Mimicry and the evolution of premating isolation in *Heliconius melpomene* Linnaeus

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Abstract

Ecological divergence can cause speciation if adaptive traits have pleiotropic effects on mate choice. In *Heliconius* butterflies, mimetic patterns play a role in mate detection between sister species, as well as signalling to predators. Here we show that male butterflies from four recently diverged parapatric populations of *Heliconius melpomene* are more likely to approach and court their own colour patterns as compared with those of other races. A few exceptions, where males were more attracted to patterns other than their own, suggest that some mimetic patterns are sub-optimal in mate choice. Genotype frequencies in hybrid zones between races of *H. melpomene* suggest that mating is random, so reinforcement is unlikely to have played a role in intra-specific divergence. In summary, co-evolved divergence of colour pattern and mate preference occurs rapidly and is likely the first step in *Heliconius* speciation.

Introduction

Many authors have highlighted the importance of mate choice in speciation (e.g. Dobzhansky, 1937; Mayr, 1942; Paterson, 1985). The observation that species can show strong assortative mating or fertilization, without evidence for hybrid incompatibility suggests that mate or gamete choice may commonly be the first step in speciation (McMillan *et al.*, 1997; Howard *et al.*, 1998; Seehausen *et al.*, 1999; Arnqvist *et al.*, 2000; Gray & Cade, 2000; Jiggins & Mallet, 2000). Nonetheless, we understand little about what drives mating preferences to diverge between incipient species.

Theoretical models suggest that sexual selection could lead to divergence in female preference and male traits through Fisherian runaway sexual selection, leading to assortative mating and hence reproductive isolation between populations (Iwasa & Pomiankowski, 1995; Payne & Krakauer, 1997; Higashi *et al.*, 1999). Sibling species of African lake cichlids that differ markedly in male colour patterns and female mate preferences, but

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little else, may have resulted from this process (Seehausen & Alphen, 1998). Recently several theoretical and comparative studies have concentrated more specifically on sexual conflict as a cause of divergence (Parker & Partridge, 1998; Gavrilets, 2000; Gavrilets *et al.*, 2001). Under certain circumstances, males and females are expected to undergo an arms race because of conflict between the sexes over traits such as mating rate (Rice, 1996). As the direction in which divergence occurs can be arbitrary, this process might lead to divergence in mating compatibility between populations and hence speciation. However, a recent comparative analysis of species diversity and mating strategy failed to provide support for these models (Gage *et al.*, 2002, but see Arnqvist *et al.*, 2000).

However even without sexual selection (i.e. differential reproductive success of different genotypes), mate preferences can diverge between populations. Genes selected for local adaptation can have pleiotropic effects on mating in a variety of ways (Schluter, 2000). For example, in phytophagous insects mating often occurs on or near the host plant, such that a shift in host preference leads directly to pre-mating isolation (Feder *et al.*, 1994; Emelianov *et al.*, 2001; Dres & Mallet, 2002). There may also be far more subtle and indirect effects of adaptation to divergent environments on mate

preference. The 'sensory drive' hypothesis of Endler (1992) describes the adaptation of mating signals and preferences to the local environment. This can lead to assortative mating between derived and ancestral populations found in different environments, leading to speciation. For example, mating calls may be adapted to the local acoustic environment (Passmore, 1985; Wiley, 1991), and similarly visual signals may be optimized to the light environment in a particular habitat (Endler, 1992; Fuller, 2002). The role of 'sensory drive' in speciation is perhaps best documented in two stickleback ecotypes, whose habitats differ in water colour (Boughman, 2001). In clear water, red is the most spectrally contrasting colour, whilst in opaque water black is a more contrasting signal (Endler, 1992). Incipient species found in limnetic and benthic habitats are known to be under strong disruptive ecological selection (Schluter, 1994). These forms also differ in male colour and female sensitivity to red, as would be predicted from their habitat type, and the differences lead to assortative mating between populations (Boughman, 2001). In addition, there is evidence that ecologically selected differences in body size are also used as mating cues (Schluter, 1998). In contrast to sexual selection models of speciation, if mate choice evolves via such pleiotropic effects there is automatically a complete association between mate preferences and divergent ecology, making speciation rapid (Maynard Smith, 1966); if common, such effects could therefore be an important contributing factor to biological diversity.

In butterflies, wing colour patterns are both used in signalling to mates and are also under selection for reasons unrelated to mate recognition, such as crypsis or mimicry (Nijhout, 1991). For example, distasteful neotropical butterflies are often adapted to local Müllerian mimicry rings, in which two or more butterfly species share similar warning colour patterns. Although even slight resemblance between model and mimic may confer some advantage (Brower *et al.*, 1971), the remarkable convergence of pattern, often between distantly related species, that includes minute details of wing pattern and even body colour, suggests very strong selection favouring the perfection of mimicry (Turner, 1971, 1976). This has been confirmed by field studies. Translocated or manipulated *Heliconius* butterflies with patterns that have not been previously encountered by the local predator community show reduced recapture rates compared with controls, suggesting strong mimetic selection (Benson, 1972; Mallet & Barton, 1989; Kapan, 2001). In addition to their role in predator avoidance, butterfly patterns are also known be used in mate detection (Crane, 1955; Brower, 1959; Emsley, 1970). For example, coloured models of the mimetic African species, *Hypolimnas misippus*, can induce chasing behaviour in males (Stride, 1957, 1958). The interaction of predator avoidance and mate detection in the evolution of butterfly wing patterns has

long been realized. For example melanic, mimetic forms of *Papilio glaucus* may suffer a mating disadvantage that could contribute to the maintenance of polymorphism in this species (Brower & Brower, 1962). However, although the suggestion that divergence in mimicry might contribute to speciation through its effect on mating signals is an old one (Bates, 1862; Turner, 1976; Vane-Wright, 1978), it has only recently been investigated experimentally (Jiggins *et al.*, 2001b).

Heliconius butterflies are involved in Müllerian mimicry rings and closely related species commonly differ in mimetic pattern (Mallet *et al.*, 1998). Previously, we have shown that the sister species *Heliconius melpomene* Linnaeus and *Heliconius cydno* Doubleday use their mimetic patterns in mate choice, leading to assortative mating (Jiggins *et al.*, 2001b). However, these sister species are around 1.5 million years old and show significant post-mating genomic incompatibility as well as marked ecological differentiation. Therefore mate preferences based on colour pattern are just one of a variety of forms of reproductive isolation that allow the stable coexistence of *H. melpomene* and *H. cydno*. Although hybridization and gene flow still occur, these taxa are therefore in the latter stages of speciation. It is an unfortunate fact that speciation occurs over many generations and it is generally not possible to study the process from start to finish in nature. Instead, steps in the process of differentiation must be reconstructed from taxa in different stages of divergence. To this end, we here describe a study of the far more closely related geographic races within *H. melpomene*. These populations are considered conspecific because they hybridize freely in narrow contact zones. However differences between races in colour patterns are striking, almost as great as between sister species. An important prediction of the proposal that divergence in mimicry causes speciation is that colour pattern change, such as that between races of *H. melpomene*, should inevitably lead to premating isolation. We here test this prediction by investigating the colour pattern preferences of races of *H. melpomene*.

The results provide strong support for our prediction, with races being more attracted to their own colour patterns in almost all comparisons. This provides several important advances on the previous study of sister species. First, divergence in pre-mating isolation because of colour pattern occurs before divergence in other traits that differ between sister species, most notably habitat preferences and genomic incompatibility (Mallet *et al.*, 1998; Naisbit *et al.*, 2002). Secondly, a far larger number of distinct populations are studied here, demonstrating that the evolution of colour-based mate-detection is ubiquitous and perhaps inevitable in *Heliconius*. Finally, the demonstration of such preferences between populations that abut and hybridize randomly suggests that reinforcement has not played a role in their divergence, and provides insights into how speciation might proceed in parapatry.

Methods

The study species

Heliconius butterflies offer useful model systems to study the evolution of mate detection. *Heliconius melpomene* is distasteful to predators (Chai & Srygley, 1990), and divided into many broadly distributed parapatric colour pattern races. While it is difficult to distinguish the model species among Mullerian mimics, some of the *H. melpomene* patterns have clearly evolved through mimetic convergence on pre-existing patterns. The Amazonian 'rayed' pattern is shared by several phylogenetically basal heliconiine species, such as *Neruda aoede* Hübner, *Heliconius xanthocles* Bates and *H. burneyi* Hübner that are the probable models for *H. melpomene* (Brown, 1981). Other *H. melpomene* colour patterns are shared only with its widespread co-mimic species, *Heliconius erato* Linnaeus, and likely represent patterns, that have arisen *de novo* in either *Heliconius erato* or *H. melpomene*. However, *H. erato* is the commoner and more ancient species and previous authors have suggested that it is the likely model for these patterns (Brower, 1996; Mallet, 1999). Hence, most of the colour pattern diversity in *H. melpomene* has arisen through selection for mimicry of pre-existing patterns in other species. Furthermore, unlike other *Heliconius* that mate at female pupation sites, *H. melpomene* mate after female emergence and are polyandrous, so adult individuals must encounter one another in order to mate (Brown, 1981). There is therefore ample opportunity for the use of mimetic colour patterns as mating cues.

Adult *H. melpomene melpomene* were collected in French Guiana (46 individuals in May 2001) at Pointe Macouria; 4.913°N, 52.360°W and Sablance; 4.963°N, 52.420°W, *H. melpomene plesseni* (20 individuals in February 2002) at El Topo (1.401°S 78.185°W), Ecuador, *H. melpomene cythera* (eight individuals in May 2002) near Mindo (0.065°S, 78.789°W) and Pedro Vicente Maldonado (0.118°N, 79.059°W), Ecuador and *H. melpomene malleti* (38 individuals in May 2001 and 41 individuals in February 2002) near San Raphael (0.182°S, 77.685°W), Ecuador. Adults were brought to Panama in envelopes and fed regularly with sugar solution. Rearing conditions described previously (Jiggins *et al.*, 2001a). In some cases natural foodplant was supplemented with artificial diet purchased from Educational Science (http://www.educationalscience.com) for first to fourth instar larvae. *Heliconius melpomene rosina* models were made from butterflies collected in the Gamboa area, Panama (Jiggins *et al.*, 2001a). *Heliconius cydno alithea* models from Western Ecuador (white morph, sites as for *H. m. cythera*) were used in experiments with *H. m. cythera* and *H. m. malleti* and models of *H. cydno chioneus* from Gamboa, Panama in experiments with *H. m. plesseni*. Experiments were carried out between May 2001 and June 2002.

Experiments using real wings and paper models

Colour pattern preference experiments were carried out as described previously (Jiggins *et al.*, 2001b) using models made from both dissected female butterfly wings (real wings) and printed paper wings. Models were made by removing wings from the body, and gluing the wing base to a small piece of stiff card (approximately 5 × 10 mm), using a water-based glue. The pieces of card were then attached to one another using thin strips of adhesive tape. The tape and card were coloured black using a permanent marker. In order to test whether responses were to colour pattern, rather than some other aspect of the wing, such as pheromones, we also carried out experiments with paper models. Paper models were made from digital photographs, taken using a Nikon Coolpix 990 (Nikon USA, Inc., Melville, NY, USA), separated from the background in Adobe Photoshop 5.5 (Adobe Corp., San Jose, CA, USA), and printed with an Epson Stylus Color 880 printer on Epson 'Photo Quality Ink Jet Paper' (Epson America, Inc., Long Beach, CA, USA). Photographs were taken in sunlight, which optimized reproduction of red and orange pigment colours and the blue iridescent structural colour. However this bleached the yellow pigment colour, so composite images were made by filling the yellow colour regions with colour from an image taken under outdoor shade light, using the 'eyedropper' and 'paint bucket' tools in Photoshop. Printed wings were then glued to a second sheet of paper to increase rigidity, cut out and attached to one another using tape as above. The underside of the wing was coloured black using a permanent marker. Models of both types were then attached to a springy wire approximately 50 cm in length with a wooden handle, to facilitate the imitation of flight.

Previous experiments have shown that species recognition in *Heliconius* involves strong male choice (Jiggins *et al.*, 2001b). Furthermore, butterfly courtship generally involves attraction of males to females using visual cues, followed by courtship behaviours involving tactile and chemical communication (Rutowski, 1991; Vane-Wright & Boppré, 1993; Fordyce *et al.*, 2002). Hence, as we are interested in the role of visual cues in mating and species recognition, we concentrate on male preferences. Female choice may play a role in inter-population divergence but is less likely to involve visual cues. Experiments were carried out with between five and 20 males in 2 × 2 × 2 m cages. Moving coloured models were presented in paired experiments, 10 min with a control model of the same pattern as the experimental butterflies and a further 10 min with an experimental pattern, the order of presentation being randomized. No more than two such replicate experiments were carried out daily. Two behaviours were recorded in each experiment. First, all butterflies passing through a sphere of 60 cm diameter around the model, demarcated by a bamboo cross, were recorded (henceforth described as 'approach'). Secondly, sustained fluttering directed towards the model was

recorded as 'courtship'. Each 'bout' of courtship was recorded as a single event. Ten replicates of the 20 min observation period were carried out for each comparison. In many cases the same individual males were present during several trials, although there was some turnover during the experiments as males died and were replaced with newly reared individuals. The statistical implications of the repeated use of the same males are discussed below. In some cases where activity was low, generally because of adverse weather conditions or small numbers of experimental males, five or 10 further experiments were carried out. In all cases results were similar in repeat experiments, so replicates are combined for analysis.

Approach and courtship probabilities and their support limits (approximately equivalent to 95% confidence intervals) were estimated using likelihood as described previously (Jiggins *et al.*, 2001b). We estimated the probability $Q_{Ai \times j}$ that males of type j would approach a model of type i relative to that of their own type j. Thus, for the experiment using *H. m. malleti* males with *H. m. malleti* vs. *H. m. cythera* models, the actual probabilities are $Q_{A \, mal \times cyt} / (Q_{A \, mal \times cyt} + 1)$ that males approach the *H. m. cythera* model and $1/(Q_{A \, mal \times cyt} + 1)$ that they approach the *malleti* model. The $\log_e$ likelihood for this experiment is therefore $\Sigma[X_{A \, mal \times cyt} ln\{Q_{A \, mal \times cyt} / (Q_{A \, mal \times cyt} + 1)\} + X_{A \, mal \times mal} ln\{1/(Q_{A \, mal \times cyt} + 1)\}]$, where $X_{A \, mal \times cyt}$ is the number of *H. m. malleti* males attracted to the *H. m. cythera* model and $X_{A \, mal \times mal}$ is the number attracted to the *H. m. malleti* model. Similarly $Q_{Hi \times j}$ parameters were estimated for probability of hovering 'courtship' of the model. $Q_{Ai \times j}$ and $Q_{Hi \times j}$ were estimated for experiments with paper as well as real wings. The summed $\log_e$ likelihood was maximized for each experiment by varying the $Q_{i \times j}$ parameters, using the Solver algorithm in Microsoft Excel. The support limits were estimated as parameter values that led to a decrease in the $\log_e$ likelihood of two units, asymptotically equivalent to 95% confidence intervals (Edwards, 1972). All Q parameters without significant support (i.e. whose support limits overlapped 1), are presented as having a value of 1 (Figs 1 and 2). It should be noted that, as the same males were used in repeated trials, each trial is not an independent data point. Hence the experiments are not designed for statistical comparison of preference parameters between experiments. The data presented therefore demonstrate whether or not a particular group of males shows significant preference for one pattern over another, and indicate the level of support for that preference. While there clearly are differences in preference strength between populations, the data should not be taken as showing statistical support for such differences.

Learning experiments

Two experiments were carried out to test the hypothesis that the response to colour pattern might be learnt through contact with conspecifics. In the first experiment, 10 male *H. m. plesseni* and 10 male *H. m. malleti* were collected in Ecuador, from populations allopatric with respect to one another (see above), and tested against one another's colour patterns. The two groups of males were then mixed and allowed to fly together for 5 days before the experiments were repeated to see whether contact with males of the other race had influenced the response. In a second experiment, newly emerged males of *H. m. malleti* were separated into two groups. The first group of five males was exposed to four virgin females of their own race for a period of at least 5 days after their emergence, whilst the second group of six males was exposed to four virgin females and four males of *H. m. plesseni* for a similar period of time. Both groups were then tested alongside one another for their response to *H. m. malleti* vs. *H. m. plesseni* patterns. If colour patterns were learnt we would expect the males that had been exposed to *H. m. plesseni* females to show a preference for the *H. m. plesseni* colour pattern over their own, or at least a reduction in the strength of preference for their own pattern relative to the control group. Preference comparisons were carried out using *G*-tests for heterogeneity on the resulting 2×2 table of summed data. For example, comparing data for total number of approaches to control and test models, from before vs. after mixing of males; or between group 1 and group 2 for the second experiment. The preference parameters calculated from these experiments are also presented as a means of visualizing the results (Fig. 2).

Hybrid male experiments

To begin to investigate the genetic basis of preference, hybrid male offspring from both reciprocal crosses between *H. m. melpomene* and *H. m. malleti* were tested separately for their response to *H. m. malleti* vs. *H. m. melpomene* patterns. Differences in the response of males from reciprocal crosses would suggest maternal (or paternal) effects or extra-chromosomal maternal inheritance of preference genes, whilst greater similarity to one or other parent in both classes would suggest dominance of alleles from one race.

Courtship and mating experiments

Mate choice experiments were carried out using two methods. First, pairwise experiments involving a single virgin female and a mature male of each genotype were carried out, and the first mating observed, at which point the experiment was terminated (Jiggins *et al.*, 2001b). Individual butterflies were not reused. However these experiments were slow because of the large number of individuals required and failed to separate male from female preference. Further comparisons were made by observing courtship and mating in male population cages. A single female was placed in a cage of five to 20 males and all courtship bouts in which one or more
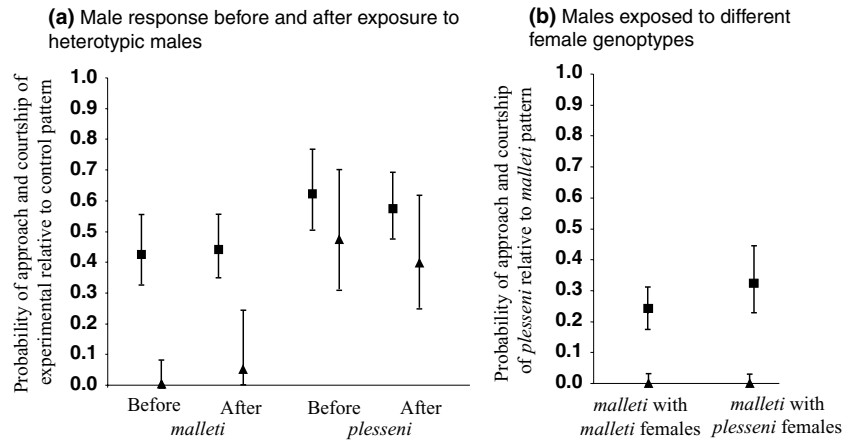
(a)

(b)
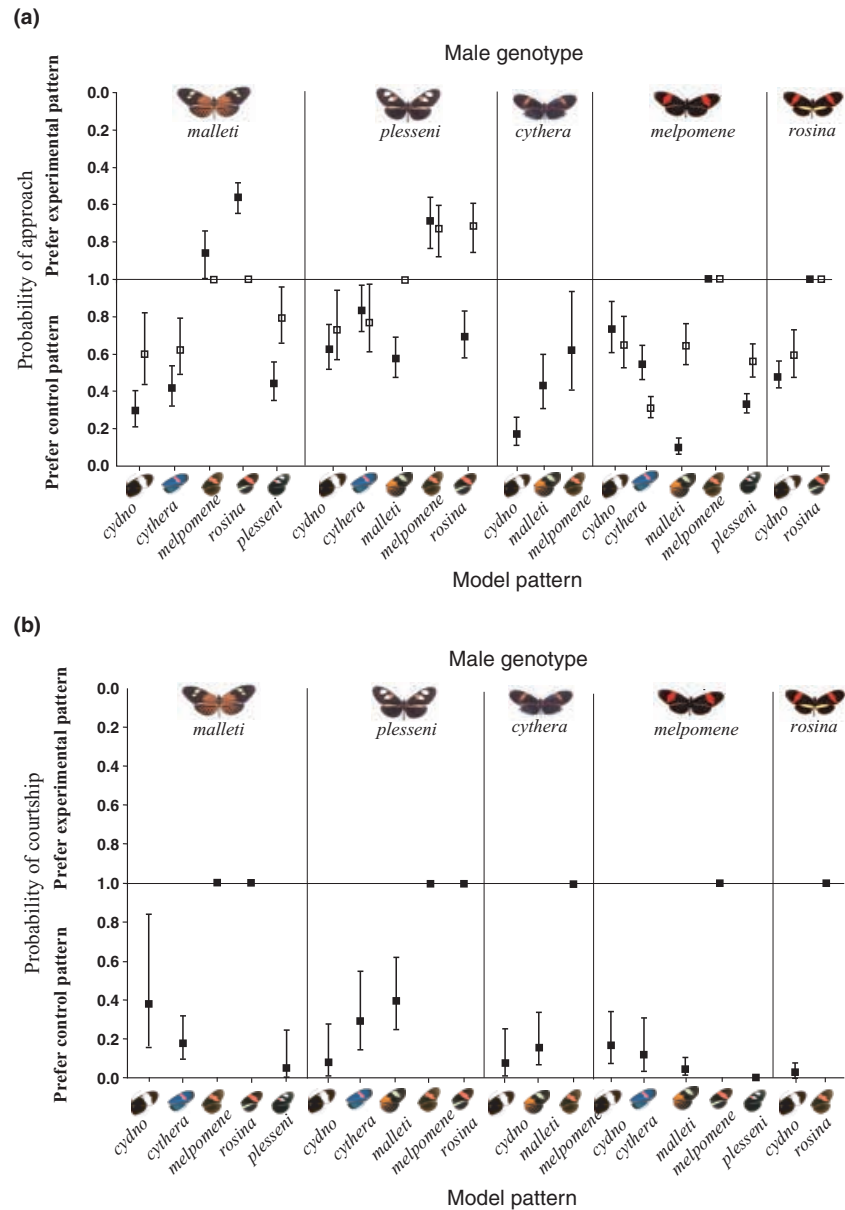
Fig. 1 (a) 'Approach' and (b) 'courtship' probabilities in pairwise comparisons of races. Preference probabilities are shown for males (whole butterflies, shown at top) tested against an experimental pattern (half butterflies, shown below), with respect to a control model of the same pattern as the males being tested. Closed points show experiments using dissected butterfly wings and open points paper models. Probabilities are shown relative to the favoured pattern, which is fixed at 1, but are plotted separately depending on whether the contypic or heterotypic pattern was favoured, above and below the axis respectively. Error bars show support limits, statistically equivalent to 95% confidence intervals, estimated by likelihood. Comparisons of *H. m. melpomene* males with *H. cydno* and *H. m. rosina* patterns and all experiments involving *H. m. rosina* males were published previously (Jiggins *et al.*, 2001b) and are shown here for comparison. Note that courtship was only rarely observed in the paper model experiments and these data are therefore not presented.

male(s) were courting the female were recorded and timed. Copula attempts where the male bent his abdomen towards the female, and successful copulas were also recorded. Copulating pairs were rapidly separated and the experiment continued without apparent disruption of courtship behaviour. Comparisons were made for 10 min with a control female of the same race as the males being studied and 10 min with an experimental female. Individual females were not reused. As with the model experiments, these trials are designed for comparison of courtship time between control and experimental females within a trial, for a particular group of males, and not between trials using different males.

Results

Experiments using real butterfly wings

Males from the four races of *H. melpomene* were tested in 16 separate comparisons representing over 53 h of observations using models made from dissected butterfly wings (Fig. 1; note that this figure also includes four previously published comparisons). In 11 of the 16 comparisons, males were significantly more likely to both approach and court their own colour pattern as compared with that of other races and species (Fig. 1a, b). The only comparisons where this was not the case

(a) Male response before and after exposure to heterotypic males

(b) Males exposed to different female genotypes

Fig. 2 Learning experiments. (a) and (b) show the results of two experiments designed to test whether butterflies learn the patterns to which they are attracted. Approach (squares) and courtship (triangles) probabilities are estimated relative to the response to control models, which is fixed at 1. In (a), wild *H. m. plesseni* and *H. m. malleti* were tested before and after contact with one another. In (b), two groups of reared *H. m. malleti* males were exposed to either *H. m. plesseni* or *H. m. malleti* females and then tested for attraction to *H. m. malleti* vs. *H. m. plesseni* patterns (see Methods). The lack of significant differences between the males before and after exposure to one another and when exposed to different females suggests that the observed preferences have a largely genetic basis. These experiments also demonstrate the repeatability of the probabilities estimated from different individuals sampled from the same population, both wild-collected and laboratory reared.

involved models of the two patterns with a broad red forewing band, *H. m. melpomene* and *H. m. rosina*, (known as the 'postman' races). In some cases the other races tested here were more or equally likely to approach the postman patterns as compared with their own. Despite in some cases being more attracted to these patterns, in none of the tests were males more likely to initiate courtship towards a pattern other than their own (Fig. 1b). These two races also have no significant preference when tested against one another's pattern (Fig. 1; Jiggins *et al.*, 2001b). Furthermore, if the two postman races are excluded, in all the remaining comparisons males show significant preferences for their own colour pattern in both approach and courtship behaviour. We also used models of *H. cydno*, the sister species to *H. melpomene*, and in all cases *H. melpomene* males were more likely to approach and court their own pattern as compared with that of *H. cydno* (Fig. 1).

Experiments using paper models

Most comparisons were repeated using models made from printed butterfly wings, representing a further 43 h of observation (Fig. 1a). These paper model experiments supported results from real wings and confirmed that the response of males was to colour pattern. Of 13 comparisons, nine were in agreement with the real wing experiments, three were non-significant and in one, when *H. m. plesseni* males were tested against the *H. m. rosina* pattern, the result was in the opposite direction to that expected from real wing experiments (Fig. 1a). Generally paper models were less attractive to males, resulting in a reduced strength of preference as

compared with real wing model tests and three cases of non-significant results in comparisons where real wing experiments showed a significant preference. The one case where paper and real wings show opposite results is harder to explain. Subsequent spectrophotometric measurement has shown a dip in reflectance of the white paper in the ultraviolet, below about 400 nm. In contrast the white butterfly pigment has a largely flat spectra from 300–700 nm. It is possible that the lack of ultraviolet reflectance of the white patch in the *H. m. plesseni* paper model led to a reduced response to the control model in these experiments, but this requires further investigation.

'Courtship' of paper models was almost never observed and data for this behaviour are not presented. Presumably, although males were attracted by paper models, at a distance of a few centimeters they could distinguish that the model was not a real butterfly and did not initiate courtship behaviour. The reduction in the strength of preference and failure to court paper models could be a result of non-colour pattern signals, such as pheromones, detected by males on the real-wing models. However the reduced response to photographed wings is more easily explained by the failure of ink-jet printed pigments to reproduce the lustre and exact spectral characteristics of *Heliconius* pigments. Whatever the explanation, the paper model experiments clearly demonstrate that a major component of the preferences demonstrated here are in response to colour and pattern.

Learning experiments

Our data showed no evidence that preferences were learnt. Wild-collected male *H. m. plesseni* and *H. m. malleti*

were tested for their response to one another's colour pattern, both before and after being exposed to males of the other race, with no change in the response observed. *G*-tests for heterogeneity carried out for *before* vs. *after* summed data were not significant for approach or courtship behaviour for either race (*H. m. malleti* males, for approach $G_1 = 0.045$, NS; for courtship, $G_1 = 1.59$, NS; *H. m. plesseni* males, for approach, $G_1 = 0.333$, NS; and for courtship $G_1 = 0.337$, NS; see also Fig. 2a). In addition, freshly emerged males of *H. m. malleti* were separated into two groups and exposed either to virgin *H. m. malleti* females or both virgin *H. m. plesseni* females and males. In experiments comparing the *H. m. plesseni* vs. *H. m. malleti* patterns conducted contemporaneously on the two groups after such exposure, there was no significant difference in response between the groups (*G*-test for heterogeneity between treatments for approach behaviour, $G_1 = 2.02$, NS; Fig. 2b). Whilst these simple experiments cannot rule out the possibility that some aspect of the response to colour pattern might be learnt, they do imply that the preferences measured in these experiments have a largely genetic basis.
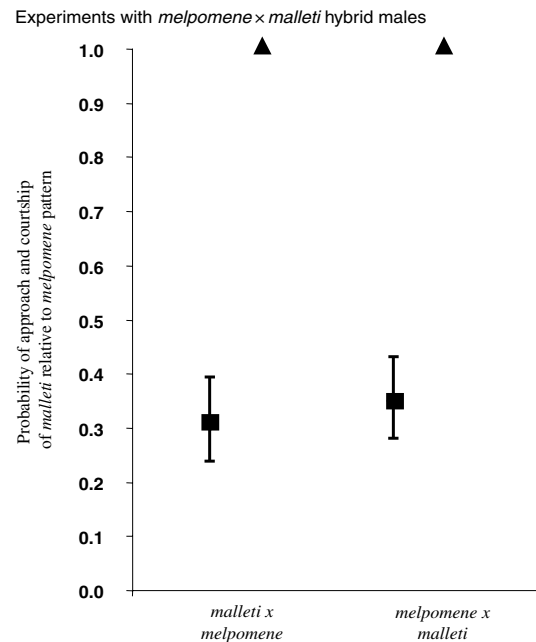
Experiments with hybrid males

Hybrids between *H. m. melpomene* and *H. m. malleti* were more likely to approach the *H. m. melpomene* than the *H. m. malleti* pattern. However, they showed no preference in courtship of the two patterns (Fig. 3). In comparison, both parental races showed some preference for the *H. m. melpomene* pattern, but only *H. m. melpomene* males preferred to court their own pattern. Hybrids are therefore somewhat intermediate in approach and courtship behaviour (Fig. 3). There was no difference between the offspring of the reciprocal crosses (Fig. 3).

Courtship and mating experiments

There was significant assortative mating in 2 × 2 mating trials involving the races *H. m. cythera* and *H. m. malleti*. In 11 trials, there were no inter-racial matings, five *H. m. cythera* × *H. m. cythera* and six *H. m. malleti* × *H. m. malleti* ($G_1 = 15.16$, $P > 0.001$). In addition over 21 h of observations were made of courtship and mating in multiple male cages (see Methods and Table 1). In general the probability that males approach and initiate courtship of live females, represented by the number of courtship bouts (Table 1), was in accordance with results from the colour pattern model experiments. *H. m. malleti* males were significantly less likely to approach *H. m. plesseni* females and *H. m. melpomene* males showed significantly fewer courtship bouts with *H. m. malleti* females. No significant differences in the number of courtship bouts were observed in the other four comparisons (Table 1).

Subsequent patterns of courtship and attempted mating were, however, not always consistent with the colour pattern model experiments. For example, *H. m. malleti*

Experiments with *melpomene* × *malleti* hybrid males

Fig. 3 Hybrid male experiments. Experiments with F1 hybrids between *H. m. melpomene* and *H. m. malleti* were carried out with off-spring of both reciprocal crosses (female genotype shown first). Both genotypes are more likely to approach (squares) the *H. m. melpomene* pattern compared with that of *H. m. malleti*. There was no difference in the probability of courtship (triangles) of the two patterns.

males in experiments with live females spent longer courting and were more likely to attempt to mate with their own females when tested with either *H. m. plesseni* or *H. m. melpomene*: in the case of *H. m. melpomene* the direction of preference is reversed as compared with the coloured model experiments (although the preference for the *H. m. melpomene* colour pattern is only borderline significant – see Fig. 1a). None of the other comparisons involving *H. m. plesseni* and *H. m. melpomene* males showed any significant differences in courtship time or number of copula attempts. In addition, although *H. m. melpomene* males were equally likely to attempt to mate with all females tested, they were significantly more likely to succeed with their own race. This could be due to female choice leading to assortative mating, but might also be due to population differences in mating vigour, as documented between populations of *H. m. melpomene* in a previous study (Jiggins *et al.*, 2001b).

Discussion

Within the last million years *H. melpomene* has evolved into a dramatic array of colour pattern races that mimic *H. erato* and other species (Turner, 1976; Brower, 1996). Here we have shown that these patterns are used as cues in mate detection, even though much, if not all of the diversity must have initially arisen through natural

Male	Female	Courtship bouts	Mean time courting (s)	Copula attempts	Copulas
malleti	*plesseni*	57*	95.0 ± 25.5*	17*	0*
	<u>*malleti*</u>	82*	233.5 ± 32.3*	66*	7*
malleti	<u>*melpomene*</u>	115	111.6 ± 22.7*	37*	5
	malleti	106	201.1 ± 20.5*	50*	4
plesseni	*malleti*	135	56.3 ± 19.2	18	1
	<u>*plesseni*</u>	130	41.1 ± 6.9	13	0
plesseni	<u>*melpomene*</u>	118	51.6 ± 17.7	10	1
	plesseni	96	32.9 ± 6.8	6	0
melpomene	*plesseni*	114	203.1 ± 37.9	76	0*
	<u>*melpomene*</u>	131	223.6 ± 41.0	95	12*
melpomene	*malleti*	119*	253.0 ± 51.9	96	5*
	<u>*melpomene*</u>	155*	221.3 ± 29.1	102	14*

Table 1 Patterns of courtship and mating of live females. The underlined female genotype shows the pattern preferred in colour pattern model experiments. Data are total counts or mean ± SE for courtship time. The * show significance ($P < 0.05$) in *G*-tests for divergence from a 1 : 1 ratio for count data and in paired *t*-tests for courtship time data. In all cases count data represent totals from 10 replicate observations of 20 min (10 min with each female), except the *H. m. plesseni* male vs. *H. m. malleti* female comparison where 15 replicates were carried out.

selection for mimicry. *H. melpomene* has therefore evolved to use the mimetic colour patterns as cues in mate detection. In some cases differences in the response to colour pattern lead to a reduced probability that males approach and court live females, suggesting that colour pattern preferences could lead to assortative mating in wild populations. A previous study of the sister species, *H. melpomene* and *H. cydno*, showed that mimetic colour patterns play a key role in species recognition (Jiggins *et al.*, 2001b). Here we demonstrate that such preferences are ubiquitous even between very recently diverged populations of the same species, showing that colour pattern based mate preferences evolve rapidly and perhaps inevitably alongside changes in colour pattern. This supports the idea that such changes commonly contribute to speciation, and leads us to propose a model of how speciation might occur in parapatry.

Nonetheless, all patterns are not equal with respect to conspecific communication. The broad red forewing band of the two 'postman' races was generally attractive to all *H. melpomene* races and in some cases led to a higher probability that males would approach a pattern other than their own. This suggests that mimetic adaptive peaks occupied by races of *H. melpomene*, such as the rayed pattern of the Amazonian races, may be suboptimal in terms of signalling to conspecifics. The results also suggest what may be generalizations regarding the patterns most attractive to *H. melpomene* males: red seems to be more attractive than orange (*H. m. malleti* was a generally rejected pattern, while *H. m. rosina* and *H. m. melpomene* were the most preferred), and large blocks of colour are more effective signals than complex patterns, as perhaps might be expected in butterflies which generally have low visual acuity (Rutowski & Kimball, 2000). Further experiments are required to investigate exactly which aspects of the patterns are being used as cues, but if such intrinsic preferences are common to all races of *H. melpomene*, they may limit or bias the direction of mimetic adaptation.

Male butterflies generally search for females using predominantly visual cues, but once a female is located,

close range courtship involves a mixture of pheromonal, tactile and visual communication (Rutowski, 1991; Vane-Wright & Boppré, 1993). Our data support this dichotomy; males of all races appear adapted to use their own patterns as cues for long-range attraction, but colour pattern appears less important in courtship of live females. The number of courtship bouts observed reflects a combination of long-range attraction and the decision to initiate courtship, and as might be expected there is general agreement between this aspect of experiments with live females and the results obtained from models. In five of six comparisons the frequency of courtship bouts is in the same direction as corresponding model experiments, with differences being significant in two cases (Table 1). It seems likely that in the wild, where conspecifics are detected over a distance of many meters, the effect of colour pattern preferences on mating probabilities would be far stronger than is observed in the enclosed space of our insectaries. However, once courtship has been initiated there is less evidence for a role of colour pattern (compare direction of preference in model experiments vs. courtship time and number of copula attempts in Table 1). In fact most comparisons show no significant differences in the time spent courting or number of mating attempts, suggesting that races generally do not differ greatly in pheromonal communication. Hence colour-based mate detection seems to diverge faster than mate recognition involving other signals.

We found no evidence that pattern preferences are learnt by contact with either females or males of other races. The possibility remains, however, that individuals might somehow observe and learn their own pattern. Crane actually investigated this possibility in *H. erato* by allowing adults to emerge in the dark and colouring their wings black before exposure to light (Crane, 1955). This treatment had no effect on the subsequent response of treated individuals to coloured models, suggesting that preferences are not induced by self-observation. We consider such self-learning improbable in *H. melpomene* also, but if it does occur to any extent it would greatly

facilitate speciation by causing an automatic self-preference among butterflies differing in colour pattern. However for the purposes of further discussion, we assume that preferences are genetically based, although this should be further tested.

No evidence for reinforcement

It seems most likely that colour pattern preferences arose between widespread populations with fixed differences at wing pattern loci, as a result of selection favouring the use of colour as a cue in mate location. However an alternative route for the evolution of mating preferences is through selection to avoid mating with incompatible individuals, or reinforcement (Butlin, 1987). Colour pattern forms are incompatible with one another because of disruptive mimetic selection, so reinforcement is plausible in *Heliconius* hybrid zones. However it may be unlikely on theoretical grounds. First, direct selection favouring mate preference genes is much more effective than indirect selection, such as occurs during reinforcement, where preferences diverge because of association with other incompatibility loci (Butlin, 1987; Servedio, 2001). Secondly, hybrid zones between the most distinct *Heliconius* races are extremely narrow relative to the species range, meaning that any selection for mate choice is likely swamped by gene flow from outside the zone (Butlin, 1987).

The empirical data also argue against reinforcement. All hybrid zones in *H. melpomene* that have been studied are in Hardy–Weinberg equilibrium such that hybridizing populations are dominated by recombinant individuals. This implies that mate preferences break down in sympatry (Turner, 1971; Mallet, 1986, 1993). The races tested here are not sampled from either side of a well studied hybrid zone, but hybrids are known to be abundant in populations where *H. m. plesseni* and *H. m. malleti*, the two East Ecuador races studied here, hybridize near Puyo in Ecuador, suggesting that strong assortative mating does not occur in that zone (pers. obs.). Hence, assortative mating breaks down in zones of contact rather than being 'reinforced'.

Nonetheless, there is evidence that sympatric *Heliconius* species show enhanced premating isolation in sympatry (Jiggins *et al.*, 2001b), suggesting that reinforcement may play a role in the latter stages of speciation. *H. m. rosina* collected from sympatry with *H. cydno* in Panama show stronger premating isolation from *H. cydno* as compared with allopatric *H. m. melpomene* from French Guiana (Jiggins *et al.*, 2001b). Although no direct test of sympatric vs. allopatric populations was made here, the data presented from a further three populations is consistent with the pattern; rejection of the *H. cydno* pattern appeared strongest in *H. m. cythera*, the *H. melpomene* race collected in sympatry with *H. cydno*, as compared with *H. m. plesseni* and *H. m. malleti* which are both allopatric to *H. cydno* (pers. obs.; Fig. 1). Further experiments designed to compare preference strengths between populations are needed, but

the patterns are consistent with theory suggesting that reinforcement is most likely between broadly sympatric species that have already evolved some degree of premating isolation because of mate- (Liou & Price, 1994; Noor, 1999) or habitat-choice (Cain *et al.*, 1999).

A likely route for parapatric speciation?

It is commonly thought that speciation occurs mostly in allopatry. A principal objection levelled at parapatric speciation via local adaptation is that strong epistasis between genes, necessary for locally favoured alleles to coalesce into a narrow hybrid zone, is unlikely (Futuyma & Mayer, 1980). Without such epistasis, local adaptation will likely produce discordant clines that do not lead to species-level differentiation. However, if ecologically selected genes have pleiotropic effects on traits involved in male–female communication, there is likely to be strong fitness epistasis between genes for mate preference and local adaptation. Hence, parapatric divergence seems plausible between the colour pattern races studied here.

Changes in colour pattern might occur initially because of geographic variation in available mimetic models. For example, mimicry rings in lowland Amazon look very different to those of adjacent Andean forests, producing a strong environmental cline in mimicry (Brown, 1979). Even if such a cline were patchy or shallow, mimetic selection against poorly adapted hybrids would mean that a steep cline would form in the mimic species because of selection against heterozygotes. An alternative outcome at this stage, if the mimetic environment is patchy at the scale of a few dispersal distances, is evolution of a single supergene controlling colour pattern differences and the establishment of a polymorphic mimic population (Joron *et al.*, 1999). A second possible starting point for colour pattern differentiation is that an entirely novel pattern might become fixed over a broad geographic range (for discussion of possible mechanisms see Mallet & Turner, 1998). Given the diversity of patterns in the *erato–melpomene* system, this must have occurred a number of times in one or both species. Hence, parapatric colour pattern races can become established either through local mimetic adaptation or shifting balance (Mallet & Singer, 1987).

Once abutting populations with distinct colour patterns are established, alleles that cause the novel colour pattern to be used as a cue in mate location are favoured, perhaps as a means of improving mate-finding efficiency. In the visually complex tropical rainforest environment, where conspecific individuals are inevitably rare and hard to encounter, it seems likely that the bright colour patterns of these butterflies would be used in finding mates. Alleles for colour pattern preferences are clearly epistatic with the colour pattern itself and will therefore only spread as far as the colour pattern hybrid zone. Thus mate preferences build up between colour pattern forms leading to populations separated by

both pre- and post-mating isolation. In *Heliconius* full sympatry likely also requires ecological divergence (Estrada & Jiggins, 2002). Hence, where mimicry rings differ ecologically, habitat preference genes that maximize overlap of mimetic species would also be epistatic with colour pattern and could accumulate in parapatry between diverging populations. One might imagine, for example, that if the model for *H. m. plesseni* were to occur only in riverine forests while that of *H. m. malleti* were in forest understorey, then habitat preference alleles would build up between these races and lead to full speciation. That this has not happened may reflect the fact that both races mimic *H. erato*, a species of open areas. Hence, in some circumstances adaptation for mimicry could incidentally lead to ecological isolation between incipient species as well as pre- and post-mating isolation.

In *Heliconius*, diversity in mimetic colour patterns is clearly maintained by natural selection through predation pressure. We have provided empirical evidence suggesting a route by which this diversity can rapidly lead to speciation. It seems likely that this process has contributed to the generation of diversity both in the genus *Heliconius* and perhaps in many other groups of tropical Lepidoptera. What is especially appealing about a role for Müllerian mimicry in speciation is that high species diversity leads to a greater diversity of mimetic patterns available as models, and hence perhaps a feedback process leading to greater speciation rates. If speciation in other taxa also depends on inter-species relationships such as mimicry, then high species diversity might similarly provide novel opportunities for more speciation. This may go some way towards a general explanation of tropical diversity.

Acknowledgments

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