybridization is rare in nature, and the few hybrids that are produced are non-mimetic, poorly adapted intermediates. Thus, the mimetic shift has caused both pre-mating and post-mating isolation. In addition, individuals from a population of *H. melpomene* allopatric to *H. cydno* court and mate with *H. cydno* more readily than those from a sympatric population. This suggests that assortative mating has been enhanced in sympatry.

Mimicry is viewed mainly as a clear, visual demonstration of natural selection within species. But this was not always so: mimicry among Amazonian butterflies was originally presented as a striking example of speciation due to natural selection[2]. More recently, it has been argued that divergence in mimetic pattern can result in intermediates having low fitness because they are non-mimetic and, if colour pattern is also used in mate recognition, assortative mating. Therefore, both pre-mating and post-mating reproductive isolation might result from the evolution of mimicry[3–5]. Here we study mate choice in *Heliconius* butterflies, a group well known for Müllerian mimicry (mimicry between distasteful species)[2,4,5]. Closely related *Heliconius* species generally differ in mimetic colour pattern, as though adaptive radiation has occurred[6,7]. The sister species *H. melpomene* and *H. cydno* are sympatric throughout Central America and the Andean foothills, where they differ in mimicry (Fig. 1) and habitat use[8]. They occasionally hybridize and backcross in nature: hybrid females are sterile, but males are fertile and can be used in the laboratory to introgress genes between the species[8–10]. In most areas, *H. melpomene* mimics the black, red and yellow pattern of *H. erato*, whilst *H. cydno* mimics the black and white pattern of *H. sapho*. *Heliconius cydno* and *H. melpomene* separated in the last 10^6 years, much more recently than the non-sister species *H. sapho* and *H. erato* (Fig. 1)[11]. This and other evidence implies that *H. cydno* and *H. melpomene* have diverged to mimic *H. sapho* and *H. erato*, rather than vice versa[12].

Sympatric Panamanian *H. melpomene* and *H. cydno* did not mate with one another in choice experiments (Tables 1 and 2), although they will do so in no-choice tests[8–10]. Males from sympatric populations spent over 25 times longer courting virgin females of their own race than heterospecifics (Fig. 2). *Heliconius* females mate soon after eclosion, when they are unable to reject males, so that courtship and assortative mating is largely due to male choice[7]. To test whether males use mimetic colour pattern as a cue in choosing mates, we investigated the response of males to moving models made with either natural wings or coloured paper. Panama *H. melpomene* males approached *H. cydno* colour patterns about half as frequently as those of their own type, and were much less likely (2–4%) to court them (Fig. 3). Similarly, *H. cydno* males were a third as likely to court a *H. melpomene* pattern as their own type, although

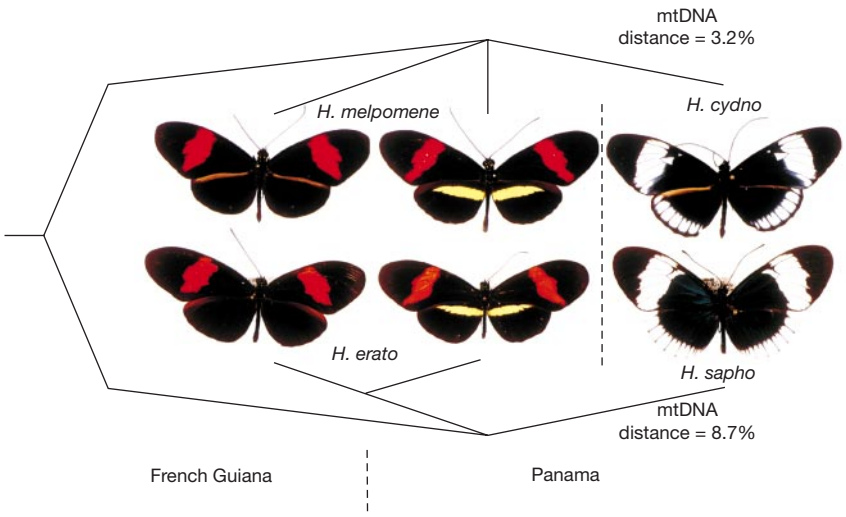

Figure 1 *Heliconius melpomene melpomene* (left, French Guiana), *H. melpomene rosina* (centre, Panama), *H. cydno chioneus* (right, Panama) are shown together with co-mimics (below) *H. erato hydara*, *H. erato cf. petiveranus* and *H. sapho sapho* respectively. Molecular phylogenies (enclosing butterflies) show that the two races of *H. melpomene* and *H. cydno* form an unresolved trichotomy. Mitochondrial sequences suggest *H. melpomene* is paraphyletic with respect to *H. cydno*[11], whereas unpublished sequences from nuclear loci show reticulate or mutually monophyletic relationships between the two species (V. Bull and M. Beltrán, personal communication). Divergence between mitochondrial sequences of *H. erato* and *H. sapho* is almost three times that between *H. melpomene* and *H. cydno* (percentage distance across 940 base pairs of the COI, leu-tRNA and COII genes)[11], suggesting that *H. melpomene* and *H. cydno* diverged to mimic *H. erato* and *H. sapho* rather than vice versa. mtDNA, mitochondrial DNA. COI, Cytochrome oxidase I; COII, cytochrome oxidase II.

the probability of initial approach did not differ from that towards conspecifics (Fig. 3). The initial attraction of male *H. cydno* to the red *H. melpomene* pattern may be due to a generalized attraction of *Heliconius* to red flowers. The butterflies clearly responded to visual cues in these experiments, as neither attraction nor courtship differed significantly in comparisons between paper models and real butterfly wings (Fig. 3).

Heliconius melpomene males from French Guiana, where *H. cydno* does not occur, courted live *H. cydno* females twenty times more vigorously than *H. melpomene* males from sympatry with *H. cydno* in Panama (Fig. 2), and the mating experiments showed a similar trend ($G_1 = 3.78$, $P \simeq 0.06$; Table 2). This was again a response to colour pattern, as *H. melpomene* males from French Guiana were also more likely than Panamanian *H. melpomene* males to court a *H. cydno* model (combined results from the coloured model and real wing experiments; $G_1 = 8.02$, $P < 0.01$; Fig. 3). In addition, *H. melpomene* males from Panama only reluctantly courted live French Guianan *H. melpomene* females, whereas French Guianan *H. melpomene* males showed no discrimination (Fig. 2); indeed all French Guiana × Panama *H. melpomene* matings in these tests involved French Guiana males (Table 1). Among *H. melpomene* races, males showed greater discrimination between live females (Fig. 2 and Table 1) than between models (Fig. 3), indicating that cues other than colour pattern, such as pheromones, may be involved. Hence, *H. melpomene* males sympatric with *H. cydno* discriminated more strongly than *H. melpomene* allopatric to *H. cydno*. This pattern is expected if mate preference has been 'reinforced' to prevent the production of unfit hybrid offspring in sympatry[13,14], although the evidence would be strengthened if replicated with other allopatric and sympatric populations[14,15]. Of course, character displacement between non-hybridizing species cannot be ruled out. For example, the presence of *H. sapho* might

Table 1 Number of matings in tetrad mate choice experiments

Female	Male	Male
Sympatric populations		
	H. melpomene (Panama)	*H. cydno* (Panama)
H. melpomene (Panama)	14	0
H. cydno (Panama)	0	11
Allopatric populations		
	H. melpomene (Panama)	*H. melpomene* (Guiana)
H. melpomene (Panama)	9.5	4
H. melpomene (Guiana)	0	13.5
	H. melpomene (Guiana)	*H. cydno* (Panama)
H. melpomene (Guiana)	14.5	0
H. cydno (Panama)	3	12.5

Mating results of 0.5 are due to two cases of virtually simultaneous mating by both pairs in a tetrad (see Methods).

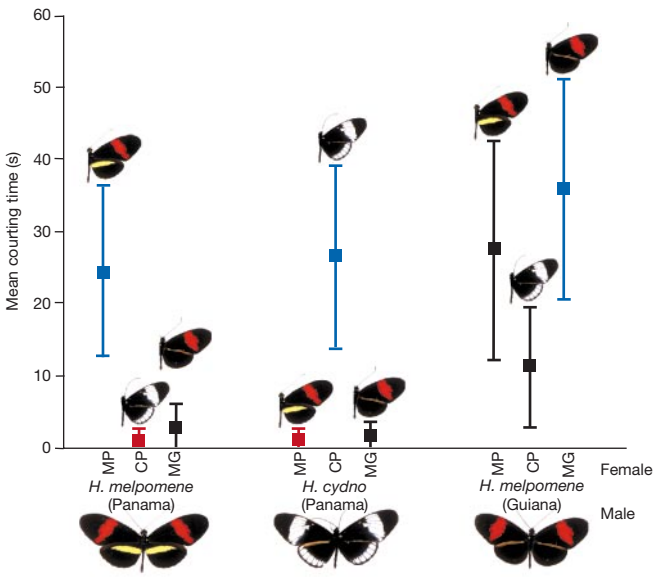

Figure 2 Time spent courting live females in 10-min trials with 95% confidence intervals. Red, comparisons between sympatric populations; black, comparisons between allopatric populations; blue, comparisons between males and females of the same genotype. MP, *H. melpomene* (Panama); CP, *H. cydno* (Panama); MG, *H. melpomene* (Guiana).

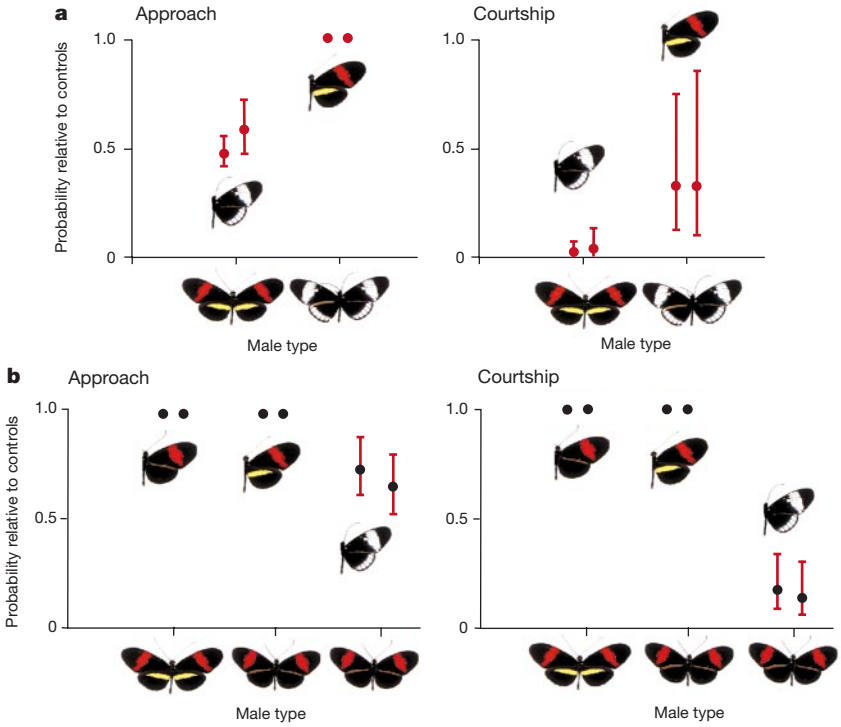

Figure 3 Relative probabilities of male approach and courtship of colour pattern models. Comparisons between Panama populations (sympatry) (**a**) and with the Guiana population (allopatry) (**b**). Values are estimated relative to within-race controls (equal to 1 in each case). Paired data points for experiments using real wings (left) and coloured paper models (right) are shown for each comparison. Values of Q_A (approach) and Q_H (hovering courtship) were estimated with support limits under the ten-parameter model. Setting real wing and paper model parameters equal gives no significant reduction of fit ($G_5 = 3.70$).

also lead to enhanced rejection of the shared *Heliconius sapho*/*H. cydno* pattern by *H. melpomene*.

Here we show that mimetic colour patterns are also important in mate recognition. Assortative mating contributes to speciation because post-mating isolation between *H. melpomene* and *H. cydno* is incomplete[8,9]. As in inter-racial hybrid zones, intermediate colour patterns are unlikely to be recognized as distasteful by predators, generating strong disruptive selection[16]. Selection on mimicry may be strong, $S \approx 0.2$–0.3 per locus in inter-racial hybrid zones giving $S \approx 0.6$ overall[8,16], comparable to that caused by F_1 female sterility ($S \approx 0.5$). Mimicry therefore provides an example of a trait under strong ecological selection that is also used as a mating cue. Such pleiotropy between mate choice and disruptive selection is an important feature of speciation theory, because it can trigger rapid speciation with a high probability[1,17,18], but only a few other examples are known[19–21].

The great diversity of colour pattern races within many *Heliconius* species shows that mimetic shifts rarely lead to speciation. Only where a shift dramatically changes colour or appearance, such as that between *H. melpomene* and *H. cydno*, will mate choice co-evolve sufficiently with mimicry to generate pre-mating isolation.

Table 2 Relative probabilities of mating between genotypes

Female	Male	Male	Male
	H. melpomene (Panama)	*H. cydno* (Panama)	*H. melpomene* (Guiana)
H. melpomene (Panama)	1	0 (0, 0.167)	0.348 (0.099, 0.921)
H. cydno (Panama)	0 (0, 0.167)	1	0.222 (0.051, 0.641)
H. melpomene (Guiana)	0 (0, 0.182)	0 (0, 0.154)	[1]

Probabilities were estimated relative to *H. melpomene* (Guiana) × *H. melpomene* (Guiana), which was set to 1 (square brackets). Support limits are shown in parentheses (values of 1 without support limits are not significantly different from 1).

In addition, *H. melpomene* and its co-mimic *H. erato* occur in light gaps and secondary forest, whereas *H. cydno* and *H. sapho* are found in more primary forest, albeit with considerable overlap[8,22]. This habitat shift, associated with mimicry, will itself contribute further to pre-mating isolation. In conclusion, pre- and post-mating isolation between *H. melpomene* and *H. cydno* has resulted from an adaptive shift in ecology and mimicry, in association with partial hybrid sterility. Subsequently, assortative mating between sympatric populations has become enhanced, possibly owing to reinforcement. This and other recent examples suggest that ecological adaptation can result in assortative mating as a byproduct and may be an important and largely overlooked cause of speciation[19–21]. □

Methods

Heliconius melpomene melpomene were collected near Cayenne, French Guiana in May 1998 (around 35 individuals) and February 1999 (58 individuals). *Heliconius cydno chioneus* and *H. melpomene rosina* were obtained continually from near Gamboa, Panama. Experiments were performed with descendents (three or fewer generations after collection), in insectaries[7] in Gamboa in 1998–1999.

Mate choice experiments

'Tetrad' experiments, consisting of a recently emerged virgin female (1 day old or less) and a mature male (more than 5 days old) of each of two genotypes, were performed in 1 × 1 × 2 m insectaries. The first mating was recorded for each experiment; individuals were not reused. On two occasions, both pairs mated simultaneously and so were scored as each having 0.5 matings. At least 25 experiments were performed per comparison.

Likelihood was used to estimate the probability $P_{i\times j}$ of a mating[7] between female type i and male type j, relative to $P_{mg\times mg}$ of a mating within Guiana *H. melpomene* (MG), which was set to 1. The overall multinomial probability of the results for each experiment were then estimated, e.g. for the Panama *H. melpomene* (MP) × Panama *H. cydno* (CP) comparison, $\Phi_{mp\times mp} = P_{mp\times mp}/(P_{mp\times mp} + P_{mp\times cp} + P_{cp\times mp} + P_{cp\times cp})$, $\Phi_{mp\times cp} = P_{mp\times cp}/(P_{mp\times mp} + P_{mp\times cp} + P_{cp\times mp} + P_{cp\times cp})$ and so on. ($\Sigma\Phi = 1$ for each tetrad). The $\log_e$ likelihood was therefore $\Sigma(X_{mp\times mp} \log_e \Phi_{mp\times mp} + X_{mp\times cp} \log_e \Phi_{mp\times cp}$ and so on) where $X_{mp\times mp}$ is the number of MP × MP matings and $X_{mp\times cp}$ the number of MP × CP matings in that tetrad. Likelihoods were summed over all experiments and maximized by

varying $P_{i\times j}$ values. Setting $P_{i\times i} = 1$ (within all genotypes) did not significantly reduce the fit ($G_2 = 0.81$, not significant), suggesting similar mating propensity among genotypes. Asymmetries ($P_{i\times j} \neq P_{j\times i}$) can therefore be presumed to be due to mate choice rather than mating propensity. Parameters and support limits (asymptotically equivalent to 95% confidence intervals[23]) were estimated under the simpler six-parameter model.

Live female courtship experiments

We placed two or three males (more than 5 days old) of different genotypes in an insectary and introduced a single virgin female (1–5 days old). Courtship (sustained hovering by the male over the female) was recorded over a period of 10 min. The female genotype was then substituted, with genotype order randomized. On mating, pairs were quickly and gently separated, which did not disrupt subsequent behaviour. Males were never reused, but females were drawn randomly from a pool of three to four individuals per genotype. In all, 840 min of observations were made in 19 replicates with all three male genotypes and a further nine with MP and MG males alone.

Colour pattern models

Between five and fifteen males in a 2×2×2 m insectary were presented with dissected natural wings or a colour pattern model, fixed to a length of flexible wire on a lightweight handle. Models were manipulated to simulate *Heliconius* flight in the centre of a spherical area (60 cm diameter) demarcated by a bamboo cross. Randomly ordered pairs of 5-min experiments were carried out: (1) a control flight with a model of the male's own colour pattern and (2) an experimental flight with a different colour pattern. Entry to the sphere was recorded as 'approach' and sustained fluttering directed at the model as 'courtship'. At least ten replicates were carried out per comparison. Each procedure was repeated with real female wings and paper models colour-matched using commercially available permanent marker pens. Reflectance spectra of real and paper models were similar (Supplementary Information), and male behaviour towards wings and models did not differ significantly (see below).

Numbers of approaches (X_A) and hovering courtship interactions (X_H) are given in the Supplementary Information. We estimated the probabilities $Q_{i\times j}$ that males of type j approached or courted models of type i relative to that of their own type j, using likelihood. Thus, for MP males with MP versus CP models, the actual probabilities are $Q_{A\,cp\times mp}/(Q_{A\,cp\times mp}+1)$ that males approach CP and $1/(Q_{A\,cp\times mp}+1)$ that they approach MP. The $\log_e$ likelihood for this experiment is therefore $\Sigma[X_{A\,cp\times mp}\log_e\{Q_{A\,cp\times mp}/(Q_{A\,cp\times mp}+1)\} + X_{A\,mp\times mp}\log_e\{1/(Q_{A\,cp\times mp}+1)\}]$, where $X_{A\,cp\times mp}$ is the number of MP males approaching CP and $X_{A\,mp\times mp}$ is the number approaching MP. Similarly $Q_{H\,i\times j}$ parameters were estimated for probability of hovering courtship of the model. Estimates were obtained for paper models as well as real wings, giving a total of 20 parameters. The summed $\log_e$ likelihood was maximized over all experiments by varying the $Q_{i\times j}$ parameters. Subsequently, all comparisons within *H. melpomene* and $Q_{A\,mp\times cp}$ parameters were set to 1 without loss of fit ($G_{10} = 11.02$, not significant). Parameter values for the resultant ten-parameter model are shown in Fig. 3. Real and paper model parameters do not differ significantly ($G_5 = 3.70$), giving a combined five-parameter model.

Received 14 July 2000; accepted 1 February 2001.

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from the London editorial office of *Nature*.

Acknowledgements

We thank C. Paresce, C. Estrada, R. Woods, M. Beltrán and L. O'Donovan for help with experiments; B. Hermiers for help in French Guiana; F. Jiggins, M. Joron, I. Emelianov, E. Bermingham and G. Hurst for discussion; and S. Upson at Perkin Elmer for reflectance measurements. This work was carried out with permission from ANAM (Autoridad Nacional del Ambiente) to work in the República de Panamá, and was funded by the Natural Environment Research Council and a BBSRC (Biotechnology and Biological Sciences Research Council) studentship.

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Lesions of the human amygdala impair enhanced perception of emotionally salient events

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Commensurate with the importance of rapidly and efficiently evaluating motivationally significant stimuli, humans are probably endowed with distinct faculties[1,2] and maintain specialized neural structures to enhance their detection. Here we consider that a critical function of the human amygdala[3,4] is to enhance the perception of stimuli that have emotional significance. Under conditions of limited attention for normal perceptual awareness—that is, the attentional blink[5,6]—we show that healthy observers demonstrate robust benefits for the perception of verbal stimuli of aversive content compared with stimuli of neutral content. In contrast, a patient with bilateral amygdala damage has no enhanced perception for such aversive stimulus events. Examination of patients with either left or right amygdala resections shows that the enhanced perception of aversive words depends specifically on the left amygdala. All patients comprehend normally the affective meaning of the stimulus events, despite the lack of evidence for enhanced perceptual encoding of these events in patients with left amygdala lesions. Our results reveal a neural substrate for affective influences on perception, indicating that similar neural mechanisms may underlie the affective modulation of both recollective[7–9] and perceptual experience.

The amygdala supports substantial projections to primary and higher-order sensory areas and the hippocampal formation[10]. Thus, the amygdala is strategically placed to allow emotional value[4,11] both to modulate perceptual sensitivity to incoming information and to bolster its post-encoding consolidation into memory. Much evidence has shown that the amygdala is involved in the latter of these

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Sex-linked hybrid sterility in a butterfly

Evolution, 55(8), 2001, pp. 1631–1638

SEX-LINKED HYBRID STERILITY IN A BUTTERFLY

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Abstract.—Recent studies, primarily in *Drosophila*, have greatly advanced our understanding of Haldane's rule, the tendency for hybrid sterility or inviability to affect primarily the heterogametic sex (Haldane 1922). Although dominance theory (Turelli and Orr 1995) has been proposed as a general explanation of Haldane's rule, this remains to be tested in female-heterogametic taxa, such as the Lepidoptera. Here we describe a novel example of Haldane's rule in *Heliconius melpomene* (Lepidoptera; Nymphalidae). Female F_1 offspring are sterile when a male from French Guiana is crossed to a female from Panama, but fertile in the reciprocal cross. Male F_1s are fertile in both directions. Similar female F_1 sterility occurs in crosses between French Guiana and eastern Colombian populations. Backcrosses and linkage analysis show that sterility results from an interaction between gene(s) on the Z chromosome of the Guiana race with autosomal factors in the Panama genome. Large X (or Z) effects are commonly observed in *Drosophila*, but to our knowledge have not been previously demonstrated for hybrid sterility in Lepidoptera. Differences in the abundance of male versus female or Z-linked versus autosomal sterility factors cannot be ruled out in our crosses as causes of Haldane's rule. Nonetheless, the demonstration that recessive Z-linked loci cause hybrid sterility in a female heterogametic species supports the contention that dominance theory provides a general explanation of Haldane's rule (Turelli and Orr 2000).

Key words.—Haldane's rule, *Heliconius*, hybrid sterility, Lepidoptera, speciation.

Received September 22, 2000. Accepted April 17, 2001.

''Future experiments—especially those dissecting the genetics of speciation in taxa with heterogametic females—will reveal if the explanation of Haldane's rule championed here fares any better than its many predecessors'' (Orr 1997, p. 215).

Recent years have seen great advances in our understanding of one particular aspect of speciation, namely the genetic basis of hybrid sterility and inviability. Most studies have concentrated on the search for an explanation of two rules of speciation (Coyne and Orr 1989): The tendency for the heterogametic sex to be preferentially affected by hybrid sterility or inviability (Haldane's rule) and the large effect of the X chromosome on incompatibility (the large X effect). After a number of years of widespread disagreement, it has recently been suggested that Muller's original explanation for Haldane's rule, known as dominance theory, largely explains both patterns (Muller 1940; Orr 1997). This posits that epistatic loss-of-function alleles causing hybrid breakdown will tend to be recessive, so that the hemizygous sex is afflicted by the expression of X-linked incompatibility genes to a far greater extent than the homogametic sex.

Over the years there has been a proliferation of proposed explanations for Haldane's rule, invoking such diverse causes as the disruption of dosage compensation, meiotic drive, cytoplasmic endosymbionts, and chromosomal rearrangements (Coyne and Orr 1989). Much of the controversy arose because Muller's original hypothesis was apparently contradicted by experiments showing that attached-X females, homozygous for two identical X chromosomes, are fertile in some *Drosophila* crosses in which males are sterile. If sterility results from recessive genes, it was thought that such homozygous females should be sterile (Coyne 1985; Coyne and Orr 1989). Subsequently, however, it was realized that dominance theory could be consistent with this result. If the loci causing male and female sterility are not the same, we do not necessarily expect that an X chromosome carrying male sterility loci will also contain female sterility loci (Turelli and Orr 1995; Orr 1997). There is now convincing evidence from *Drosophila* that loci causing hybrid inviability act in both sexes, whereas loci causing sterility are sex-specific (Hollocher and Wu 1996; True et al. 1996). In addition to dominance theory, there may be other effects that exaggerate Haldane's rule, notably faster-male evolution and possibly also faster-X evolution (Charlesworth et al. 1987; Wu et al. 1996). However, dominance theory appears to provide the only generally applicable explanation of Haldane's rule for both sterility and inviability in all taxa with sex chromosomes (Muller 1940; Turelli and Orr 1995, 2000).

The genetic data that led to this consensus have come almost exclusively from studies of *Drosophila* and other Diptera (Dobzhansky 1951; Coyne and Orr 1989; Orr 1997; Presgraves and Orr 1998). Crosses in female-heterogametic taxa such as birds and butterflies follow Haldane's rule (Coyne 1992; Laurie 1997), but virtually nothing is known about the genetic basis of hybrid dysfunction in such taxa. There is thus a need for studies examining the genetic architecture of hybrid sterility in birds and butterflies. Such studies will test whether the patterns observed in *Drosophila* are indeed generally applicable, as predicted by dominance theory. Here we describe and perform genetic analyses on a previously unknown, asymmetrical Haldane's rule effect between races of *Heliconius melpomene* (Linnaeus).

MATERIALS AND METHODS

Heliconius melpomene melpomene individuals were collected near Cayenne, French Guiana, in May 1998 and Feb-

ruary 1999 (Pointe Macouria, 4°54.8′N, 52°21.6′W; Sablance, 4°57.8′N, 52°25.2′W, elevation at sea level for both sites) and from eastern Colombia between April 1998 and January 1999 (Virgen de Chirajara, 4°12.8′N, 73°47.9′W, elevation 1150–1450 m; Pajarito, 5°17.5′N, 72°42.5′W, elevation 940 m). *Heliconius melpomene rosina* individuals were collected from Gamboa (9°7.4′N, 79°42.2′W, elevation 60 m) and the nearby Parque Nacional Soberanía, República de Panamá, during the course of the experiments.

Crosses were carried out between these races of *H. melpomene* in insectaries sited in Gamboa, Panama (August 1998–October 1999), and La Vega, Colombia (May 1998–January 2000). Brood females were kept in individual cages at least 1 × 1 × 2 m with access to ample *Psiguria* flowers, occasionally supplemented by hand-feeding with commercially available bee pollen. In Panama, larvae were reared individually from egg to third instar to prevent cannibalism, and subsequently in groups of two to five individuals from the same brood, fed on shoots of *Passiflora biflora.* Eggs were collected daily and placed individually in plastic containers with damp cotton to maintain moisture. These were inspected daily and hatch rates recorded. In Colombia, larvae were reared on *P. edulis* and *P. oerstedii* plants in the insectaries. All eggs present were collected from each female every 15 days and their hatch rates recorded. In all cases, females were provided with healthy *Passiflora* plants on which to lay, either *P. menispermifolia,* the primary host of *H. melpomene* around Gamboa, or *P. edulis* (maracuyá). Control females were kept alongside crosses and reared under identical conditions. In text and tables, the genotypes of *H. melpomene* from French Guiana, Panama, and Colombia are abbreviated as MG, MP, and MC, respectively, and all crosses are given with the female genotype first. Eggs laid by several females representing each control genotype and the sterile F_1 class were measured under a binocular microscope using a 5 mm miniscale. Sample sizes for egg measurements are MP, $N = 10$; MG, $N = 15$; and F_1, $N = 28$.

Hatch rate data were analyzed using likelihood-ratio tests based on the beta-binomial distribution. Our problem is to compare survival between broods of different types (e.g., pure vs. hybrid). Traditionally, one might use analysis of variance with percent survival as the variate. However, this method is inefficient, and variation in brood size can cause heteroscedasticity. An alternative might be to assume a binomial distribution within broods of the same type. However, this method ignores real differences in survival between replicate broods, which may be due to genetic or environmental variation. Here we assume that the count within each brood has a simple binomial distribution, while the binomial probability p varies according to a beta distribution among replicate broods. This leads to a beta-binomial distribution of the counts overall (Johnson et al. 1993). The beta distribution is particularly relevant in this context because it can approximate unimodal (e.g., normal or binomial) distributions or U-shaped, L-shaped, or reverse-L-shaped distributions, any of which might be expected in extreme cases of variation within brood classes (e.g., in backcrosses involving the segregation of sterility, as here). The beta-binomial is thus useful for a very general class of problems in which variable count data is used to compare fractional parameters between data classes. We analyze our data using the program BETABINO written by Z. H. Yang. BETABINO is described more fully in the Appendix and is available via ftp://abacus.gene.ucl.ac.uk/pub/ or on request from C. D. Jiggins.

Genetic Analysis

Primers for an approximately 470-bp fragment of the triose phosphate isomerase (*Tpi*) gene were developed by W. O. McMillan and D. Heckel for *Heliconius erato* from sequences of the noctuid moth *Heliothis* (Logsden et al. 1995). This locus is sex linked in *Heliconius* as in many other Lepidoptera (Turner et al. 1979). Primer sequences, sited in coding regions, are 5′-GGTCACTCTGAAAGGAGAACCATCTT-3′ (forward) and 5′-CACAACATTTGCCCAGTTGTTGCCAA-3′ (reverse), which amplify a highly variable intron of approximately 470 bp in the *Tpi* gene. Using MacVector (Eastman Kodak, Rochester, NY), sequences from individuals collected in Guiana and Panama (M. Beltrán, unpubl. ms.) were searched for diagnostic restriction enzyme sites. One site approximately in the center of the sequence was found; it differs between the two races by a single base-pair substitution and corresponds to the restriction site of the enzyme Dde1 (5′ to 3′ CTNAG). Total genomic DNA was extracted from frozen tissue (one-third of a thorax) homogenized in 500 μl CTAB buffer, and digested overnight at 55°C with 0.1 mg proteinase K. After three extractions (twice with phenol:chloroform: isoamyl alcohol and once with chloroform : isoamyl alcohol), the DNA was precipitated with ethanol (Sambrook et al. 1989). The *Tpi* fragment was amplified using a polymerase chain reaction protocol of 94°C for 7 min, followed by 10 cycles of 94°C for 45 sec, 58°C for 45 sec falling by 0.5°C per cycle, and 72°C for 105 sec and finally 25 cycles of 94°C for 45 sec, 53°C for 45 sec, and 72°C for 105 sec. Amplified DNA was digested with Dde1 (0.05 units/μl) for 4 h at 37°C. Digestion products were separated by electrophoresis on 1.5% agarose gel and stained with ethidium bromide. Sex linkage of the locus was confirmed by restriction analysis of 12 individuals from F_1 brood 128: All male offspring were heterozygotes, and all females appeared to be homozygotes (i.e., were actual hemizygotes) of the paternal allele (six males : six females, $G_1 = 16.6$, $P < 0.001$). Backcross broods were then analyzed to investigate possible linkage with sterility phenotypes.

RESULTS

Panamanian Crosses

Female hybrids between *H. melpomene* from French Guiana (MG) and Panama (MP) show asymmetrical sterility. The female offspring of a cross between a female from Panama and a male from Guiana (MP × MG) lay eggs that are significantly smaller than normal (egg sizes with standard errors are: MG, 1.3 ± 0.02 mm × 0.8 ± 0.01 mm; MP, 1.3 ± 0.01 mm × 0.9 ± 0.02 mm; MP × MG, 0.9 ± 0.02 mm × 0.5 ± 0.01 mm), and never hatch (250 sterile eggs laid from 19 broods). In contrast, the reciprocal cross (MG × MP) produces female offspring that lay fertile eggs with a hatch rate of 0.88 ± 0.02 when mated to MG and 0.90 ± 0.04 when mated to MP (Table 1). This compares with control hatch

TABLE 1. Hatch rates in control, F_1, and backcross broods. MP are *Heliconius melpomene* from Panama, MG are from French Guiana, and MC are from Colombia. Cross types are given with the female genotype first. Values shown are derived from a beta-binomial model fitted to the data over all broods, excluding females that laid no eggs and brood classes that were completely sterile. Broods with high variance and intermediate mean are primarily backcross classes that show segregation of sterility between individuals (see Results). In some cases, broods sharing one parental genotype have been combined in the analysis because hatch rate did not differ significantly between crosses (shown as MG/MP or ?). Note that where the number of broods in a crosstype is low, exact parameter estimates may vary slightly between runs of the BETABINO program.

Female genotype	Male genotype	No. broods	No. eggs	Mean hatch rate	SE	Variance	SE
Panama crosses							
MG	MG	18	863	0.90	0.03	0.012	0.007
MP	MP	14	577	0.93	0.02	0.003	0.002
MG	MP	6	180	0.94	0.02	0.001	0.001
MP	MG	8	265	0.85	0.06	0.020	0.018
MG/MP	MG × MP	7	275	0.71	0.14	0.120	0.056
MG/MP	MP × MG	4	347	0.95	0.01	0.000	0
MG × MP	MG	3	257	0.88	0.02	0.000	0
MG × MP	MP	3	501	0.90	0.04	0.004	0.005
MP × MG	MG	13	144	0	—	—	—
MP × MG	MP	6	106	0	—	—	—
(MG × MP) × MG	MG	18	383	0.45	0.08	0.115	0.023
(MG × MP) × MG	MP	5	52	0.10	0.04	0.000	0
(MG × MP) × MP	MG	7	205	0.94	0.02	0.001	0.002
MP × (MG × MP)	MG/MP	10	225	0.49	0.14	0.206	0.023
MP × (MP × MG)	MP	6	174	0.57	0.16	0.164	0.041
Colombia crosses							
MC	MC	7	285	0.55	0.09	0.051	0.023
MG	MG	3	159	0.60	0.10	0.023	0.021
MC	MG	2	81	0.77	0.20	0.080	0.081
MG	MC	1	41	0.15	0.05	0.000	0
MC × MG	?	2	31	0.36	0.08	0.000	0
MC × MG	?	8	0	—	—	—	—
MG × MC	MC	6	255	0.65	0.11	0.050	0.031
MC	MC × MG	3	115	0.53	0.15	0.068	0.040
MC × (MC × MG)	?	6	140	0.57	0.09	0.017	0.024

rates of 0.90 ± 0.03 for MG and 0.93 ± 0.02 for MP (Table 1).

In *Heliconius* the ovarioles develop fully only after emergence from the pupa. Females were therefore dissected to investigate the morphology of sterile phenotypes once they had begun laying or after several days of access to pollen (Dunlap-Pianka et al. 1977). Sterile F_1 females showed no obvious abnormalities in ovarian development, except that eggs entering the oviduct were considerably smaller than in normal females (C. D. Jiggins, pers. obs.). Unfortunately, this meant that sterility was not reliably identifiable in dissections of backcross females and had to be estimated directly using hatch rates. However, the abnormal egg size observed prior to fertilization demonstrates that failure to hatch is due to F_1 female sterility rather than gametic incompatibility or zygote inviability.

Male F_1 hybrids are fertile (Table 1). One F_1 male genotype (MP × MG), when backcrossed to parental females, has a high hatch rate of 0.95 ± 0.01, which is similar to controls. The reciprocal F_1 male (MG × MP) has a reduced hatch rate of 0.71 ± 0.14. Combining these two male F_1 classes results in a significant reduction of fit of the model ($G_2 = 8.4$, $P < 0.02$), implying a difference in male fertility between F_1 classes. However, this results from a drastic reduction of hatch rate in just two of seven broods (brood 199, 16/39 hatched; brood 277, 0/16 hatched). If these two broods are excluded, the 220 eggs from the remaining five broods gave a normal hatch rate of 0.95 ± 0.02. It is therefore unclear whether this reduction in hatch rate is due to partial male sterility or other environmental or genetic variation.

Thus, female F_1 hybrids are completely sterile in only one direction of cross, implying sex-linked or cytoplasmic incompatibility. Maternal effects might also cause asymmetry, but generally affect early life stages and are therefore not considered likely to cause sterility (Turelli and Orr 2000). More specifically in this case, the sterile females have cytoplasm and a W chromosome from MP, but a Z chromosome from MG. Backcrosses can be used to differentiate between cytoplasmic (or W-chromosome) and Z-chromosome effects. When the fertile F_1 females (MG × MP) are backcrossed to MG males, their female offspring possess the MG cytoplasm ultimately from an MG mother, an MG Z chromosome from their father, and on average 75% MG autosomes. Because sterility in the F_1 generation is associated with the MP cytoplasm, these females should be fertile if sterility results from cytoplasmic effects. In fact, this cross results in segregation of sterility phenotypes, ranging from fully fertile to fully sterile females with a number of intermediate partially sterile phenotypes (Fig. 1a). The expression of sterility in this cross implies that interactions between the MG Z chromosome and MP autosomal genes, and not cytoplasmic or W-chromosome factors, are the primary cause of the incompatibility.

The fertile female F_1 (MG × MP) was also backcrossed in the other direction, to MP males, whereby all female offspring inherit the MP Z chromosome. The offspring of this

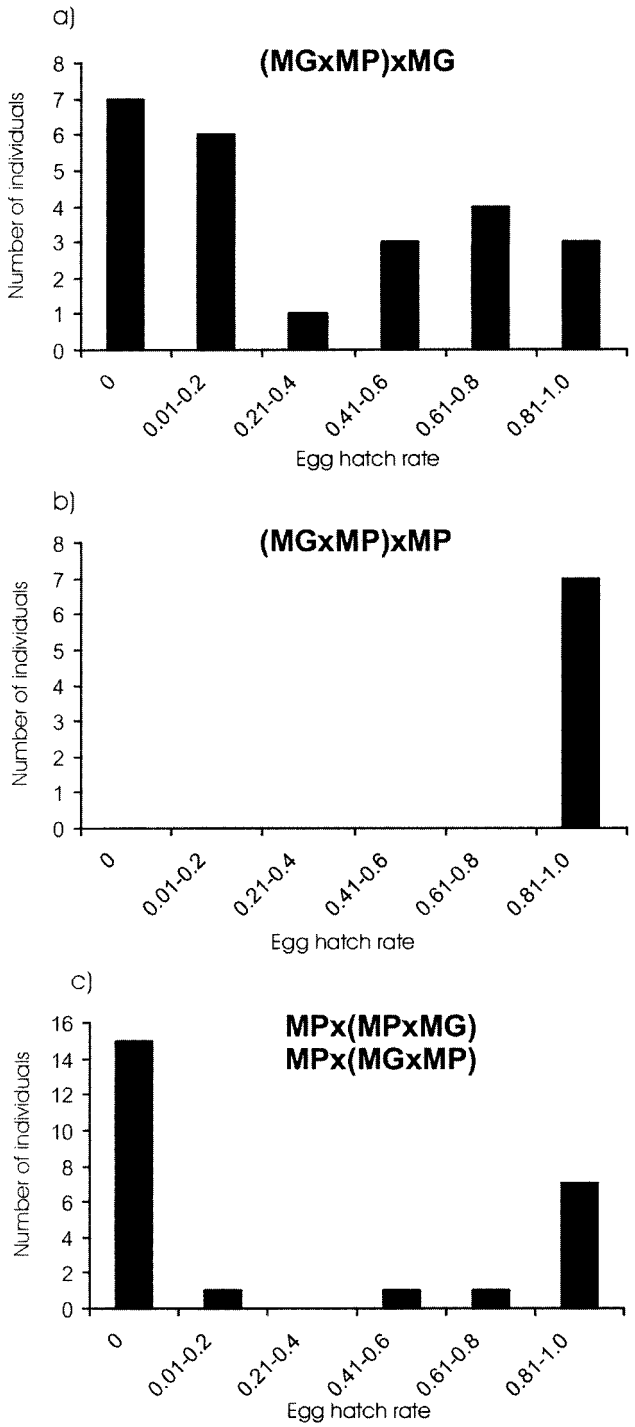

FIG. 1. Segregation of sterility phenotypes in backcross broods. The distribution of hatch rates for the female offspring of each cross type are shown. Females shown either survived ≥ 20 days after mating and laid no eggs or laid at least five eggs (although the majority laid far more than this). Female genotypes are shown first. MG is *Heliconius melpomene* from Guiana and MP is *H. melpomene* from Panama.

cross were all fully fertile (Fig. 1b), in spite of the presence of MG cytoplasm and W chromosomes on MP chromosomal backgrounds. The lack of sterility in these females is expected if MG W chromosomes and cytoplasm are not causes of the sterility phenotype.

To confirm the role of the Z chromosome, male F_1s were backcrossed to MP females, producing offspring in which segregation of MG and MP Z chromosomes occurs on a largely Panama genetic background. There was striking bimodality in the incidence of sterility in this cross, with a ratio of 15: 1 : 9 sterile : partially sterile : fertile females (combining crosses involving both F_1 male genotypes; see Fig. 1c). Sterile females were either kept for at least 20 days after mating and laid no eggs or laid at least five eggs that failed to hatch. Partially sterile and fertile females laid at least five eggs and showed a hatch rate of < 0.2 and > 0.5, respectively.

Eight additional females from these crosses survived between 10 and 20 days but never laid any eggs. Fertile females almost invariably began egg laying within five to 10 days of mating, but very occasionally only began to lay after 15 to 20 days (pers. obs.). Thus, the females that survived more than 10 days are most likely to have been sterile but cannot with certainty be assigned to any class. The above ratio of sterile : fertile females may therefore underestimate the number of steriles.

The segregation of sterility phenotypes in these backcrosses showed a highly significant association with *Tpi*, a Z-linked protein-coding gene. Using a restriction digest diagnostic for the MG allele, segregation of this marker could be followed in backcross broods. Of 16 individuals genotyped, all seven fertile phenotypes had the MP genotype at *Tpi*, whereas of nine sterile phenotypes eight had an MG and one an MP genotype (test of heterogeneity: $G_1 = 15.9$, $P \ll 0.001$).

Colombian Crosses

Crosses between *H. m. melpomene* from Colombia and French Guiana show a hybrid sterility effect similar to that observed between Panama and French Guiana (Table 1). When a male from French Guiana and a female from Colombia are crossed, the female F_1 offspring are largely sterile. The reciprocal cross produces fertile female offspring and all F_1 males are fertile, following Haldane's rule (Table 1). In contrast to the Panamanian crosses, most sterile F_1 females never laid eggs. However, Panama × Guiana sterile females were also reluctant to lay eggs unless maintained with abundant pollen supplies, so it seems likely that the sterility phenotype in Colombian crosses was similar to that of the Panamanian crosses. The egg hatch rate is also considerably lower, averaging only 0.55 ± 0.092 and 0.60 ± 0.10 in MC × MC and MG × MG pure broods, respectively, presumably for environmental reasons. Two of 10 F_1 females that were expected to show sterility laid some fertile eggs (females 861 and 9 laid a total of 31 eggs, 11 of which hatched), implying reduced incompatibility in Colombia × Guiana hybrids as compared to Panama × Guiana. Hatch rates of seven backcross females, offspring of an F_1 male backcrossed to a Colombia *melpomene* female, were also measured. These crosses gave a ratio of 6:0 fertile/partially sterile : sterile (excluding

one sterile female that lived only for nine days), differing significantly from the ratio of 10:15 shown in the equivalent Panama × Guiana cross; $G_1 = 9.3$, $P < 0.01$). Additional crosses will be needed to study how the genetic architecture of sterility in Colombia × Guiana crosses differs from that shown for Panama × Guiana.

Discussion

The genetic basis of hybrid incompatibility is one of the best understood aspects of speciation. In particular, there is now a general consensus over the cause of Haldane's rule—the tendency for hybrid breakdown to affect preferentially the heterogametic sex (Orr 1997; but see Wu and Davis 1993). Nonetheless, there have been virtually no studies dissecting hybrid incompatibility in female-heterogametic species. Here we have identified a novel example of hybrid sterility in a butterfly, *H. melpomene,* which follows Haldane's rule (Haldane 1922). Including this example with the results of a recent literature survey (Laurie 1997) makes a total of 44 known examples of sex-specific hybrid sterility in birds and Lepidoptera, 14 of which are in Lepidoptera. Of these 44 cases, only one does not follow Haldane's rule. Therefore hybrid sterility overwhelmingly follows the rule in these taxa.

Sterility Involves a Large Z Effect

Sterility of *H. melpomene* backcross hybrids is strongly associated with the Z-linked *Tpi* gene. This provides the best evidence yet for a large Z (or X) effect on hybrid sterility in female-heterogametic taxa.

Two previous studies have demonstrated Z effects on incompatibility in Lepidoptera. Crosses between two species of *Colias* butterflies show asymmetric female sterility similar to that described here (Grula and Taylor 1980). In this case the segregation of Z chromosomes in backcrosses could be followed using a sex-linked color-pattern trait, showing that the Z chromosome was associated both with reduced male mating vigor and with inviability. Unfortunately, sample sizes were too small to provide much information on the inheritance of female sterility, although both autosomal and Z-linked loci are likely involved. Similarly, two races of *Papilio glaucus* differ at a number of Z-linked loci including allozyme, color pattern, and life-history characters (Hagen and Scriber 1989). These traits showed significant deviations from Mendelian ratios in backcrosses, associated with reduced female pupal survival. Deficits of certain genotypes are most readily explained by a reduction in hybrid viability due to introgression of the foreign Z chromosome. Thus, in the handful of studies where backcross analysis has been carried out, a large Z effect seems to be the rule in Lepidoptera as it is in *Drosophila* (Coyne and Orr 1989).

Number of Loci Involved in Sterility

In common with a number of cases of hybrid incompatibility, the effect demonstrated here is asymmetric (Coyne and Orr 1997). Thus, a Guiana male crossed with a Panama female produce sterile hybrid females, but in the reciprocal cross the F_1 females are fertile. Muller (1942) argued that the common occurrence of asymmetry implies that relatively few genes are involved in causing incompatibility. If many loci were involved, there should be greater similarity in the number of loci that accumulate in the two diverging taxa. However, depending on the form of the interactions, asymmetry could theoretically occur even if up to 100 loci were involved in causing incompatibilities (Turelli and Orr 1995; M. Turelli, pers. comm.). Thus, although asymmetry is more probable when a few major loci are involved, asymmetric hybridizations such as ours provide only weak evidence that this is the case.

A better way to determine the number of loci involved would be by linkage analysis, and the close association of sterility with the *Tpi* gene would seem to imply that this region of the Z chromosome has a strong effect on sterility. However, Maside and Naveira (1996a,b) have cautioned against inferring single genes with major effects on sterility using linkage analysis. In crosses between *Drosophila buzzatii* and *D. koepferae* sterility results if greater than 40% of any autosome is introgressed (Naveira and Maside 1998). Introgressions of less than 30% result in fertile males. Thus, many interacting loci cause sterility and any one locus alone has little or no effect. Similar results are seen in analysis of X-linked factors such as the Odysseus gene, a sterility factor of major effect detected in crosses between *D. simulans* and *D. mauritiana* (Perez et al. 1993). Subsequent analysis has shown that if smaller fragments of the Odysseus region are introgressed, no one fragment alone can cause sterility. Again, many interacting genes are clearly involved (Wu and Hollocher 1998).

In *H. melpomene,* the backcross showing segregation of Z chromosomes has a 1:1 ratio of sterile : fertile females (16: 9 when sterile and partially sterile females are combined; $G_1 = 1.99$, n.s.; see Fig. 1c), compatible with a single-gene hypothesis. This is a weak test, however, and sterility might still be polygenic, with a threshold fraction of the Guiana Z chromosome required for sterility. In first generation backcrosses an association of sterility with *Tpi* would be expected if the marker were situated near the center of the Z chromosome, such that the Guiana *Tpi* allele was commonly associated with a predominantly Guiana Z chromosome. In both *Heliconius erato* and *Helicoverpa armigera,* the *Tpi* gene is at one end of the Z-chromosome linkage group (D. Heckel, pers. comm.; N. Flanagan, pers. comm.). If this is also the case in *H. melpomene,* then the association of sterility with *Tpi* suggests that sterility factor(s) are localized toward one end of the chromosome. However, more Z-linked markers are needed to confirm this.

The backcross of the female F_1 to Guiana provides some information on the main autosomal factors in the MP genome that interact with the MG Z chromosome (Fig. 1a). The situation is simplified as there is no recombination in females (Turner and Sheppard 1975), so in this cross introgressed chromosomes are inherited intact. All females therefore possess a complete MG Z chromosome and the segregation of phenotypes depends on which MP chromosomes are inherited. The observed variation from sterile to fertile phenotypes implies that many autosomes are involved, several of which are needed for complete sterility.

Evidence in Support of the Dominance Theory

The large Z (or X) effect demonstrated here suggests that sterility factors are recessive as predicted by dominance theory. Similarly, in *Drosophila* the introgression of hemizygous X-linked fragments often leads to far greater hybrid dysfunction than introgression of similar-sized heterozygous fragments on the autosomes. Because many introgressed autosomal regions also cause male sterility when homozygous, this implies that the large X effect is due mainly to the recessiveness of sterility genes, rather than a greater preponderance of loci on the X chromosome (Hollocher and Wu 1996; True et al. 1996). The large X effect therefore provides support for dominance theory (Turelli and Orr 2000) and is not just an artifact of backcross analysis (Wu and Davis 1993; Wu et al. 1996).

Some Panama × Guiana backcross females have a more extreme sterility phenotype than is ever expressed in F_1 crosses. Sterile F_1 females invariably laid small eggs that never hatched. In contrast, some backcross female genotypes never laid eggs, despite living for more than 50 days in some cases. One likely explanation is that more incompatible genetic interactions are expressed in these individuals than in the F_1s. Interactions between two or more incompatible homozygous (or hemizygous) loci (H_2 interactions in the terminology of Turelli and Orr 2000), occur in backcross but not F_1 genotypes. In this case, increased sterility in a backcross might result from interactions between the hemizygous Guiana Z chromosome and homozygous Panamanian autosomal loci. This would imply that the Panama genome contains autosomal recessive alleles not expressed in the F_1 that contribute to sterility. This provides a further line of evidence supporting the recessive nature of sterility factors.

Lepidoptera and Alternative Causes of Haldane's Rule

In addition to dominance, two further effects are considered likely to contribute to Haldane's rule (Orr 1997), and studies of female heterogametic taxa such as the Lepidoptera may hold the key to understanding their relative importance. First, genes for male sterility appear to diverge faster than those that affect females (Wu and Davis 1993), perhaps due to rapid evolution of the male reproductive system driven by sexual selection (Chapman et al. 1995). Indeed, introgression experiments have shown many more male than female sterility factors in *Drosophila* crosses (Hollocher and Wu 1996; True et al. 1996), which likely explains the frequent occurrence of male sterility in *Drosophila* (Wu et al. 1996). However, faster-male evolution should cause anti-Haldane effects in female-heterogametic taxa such as butterflies and would therefore seem to be contradicted by the observation that Haldane's rule for sterility is overwhelmingly obeyed in Lepidoptera and birds (Laurie 1997). This is most likely because male sterility factors are not expressed in homogametic F_1 males if they are recessive, and faster-male evolution is therefore masked by dominance in female-heterogametic taxa (Turelli 1998). To confirm this, future studies need to investigate whether there is a similar preponderance of male versus female sterility factors in Lepidoptera as has been observed in *Drosophila*.

If the X chromosome were to evolve faster than the autosomes, this could also contribute to Haldane's rule (Charlesworth et al. 1987). Faster evolution of the X chromosome might result from fixation of favorable recessive alleles, although such beneficial mutations must be extremely recessive for this effect to be strong (Charlesworth et al. 1987). Alternatively, mutation rates likely vary between sex chromosomes and autosomes, because males have higher per-generation mutation rates than females (Miyata et al. 1987). In male-heterogametic taxa, X chromosomes spend 75% of their time in females, and are thus expected to show lower mutation rates than autosomes, but in female-heterogametic taxa this situation is reversed. In Lepidoptera, therefore, either of these effects might cause faster divergence of X (or Z) chromosomes, which could exaggerate Haldane's rule (Orr 1997). There is tentative evidence that lepidopteran Z chromosomes do diverge faster than the autosomes, as many species-diagnostic traits are Z linked, including 24% of differentiated allozyme loci across 11 species comparisons (Sperling 1994; Prowell 1998). However, in Lepidoptera, which generally have chromosome numbers around 30, between 10% and 25% of all polymorphic allozyme loci are Z linked (Mallet et al. 1993; Raijmann et al. 1997; D. Heckel, pers. comm.). This might mean that sex-linked loci are both more polymorphic and diverge faster, but could reflect a sampling bias in the enzyme loci studied. In conclusion, it seems plausible but by no means proven that faster-X evolution contributes to Haldane's rule. Direct estimates of the relative abundance of Z-linked versus autosomal sterility factors are needed in Lepidoptera.

Genetic Compatibility Is Not Concordant with Color Pattern

Species of *Heliconius* consist of multiple geographic populations, typically separated by incompatibility in mimetic pattern, rather than hybrid fertility (Sheppard et al. 1985; Mallet and Barton 1989). In the case of mimicry, the adaptive peaks can be clearly identified as color patterns that are adapted to local mimicry rings. In contrast, we know nothing about the adaptive value or otherwise of the genes causing hybrid sterility. Nonetheless the effects are analogous, in that both represent divergent adaptive peaks separated by troughs of reduced hybrid fitness. Interestingly, hybrid sterility is not concordant with color pattern: Crosses between Colombian and French Guianan populations of the race *H. m. melpomene* produce sterile hybrids (Table 1). All previous races of *H. melpomene* that have been crossed are genetically compatible. Sheppard et al. (1985) crossed *H. melpomene* from Belem, to the east of French Guiana, with populations from Venezuela, eastern Brazil, Trinidad, central Amazonia, and eastern Ecuador and found no evidence for hybrid breakdown. Similarly, crosses of Costa Rican females with a male from eastern Colombia and another from Trinidad both produced fertile female offspring (J. Mallet and L. E. Gilbert, unpubl. data). These latter crosses in particular might have been expected to show sterility if the incompatible Z chromosome were more widespread. Notably, *H. m. melpomene* from Trinidad are similar in appearance to *H. m. melpomene* from French Guiana, but show no incompatibility with either Be-

lem or Costa Rica. Thus, the distribution of hybrid sterility does not obviously correspond to color pattern boundaries.

Hybrid compatibility seems more concordant with the geographic structure of mitochondrial DNA haplotypes. Apart from divergence across the Andes, most adjacent color-pattern races are little differentiated at mitochondrial DNA (Brower 1996) or allozymes (Turner et al. 1979; Jiggins et al. 1997). In contrast, the French Guiana populations form the most divergent mitochondrial lineage within *H. melpomene* (Brower 1996). Hybrid female sterility, even in only one direction of cross, will strongly impede mitochondrial gene flow (Sperling 1994). It is perhaps unsurprising, therefore, that the incompatible Z-chromosome distribution corresponds with mitochondrial DNA rather than color-pattern differentiation.

Implications for Speciation

To demonstrate that any process contributes to speciation, it must be shown to play a role in reproductive isolation between species that still hybridize and exchange genes. At least one *Heliconius* species pair, *H. himera* and *H. erato,* show no hybrid incompatibility, demonstrating that hybrid sterility is not essential for speciation (Jiggins et al. 1996; McMillan et al. 1997). Nonetheless, *H. melpomene* and its sister species, *H. cydno,* hybridize occasionally in the wild to produce sterile F_1 female hybrids (Linares 1989; Mallet et al. 1998; Gilbert 2000; R. E. Naisbit, C. D. Jiggins, M. Linares, C. Salazar, and J. Mallet, unpubl. ms.). Male hybrids are fertile, and there is evidence of recent gene flow (V. Bull, unpubl. ms.). Thus, hybrid sterility must play a role as a current barrier to gene flow in this case. However premating isolation is probably more important: There is strong assortative mating between *H. melpomene* and *H. cydno,* using color pattern as a mating cue (Jiggins et al. 2001), and habitat segregation of mimicry rings leading to ecological isolation (Smiley 1978; Srygley and Ellington 1999). As in hybrid zones, premating isolation is likely to be more effective than hybrid incompatibility in maintaining species differences despite gene flow (Jiggins and Mallet 2000). Thus, in *Heliconius* butterflies, ecology and mimicry likely play the key roles in speciation (Jiggins et al. 2001), such that studies of genomic incompatibility alone cannot be considered sufficient to understand the process.

Acknowledgments

We thank M. Turelli for comments on the manuscript and D. Heckel for valuable discussion; M. Beltrán, C. Paresce, C. Estrada, L. O'Donovan, and R. Coe, who reared butterflies in Panama; G. Fajardo, who reared butterflies in Colombia; B. Hermiers for help in French Guiana; and W. O. McMillan, D. Heckel, and L. Gahan for use of the *Tpi* primers. This work was carried out in Panama at the Smithsonian Tropical Research Institute with permission from the Autoridad Nacional del Ambiente and funded by the Natural Environment Research Council, and in Colombia by grants from Colciencias and Banco de la República to ML (Grants CO-1204-05-510-93 and 734 respectively).

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Corresponding Editor: C. López-Fanjul

Appendix

The BETABINO program, written by Z. H. Yang, fits beta-binomial models to data of count (available via ftp://abacus.gene.ucl.ac.uk/pub/). We want to compare the probability of an event (e.g., success of hatching) across different classes (e.g., mating types). In each class, multiple families are tested, serving as multiple replicates. The problem with using a binomial model is that the success rate varies among families in each class. In a beta-binomial model, the probability of observing k_{ij} successes in n_{ij} trials in the jth family of the ith class is given by the binomial distribution Bino(k_{ij}; n_{ij}, p_{ij}):

$$\text{Prob}(k_{ij};\, n_{ij},\, p_{ij}) = \binom{n_{ij}}{k_{ij}} p_{ij}^{k_{ij}} (1 - p_{ij})^{k_{ij}},$$

$$0 \le k_{ij} \le n_{ij}. \qquad \text{(A1)}$$

However, the probability parameters p_{ij} (i.e., success rates) vary according to a beta distribution Beta(α_i, β_i) with shape parameter α_i and scale parameter β_i. The density of the beta distribution is

$$f(p;\, \alpha,\, \beta) = p^{\alpha-1}(1 - p)^{\beta-1}/B(\alpha,\, \beta), \qquad 0 \le p \le 1, \qquad \text{(A2)}$$

where $B(\alpha, \beta)$ is the beta function. The counts of successes among families from the same class then follow a beta-binomial distribution, also known as the negative-hypergeometric distribution (Johnson et al. 1993, pp. 242, 264–266):

$$\text{Prob}(k_{ij};\, n_{ij},\, \alpha_i,\, \beta_i) = \binom{-\alpha_i}{k_{ij}} \binom{-\beta_i}{n_{ij} - k_{ij}} \Big/ \binom{-\alpha_i - \beta_i}{n_{ij}},$$

$$0 \le k_{ij} \le n_{ij}. \qquad \text{(A3)}$$

The likelihood is calculated as the product of probabilities of the counts over families. To facilitate comparison among different classes, we use the mean of the p_{ij} values, $m = \alpha/(\alpha + \beta)$, and their variance, $v = \alpha\beta/[(\alpha + \beta)^2 (\alpha + \beta + 1)]$ as parameters instead of α and β, and thus specify the beta distribution as Beta(m, v). Parameters *alpha;* and β are given as

$$\alpha = m(m - m^2 - v)/v \quad \text{and} \qquad \text{(A4a)}$$

$$\beta = (1 - m)(m - m^2 - v)/v, \qquad \text{(A4b)}$$

with $0 \le m \le 1$, and $0 \le v \le m(1 - m)$.

The BETABINO program obtains maximum-likelihood estimates of parameters and calculates the optimum log likelihood under four models concerning differences among classes: the same mean and variance among classes (model 0), the same mean but different variances (model 1), different means and the same variance (model 2), and different means and variances (model 3). The models can be compared using the likelihood-ratio test to examine whether the different classes have the same average probability of success. The analysis is rather similar to a one-way analysis of variance, where the class is the main effect and the multiple families with each class are the replicates.