Interspecific sexual attraction because of convergence in warning colouration: is there a conflict between natural and sexual selection in mimetic species?

C. ESTRADA*† & C. D. JIGGINS*‡

*Smithsonian Tropical Research Institute, Balboa, Panama, Republic of Panama
†Section of Integrative Biology, The University of Texas at Austin, TX, USA
‡Department of Zoology, University of Cambridge, Cambridge, UK

Abstract

When species converge in their colour patterns because of mimicry, and those patterns are also used in mate recognition, there is a probability of conflicting selection pressures. Closely related species that mimic one another are particularly likely to face such confusion because of similarities in their courtship behaviour and ecology. We conducted experiments in greenhouse conditions to study interspecific attraction between two mimetic butterfly species, Heliconius erato and Heliconius melpomene. Both species spent considerable time approaching and courting females of the co-mimic species. Experiments using wing models demonstrated the importance of colour pattern in this interspecific attraction. Although males of H. melpomene were attracted to their co-mimics as much as to their own females, H. erato males were more efficient at distinguishing conspecifics, possibly using wing odours. Although preliminary, these results suggest that the use of additional cues may have evolved in H. erato to reduce the cost of convergence in visual signals with H. melpomene. Overall, our results showed that there might be a cost of mimetic convergence because of a reduction in the efficiency of species recognition. Such cost may contribute to explain the apparently stable diversity in Müllerian mimetic patterns in many tropical butterfly assemblages.

Introduction

Phenotypic traits that are used as cues in species recognition or mate choice can evolve and be maintained by selection for other purposes. In particular, defence mechanisms and sexual communication in insects are connected in many ways. For example, some butterflies and moths collect pyrrolizidine alkaloids for both defence and male pheromone production, tiger moths (Lepidoptera: Arctiidae) produce ultrasound both to avoid bat predation and for courtship, and both cryptic and aposematic colourations are also often used in species recognition (Boppré, 1978; Brown, 1984; Eisner & Meinwald, 1995; Conner, 1999; Weller et al., 1999; Jiggins et al., 2001; Nosil et al., 2002). This dual role of

Correspondence: C. Estrada, Section of Integrative Biology, The University of Texas at Austin, 1 University Station CO930, Austin, TX 78712, USA.
Tel.: +15124714705; fax: +15124713878;
e-mail: catalinaestra72@hotmail.com

traits in signalling to conspecifics and defence against predators might lead to conflicting evolutionary pressures. In particular mimicry, where species converge to a common warning pattern (Bates, 1862; Müller, 1879) is likely to lead to mating confusion as signals are shared among coexisting species with similar phenotypes.

Similar to other traits with sexual and nonsexual functions, warning colouration in mimetic species could be under opposing selection pressures if sexual and natural selection favour different phenotypes (Endler, 1980; Ellers & Boggs, 2003; Stuart-Fox et al., 2004). On one hand, shape, colouration and behaviour evolve to resemble closely those of coexisting mimetic species to minimize the chances of mistakes by predators (Müller, 1879; Brower et al., 1963; Benson, 1972; Mallet & Barton, 1989; Kapan, 2001). Although close similarity may not always be expected or necessary (Johnstone, 2002; Holen & Johnstone, 2004; Rowe et al., 2004; Darst & Cummings, 2006), nonetheless the remarkable convergence of such traits among many mimetic species

suggests that specialized or experienced predators can select for precise mimicry (Mallet & Gilbert, 1995; Devries et al., 1999; Srygley & Ellington, 1999; Langham, 2004; Rowe et al., 2004; Moland et al., 2005). On the other hand, selection might favour differences in colouration for species recognition in order to prevent energetically costly chasing and courtship displays between species. Both male harassment and courtship often result in reductions in individual fitness (Clutton-Block & Langley, 1997; Bateman et al., 2006; Gosden & Svensson, 2007). Selection pressures for reductions of this effect, for example might in part explain the maintenance of male-like female morphs and female polymorphism in resistance or tolerance of mating attempts by males (Cook et al., 1994; Gosden & Svensson, 2007).

Butterflies are a good system to explore these conflicts because male searching and species recognition are mediated by visual cues (Scott, 1973; Rutowski, 1991). Although chemical, behavioural and even acoustic signals are involved (Boppré, 1984; Rutowski, 1984; Costanzo & Monteiro, 2007), recognition by sight is what brings individuals together in the initial stages of courtship (Scott, 1973; Silberglied, 1977). Furthermore, prezygotic isolation between closely related species commonly involves changes in wing colour patterns that include ultraviolet reflectance, colour bands and degree of melanization (Silberglied & Taylor, 1973; Shapiro, 1983; Wiernasz, 1989; Cook et al., 1994; Jiggins et al., 2001; Fordyce et al., 2002; Lukhtanov et al., 2005; Kronforst et al., 2006; Mavárez et al., 2006). However, wing colour patterns evolve not only to serve in intraspecific communication, but also for thermoregulation (True, 2003) and protection against predators through crypsis, disruptive patterns or warning colouration.

The potential for conflict between mimicry and sexual communication has attracted scientific attention ever since the role of wing colouration in butterfly sexual behaviour was recognized (Poulton, 1907; Brower et al., 1963; Silberglied, 1977; Boppré, 1978). For example, female-limited Batesian mimicry has been explained as the result of strong sexual selection by female choice against male wing phenotypic changes (Turner, 1978) (but see Turner, 1978; Ohsaki, 2005; Lederhouse & Scriber, 1996 for alternative hypothesis). Similarly, interspecific interference in courtship among similar species was proposed as one of the reasons for the lack of convergence to a single warning colouration among sympatric unpalatable species (Brower et al., 1963). Such interference has been rarely studied among mimetic species (Cook et al., 1994), but evidence for interspecific courtship among species with similar colouration has been reported several times in insects (Brower, 1959; Nielsen & Watt, 2000; Hochkirch et al., 2007; Svensson et al., 2008). In those cases, cost associated with interspecific courtship might

explain geographic and temporal separation of similar species (Brower, 1959; Hochkirch et al., 2007), or, together with intraspecific male harassment, contribute to explain the maintenance of female colour polymorphisms (Nielsen & Watt, 2000; Svensson et al., 2008).

Previous work on sexual attraction between mimetic butterflies has concentrated on Batesian mimics that are rather distantly related. However, recognition problems are likely to be more acute in closely related mimics that exhibit similar courtship behaviours and use similar resources (Vane-Wright & Boppré, 1993). Here, we investigate interspecific sexual attraction in congeneric Müllerian mimics, *Heliconius melpomene* Linnaeus and *Heliconius erato* Linnaeus (Nymphalidae: Heliconiinae). These species exhibit a series of races that, in sympatry, converge in colour pattern as well as in habitat use across their geographic range (Brown, 1979; Turner, 1981). Races that belong to each mimetic pair appear identical except for minor differences in pattern, and there is no evidence for differences between either sexes or co-mimic species in their visible range or ultraviolet spectral reflectance (Crane, 1954). *Heliconius* are also known to use wing colouration as mate recognition cues (Crane, 1955; Jiggins et al., 2001; Mavárez et al., 2006). In these butterflies, male mate preferences have coevolved with changes in mimetic colour patterns, (Jiggins et al., 2004) probably facilitated by a strong genetic association between these traits (Kronforst et al., 2006). Furthermore, patrolling males of several *H. melpomene* geographic races are more likely to approach and court females of their own race than those of races with different colouration (Jiggins et al., 2004).

Similarly to *H. melpomene*, at least one race of *H. erato* is also known to use wing colour and pattern as visual stimuli during courtship in both sexes (Crane, 1955). However, males of this species are not exclusively attracted to adult females but also search for pupae close to host plants and mate with them during eclosion (pupal mating) (Gilbert, 1976; Deinert, 2003). In this mating system chemical signals must play a primary role in species recognition. It is not known what proportion of mating in the wild occurs on pupae, but there is a possibility of courtship interference between these co-mimic species given that, at least in some cases, *H. erato* males are attracted to colour, and initiate courtship toward females encountered in flight.

Here, we investigated the potential for confusion in mate recognition between *H. melpomene* and *H. erato*. We first examined male courtship preferences between *H. erato* geographic races with different wing colour pattern to test whether the use of warning colouration in recognition is widespread in this species, as has already been demonstrated for *H. melpomene* (Jiggins et al., 2004). Then, we investigated interspecific attraction by measuring the extent to which males of both species approached and courted live females and wing models of their own and their co-mimic species. Finally, we begin to explore

which traits might be used in *Heliconius* to overcome signal confusion.

Materials and methods

Butterfly collection and rearing

All experiments were performed between August 2001 and June 2002 in Gamboa, Panama. We used adults of three geographic races of *H. erato* and four of *H. melpomene* reared from wild caught individuals from Ecuador, Panama and French Guiana (Table 1). Wings of two additional *H. erato* races and one *H. melpomene* were also used (Table 1). From Gamboa and surrounding areas, we collected *H. erato* cf. *petiverana* (*H. e. petiverana*), and *H. melpomene rosina*. Remaining races were collected as adults in May 2001 (*H. m. cythera, H. e. cyrbia, H. m. malleti, H. m. melpomene*) and March 2002 (*H. m. plesseni, H. e. notabilis, H. m malleti*) and brought to Panama in glassine envelopes. Both species were reared on *Passiflora biflora* or, from first to fourth instar, on *P. biflora* leaves mixed with artificial diet purchased from Educational Science Co (http://www.educationalscience.com/). The type of diet did not have any detectable effect on the colour of the wings or behaviour. Males from each race were kept in $2 \times 2 \times 2$ m^3 outdoor insectaries isolated from females and were fed with sugar water solution (10%) and pollen from *Psiguria* sp. and *Lantana camara* flowers.

Experiments among *H. erato* races

Males were tested specifically for their response to models with different colour patterns inside their insectaries. For each test, two butterfly models were presented to the males sequentially and in random order. First, a

model with the same colour pattern as the males (control), and second a colour pattern from a different race (experimental). Each model was presented for 10 min with a resting period of 10 min between the two. We tested males once or twice a day until completing 10 tests for each particular colour pattern comparison. Experiments were carried out in stock cages containing at least five males. Males were replaced when fresh individuals became available and to replace any dead individuals. Although it would have been optimal to use different males for each replicate, this was not possible because of limitations on rearing capacity. As in previous experiments, these tests should therefore be treated as replicate experiments on a common set of males, rather than independent population replicates. Nonetheless, previous data show that the results of these experiments are highly repeatable when independent sets of males are tested, or when carried out by different observers (Jiggins *et al.*, 2004; C. Estrada & C.D. Jiggins, unpublished).

Butterfly wing models were made with wings dissected from female bodies and glued to a piece of adhesive tape which was coloured black with a permanent marker. The adhesive tape kept the wings together in the normal position but also allowed for the movement of the model in a mode that simulated flight. The model was then attached to the tip of a 50 cm flexible wire and presented to males in the centre of a sphere 60 cm in diameter marked by a wooden cross (as in Jiggins *et al.*, 2001). During tests the wire was moved gently to simulate wing flapping. When a male flew toward the model and passed through the virtual sphere during the 10 min observation period, the behaviour was recorded as 'approach', and when a male came close to the model and fluttered in a sustained manner, the behaviour was recorded as 'courtship'. A summary of the tests carried out is given in Table S1 in the supplementary material.

In order to confirm that observed preferences were indeed because of colour pattern, comparisons in which significant preferences were found were repeated using models made from printed photographs (hereafter called paper models). Models consisted of digital photographs taken with a *Nikon Coolpix* 990 (Nikon USA, Inc., Melville, NY, USA) under sunlight, separated from the background in Adobe Photoshop 5.5 (Adobe Corp., San Jose, CA, USA), and printed using an Epson Stylus Colour 880 (Epson America, Inc, Long Beach, CA, USA). Red, blue and orange were reproduced in the photographs in a manner similar to real wing colours. Yellow bands, on the other hand, required shaded lighting conditions to be reproduced accurately, so images were manipulated in Photoshop to combine colours from different digital images. The similarity of reflectance spectra of colour bands from wing and paper models was confirmed using an Ocean Optics, Inc. spectrophotometer, and are shown in Table S2 in the supplementary material. Table S2 shows the wavelength of the

Table 1 Geographic races of *Heliconius melpomene* and *Heliconius erato* used in experiments.

H. melpomene races	*H. erato* races	Collection data
cythera	cyrbia	Mindo (0.065 ° S, 78.789 ° W) and Pedro Vicente Maldonado (0.118 ° S, 77.685 °W), Ecuador.
plesseni	notabilis	El Topo (1.401 ° S, 78.185 °W), Ecuador.
malleti	lativitta†	San Rafael (0.182 ° S, 77.685 ° W), Ecuador.
melpomene	hydara†	Pointe Macouria (4 ° 54.8′ N, 52 °21.6′ W) and Sablance (4 °57.8′ N, 52 °25.2′), French Guiana.
rosina†	petiverana	Gamboa (9 ° 7.33′ N, 79 ° 42.90′ W), Panama.

Co-mimics in the text refer to pairs of geographical races of the two species that have similar wing colour pattern and coexist in sympatry (pairs of races in each row). Conspecifics refer to races that belong to the same species but occupy different geographic ranges (each column).
†Indicate races where only wing models but not males were used.

maximum and medium peak reflectance for each colour band. The peak represents the hue at which the colour band has its maximum reflectance. Different peaks correspond to difference hues. The mean wavelength represents the point on the curve where the reflectance is changing faster. These are commonly used measures to compare reflectance spectra (Keyser & Hill, 1999). Certainly, printed models are likely to differ from real wings in aspects of both hue and brightness, but where preferences can be replicated using printed models this provides confirmation that butterflies are indeed using colour pattern as a cue and not some other aspect of our dissected wing models. Printed wings were glued to cardboard to increase their firmness and were coloured black on the underside with a permanent marker. They were attached to a wire and shown to males in the same way as real wing models.

Experiments between co-mimics

To investigate whether males were attracted to their co-mimic females a series of experiments were carried out in which males were presented with these females as well as conspecifics. Co-mimic refers to a pair of geographical races of *H. erato* and *H. melpomene* that coexist and have similar wing colour pattern (rows in Table 1). Conspecific here is used to indicate that females or models used were from the same species and colour pattern as the males being tested. We tested males with live females and wing models.

Experiments with live females
In the first set of experiments the mimetic pair *H. m. plesseni* and *H. e. notabilis* were tested with live females in a $2 \times 2 \times 2$ m^3 insectary. In each no-choice test a group of males was presented with a conspecific and a co-mimic female. A single virgin female was introduced to the insectary for 10 min, followed by a 10-min interval and then a female of the second species. The number of times males approached the female and initiated courtship was recorded as 'courtship bouts', and the time spent by at least one male hovering over the female as 'courtship time'. Copulation attempts, identified as a male bending his abdomen in search of the female abdomen, were also recorded. We carried out 10 replicates of this comparison with males of *H. m. plesseni* and 10 with *H. e. notabilis* males. Virgin females were 1–4 days old and they were used only once. The order of presentation of females was randomized. Only one copulation occurred between two *H. e. notabilis* individuals; the pair was immediately separated and the trial continued without apparent effect on the butterflies involved. For these tests, groups of males contained about 13 (±2) individuals of *H. m. plesseni* and about 7 (±1) of *H. e. notabilis*.

A further set of experiments were designed to compare co-mimics and conspecific attraction to live females. One male of each of the co-mimic pair *H. m. cythera* and *H. e. cyrbia* and one male of the *H. melpomene* race, *malleti*, were placed in an $1 \times 1 \times 2$ m^3 insectary. The three males were then presented with a single virgin female of any of the three races and the same behaviours described above were recorded for 10 min. Females were introduced at random and males were not reused after trials. In total 12 tests were carried out. These tests failed to measure attraction because males of the two *H. melpomene* races had very low activity levels. *H. erato* males (*cyrbia*), however, were very active and showed attraction to females. We therefore included the results here to show the behaviour of the *cyrbia* males.

Experiments with wing models
The attraction between conspecifics and co-mimics was then tested using wing models. Each group of males was tested for their response to a conspecific female wing model vs. a co-mimic model. The experiments were carried out in the same way as described above for tests among *H. erato* races. We used males of four *H. melpomene* and three *H. erato* races (Table S1, supplementary material).

Whenever significant differences were found in the attraction to conspecific vs. co-mimic wing models, at least 10 further tests were carried out using printed paper models and at least 10 more using hexane-washed dissected wing models. For the latter, wing models were immersed in HPLC-grade hexane for 1 h. These tests were done to examine whether preferences were because of subtle differences in colour patterns between co-mimics, or because of cuticular odour cues. Since washing wings with hexane did not produce any change in the colour of major bands (Table S2, supplementary material), results obtained with these models complement those found with printer paper models but corrected for problems in reproducing accurately the colours in printed photographs.

Statistical analysis

The probability of a group of males approaching and courting each of the two wing models for each 10 tests was estimated using likelihood as described previously (Jiggins *et al.*, 2001). The expression mLn $P_{ij} + n$ Ln $(1 - P_{ij})$ was calculated for each test where P_{ij} is the probability of the *j*-type males approaching or courting an *i*-type model. *m* and *n* are the number of approaches or courtship events towards an *i*-type model or to their own colour pattern model (*j*) respectively. First a probability of $P_{ij} = 0.5$ was assigned for each test and the expression calculated for the ten tests performed for every comparison (e.g. males *H. e. notabilis* with *H. e. notabilis* and *H. e. cyrbia* models). The summed likelihood for the ten trials was then maximized by changing the P_{ij} parameter using the Solver algorithm in Microsoft Office Excel 2003. Once the best probability P_{ij} for the comparison was estimated, it was transformed so

attraction of males to an *i*-type model is given relative to the attraction to the male colour pattern *j*, set as one. Support limits for P_{ij}, which are asymptotically equivalent to 95% confidence intervals, were also obtained by varying P_{ij}. So the summed Ln for the comparison increased or decreased 2 units from the maximum (Edwards, 1972). Since males were often reused in several tests, each test is not an independent point. These experiments are not designed for statistical comparison of preference between experiments and data should be interpreted as indicating whether or not a particular group of males show a significant preference for one pattern over another. Therefore, confidence intervals indicate whether attraction to an *i*-type model (species or race different to the one males belonged to) is significantly different from 1, or the probability of approaching the control model. Statistical significance estimated in this way is equivalent to a *G*-test.

For experiments between co-mimics using live females, we used paired Wilcoxon signed-rank test to compare mean courtship times and courtship bouts, and a *G*-test with Williams' correction to test for divergence of 1 : 1 ratios for number of copulation attempts. As with model experiments, these tests are designed for comparison between control and experimental females within a test, for a particular group of males, and not between trails using different males. Both tests were performed using the statistical computing program R 2.2.1.

Results

Experiments among *H. erato* races

We tested males from three *H. erato* races for their attraction to models of other geographic races relative to their own. Nine pairwise comparisons using both wing and paper models were carried out, representing a total of 50 h of observation. Each point in Fig. 1 represents the probability of a population of males approaching or courting a model of another race relative to their own. Probabilities below the line $P = 1$ show that males approached or courted the control pattern more often than the experimental, whereas probabilities above the line indicate that males approached or courted the experimental pattern more. Table S1 in the supplementary material shows the mean approach and courtship behaviours for each pairwise comparison.

Considering all comparisons, in the course of 10 tests, males approached control and experimental models an average (±SD) of 243.78 ± 79.98 and 158.11 ± 102.59 times respectively when using wing models, and 201.33 ± 52.18 and 121.5 ± 69.41 respectively when using paper models. In most cases males were more likely to approach their own colour pattern model than those of other races; although in a few cases the effect was not significant (when the 95% confidence intervals include preference $P = 1$) (Fig. 1a). The proba-
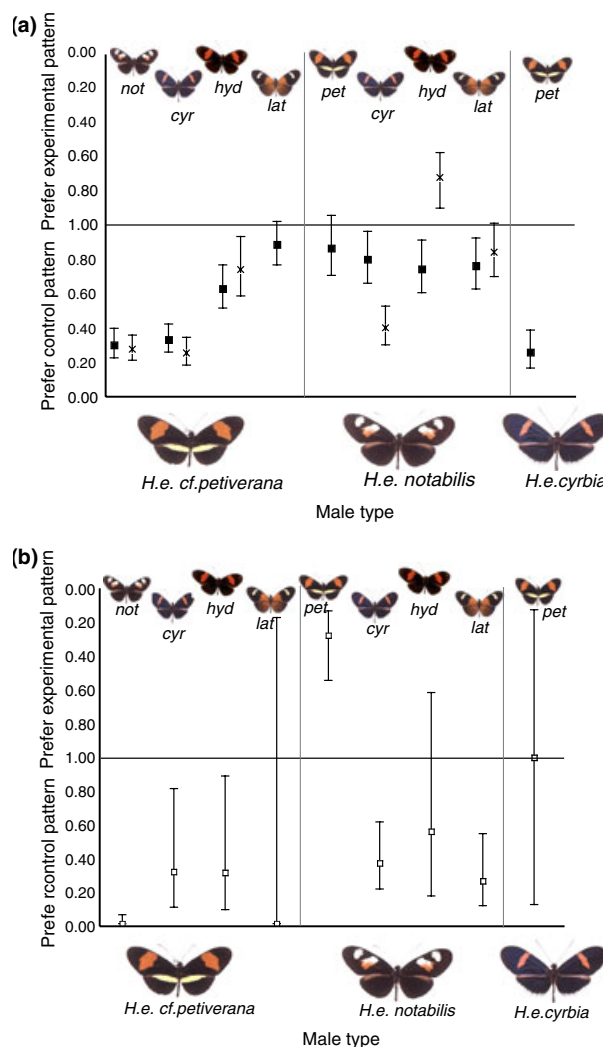
Fig. 1 Approach (a) and courtship (b) probabilities of pairwise tests among *Heliconius erato* races. Males were tested for their attraction to wing models of races with a different colour pattern relative to attraction to their own race pattern. Pictures of the races whose males were tested are on the bottom of the figure whereas pictures on the top represent the races used as experimental models. Each point in the graph represents the probability of a population of males approaching or courting a model for another race compared with their own race set as $P = 1$. Therefore, probabilities below the line at 1 represent preferences for models with the male colour pattern, whereas probabilities above the line at 1 indicate preference for the other race models. Error bars show support limits equivalent to 95% confidence intervals. Probability and error bars were estimated using likelihood. Models were made with real wings (■/□) and printed photographs (*). Race name abbreviations are *not* for *notabilis*, *cyr* for *cyrbia*, *hyd* for *hydara*, *lat* for *lativitta* and *pet* for *petiverana*. As courtship behaviour toward paper models were almost absent results from these experiments are not shown.

bility of males approaching other races was higher (or close to $P = 1$) when the other model involved in the comparison had wide red or orange bands in the

forewing (e.g. *H. e. petiverana, H. e. lativitta* and *H. e. hydara*). The exception to this was *H. e. cyrbia* males tested with *H. e. petiverana* models. Here males were less than half as likely to approach models of *H. e. petiverana* relative to their own model.

In seven of the nine comparisons among *erato* races, males approached wing models of their own race significantly more often than those of other races. In each case where a significant preference was found, we performed 10 additional tests using paper models to examine whether the preference was indeed because of colour pattern and not to other factors such as chemical cues. Patterns of approach to paper models were generally similar to those found with dissected wings, showing the importance of the wing colouration in model attraction. The differences in attraction between control and experimental paper models were however often weaker with paper models, and in one comparison, between *notabilis* males with the *hydara* model, attraction went in the opposing direction favouring the models of the latter race (Fig. 1a). It is likely that the reduced brightness of printed photographs as compared to real wings explained the generally reduced levels of response in these experiments. Indeed, reproduction of the white band in the *notabilis* pattern was the least accurate, because of the absence of UV reflectance in paper models (Table S2, supplementary material), perhaps explaining the reduced attractiveness of *notabilis* paper models to their own males when tested against *hydara* models.

The frequency of courtship behaviour, particularly towards paper models, was in general low (Fig. 1b). This probably accounts for the larger confidence intervals observed for 'courtship' compared with 'approach' behaviours. Courtship behaviours toward control and experimental models in 10 tests were displayed on average 19 ± 17.51 and 8.56 ± 11.14 times respectively when wing models were used. Courtship to paper models was almost absent and results are not reported. In spite of the low frequency of courtship events toward models, with the exception of the pair *H. e. notabilis* vs. *H. e. petiverana*, all comparisons showed males courting significantly more control than experimental models (Fig. 1b). It seems likely that males are initially attracted to models by their colouration, but once at close range, the lack of further cues or the crudeness of the model deterred them from engaging in courtship behaviour.

Experiments between co-mimics

Here, we tested the attraction of patrolling males to females and wing models of their co-mimic species. Courtship toward co-mimics occurred frequently. When males were presented with virgin females, they were more than half as likely to initiate courtship towards a co-mimic as compared to a conspecific (Table 2). Although males were always more likely to approach, court and attempt to copulate with females of their own species than with their co-mimics, such differences were not always significant. These results suggest that males are initially attracted to females of both species using visual cues. However, as might be expected extended courtship and copulation attempts towards co-mimics were rare and likely require additional species-specific cues from both sexes (Table 2). Evidence that initial attraction to females is because of similarity in colour patterns instead of a general attraction to flying live butterflies came from experiments carried out with the blue race *H. e. cyrbia*. Males from this race initiated courtship toward their co-mimic females (*H. m. cythera*) on 29 occasions, about half as frequently as towards conspecific females (Table 2), but courted *H. m. malleti* females, an orange rayed butterfly, only eight times (*G*-test, $G_1 = 21.034$, $P < 0.01$) and for a shorter time (courtship time toward *H. m. malleti* females = 3.234 ± 6.583 s, Wilcoxon signed-rank test, $V = 36$, $P < 0.05$).

Table 2 Patterns of courtship and copulation attempts when males were presented with co-mimic and conspecific live females for a period of 10 min each.

Males	Female	Courtship bouts	Mean courtship time (s)	Copulation attempts
H. m. plesseni	*H. m. plesseni*	80 (8 ± 3.83)	78.3 ± 75.17	23 (2.3 ± 3.37)
	H. e. notabilis	50 (5 ± 2.67)	14.8 ± 14.98	3 (0.3 ± 0.67)
		$V = 36^{NS}$	$V = 5*$	$G_1 = 17.12**$
H. e. notabilis	*H. e. notabilis*	95 (9.5 ± 2.76)	203.8 ± 139.68	43 (4.3 ± 4.32)
	H. m. plesseni	87 (8.7 ± 3.68)	36.8 ± 24.82	0
		$V = 32.5^{NS}$	$V = 54**$	$G_1 = 58.93**$
H. e. cyrbia	*H. e. cyrbia*	59 (4.92 ± 4.74)	29.48 ± 40.09	7 (0.64 ± 1.50)
	H. m. cythera	29 (2.42 ± 2.5)	4.69 ± 5.35	2 (0.80 ± 0.6)
		$V = 64.5*$	$V = 75**$	$G_1 = 2.79^{NS}$

Result of 10 trials for *H. m. plesseni*-*H. e. notabilis* and 12 for *H. e. cyrbia*-*H. m. cythera* comparisons. Paired Wilcoxon signed-rank test were done to compare courtship time and courtship bouts, and *G*-test with Williams' correction were carried out to test for heterogeneity in the copulation attempts. Total for all tests and average per test ± standard deviation are given for courtship bouts and copulation attempts.
*$P < 0.05$, **$P < 0.01$, 'NS' $P > 0.05$.

Males presumably distinguish conspecific females at close range using cues such as species-specific phero-mones. They could also be using minor colour pattern differences only noticeable at close range. Experiments with wing models, however, do not support this possi-bility. Seven pairwise comparisons between co-mimic species using wing, paper, and hexane-washed models were carried out for a total of 48 h of observation (Table S1, supplementary material).

Males of the four *H. melpomene* races tested (*H. m. mal-leti, H. m. cythera, H. m. melpomene* and *H. m. plesseni*) approached and courted wing models of their own species as much as those of the *H. erato* co-mimic races (Fig. 2). Considering all comparisons, males approached control and experimental models an average (±SD) of 299.75 ± 218.09 and 310.5 ± 243.89 times respectively when using wing models. Courtship frequency averages (±SD) were 12.5 ± 17.71 and 12.75 ± 15.26 toward control and co-mimic models respectively. In Figs 2 and 3, probabilities below the line at $P = 1$ indicate that males approached or courted conspecific models more often than co-mimic model, whereas probabilities above the line, show that males were more attracted to co-mimics. When 95% confidence intervals intersect $P = 1$ males were as likely to approach or court either model.
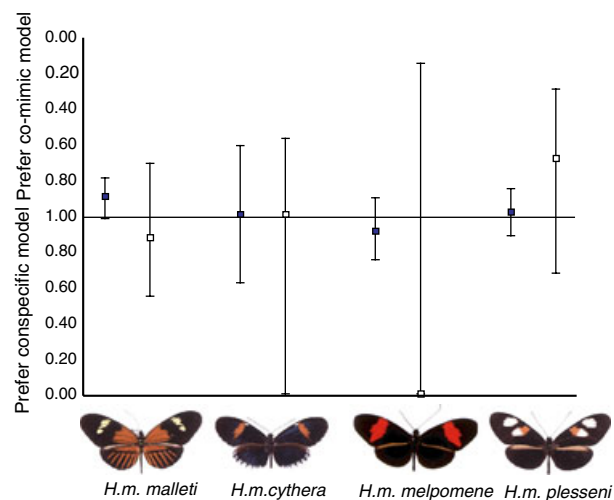
Fig. 2 Probabilities of *Heliconius melpomene* males approaching (■) and courting (□) models of their co-mimic races relative to their own female models. Pictures of *H. melpomene* males are shown on X-axis. Males were tested with their own species as well as with their co-mimic models for a period of 10 min each and 10 (or more) trials were carried out for every possible comparison. Each point in the graph represents the probability of a population of males approaching or courting a *H. erato* co-mimic model compared with their conspecific model set as $P = 1$. Therefore, probabilities below the line at 1 show preference for conspecifics, whereas probabilities above the line at 1 indicate preference for co-mimic models. Error bars show support limits equivalent to 95% confidence intervals. Probability and error bars were estimated using likelihood. Models were made with real butterfly wings.
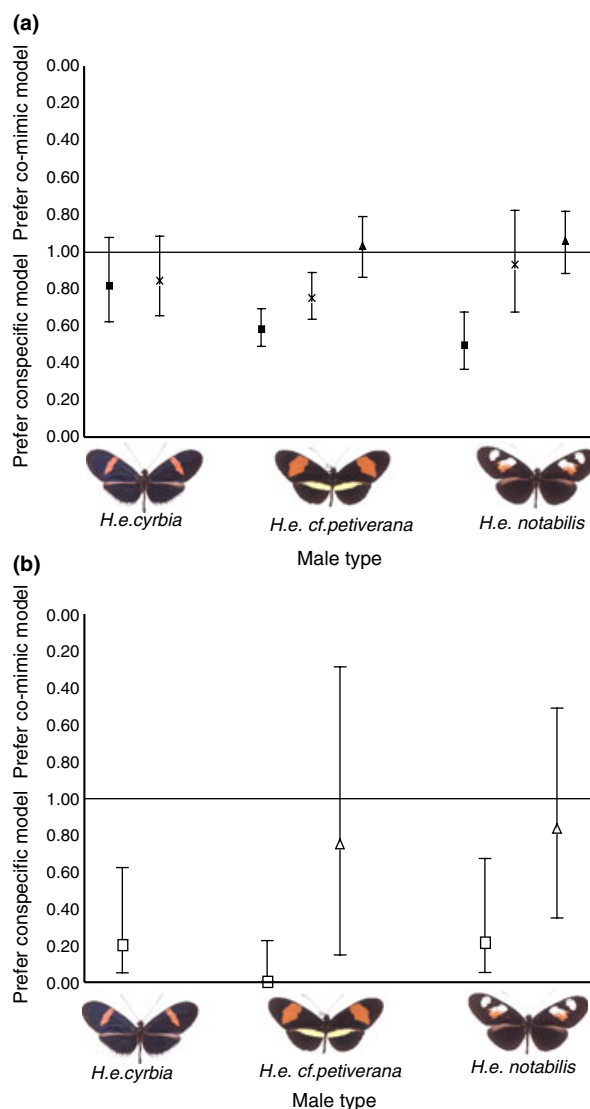
Fig. 3 Probabilities of *Heliconius erato* males approaching (a) and courting (b) models of their co-mimic races relative to their conspecific female models. Pictures of *H. erato* males are shown on the bottom of the figure. Males were tested with their own species as well as with their co-mimic models for a period of 10 min each and 10 (or more) trials were carried out for every possible comparison. Each point in the graph represent the probability of a population of males approaching or courting a *H. melpomene* co-mimic model compared with their conspecific model set as $P = 1$. Therefore, probabilities below the line at 1 show preference for conspecifics, whereas probabilities above the line at 1 indicate preference for co-mimic models. Error bars show support limits equivalent to 95% confidence intervals. Probability and error bars were estimated using likelihood. Models were made with butterfly real wings (■/□), printed photographs (*) and wings washed with hexane (▲/Δ). Courtships toward photograph models were almost absent and those results are not shown.

In contrast to *H. melpomene, H. erato* males (*H. e. cyrbia, H. e. petiverana* and *H. e. notabilis*) were more likely to approach and court conspecific dissected wing models (Fig. 3). However, when experiments were repeated using paper models *H. e. cyrbia* and *H. e. notabilis* males approached co-mimics as frequently as conspecifics. Furthermore, when the real wings were washed in hexane, males similarly failed to distinguish conspecifics from co-mimics and approached and courted both models alike (Fig. 3). These patterns suggest a potential role of wing chemical odours in early states of species recognition for *H. erato* males. The average (±SD) number of approaches to conspecific and co-mimic models were 194.67 ± 133.77 and 117 ± 73.53 respectively for wing models, 176.33 ± 128.70 and 139.67 ± 87.89 respectively for paper models, and 231.5 ± 13.44 and 238 ± 5.66 respectively for hexane-washed models. The frequency of courtship toward models was very low (Fig. 3b). The average number of courtship behaviours exhibited toward conspecific and co-mimic models were 13 ± 2.64 and 2 ± 1.73 respectively when using wing models, and 8 ± 5.66 and 6.5 ± 4.95 respectively for hexane-washed models. Courtship towards paper models was again rare and results are not shown.

H. e. petiverana males differ from the other two *H. erato* races, as they were still more attracted to their own species than their co-mimics when tested with paper models. It is unclear why this was the case given that the same males were unable to distinguish conspecific and co-mimic wings after washing them with hexane. It seems most likely that a slight difference in the hue of the red band between the paper models or some other aspect of the pattern reproduction during printing led to this result. *H. m. rosina* wings used for the photograph came from a slightly older individual compared to the one used for *H. e. petiverana*. A change in colour from bright red to orange is common in both species when individuals age (Crane, 1954) as a consequence of oxidation of the red xanthommatin pigment (Gilbert *et al.*, 1988). Therefore, preferences for younger females may have affected our results in the comparison between co-mimics attraction. More experiments need to be done in order to confirm this observation.

Discussion

Our experiments show that *H. erato* males use wing colour patterns in mate recognition and that in general are more likely to approach and court models of their own colouration than those of other races of the same species. Thus, extending previous work, we have shown that both co-mimics, *H. erato* and *H. melpomene* use the same signals to find and recognize mates (Crane, 1955; Jiggins *et al.*, 2004). In experiments between co-mimics, we found that both species spent considerable time approaching and courting one another, suggesting that there is a genuine cost of mimicry because of a reduction

in the efficiency of species recognition. Males approached co-mimic females more than half as frequently as their own females and even attempted copulation with them in few cases (Table 2). However, in spite of the initial attraction, at close range both species could distinguish conspecifics from co-mimics probably using additional signals. Thus, reproductive interference because of signal confusion during mate attraction might result in fitness lost involving waste of energy or time but not waste of gametes. Mating between these species seems unlikely, since, to the best of our knowledge, mating or hybrids between *H. melpomene* and *H. erato* group species has never been seen in nature or captivity (Mallet *et al.*, 2007).

The behaviour of males toward wing models was different in the two species. In general males of the four races of *H. melpomene* were initially attracted to co-mimic models as much as those of their own females and therefore appeared to be using visual recognition signals only. In contrast, when models made of real dissected wings were used, *H. erato* males were more likely to approach and court their own female than those of *H. melpomene*. However, the ability of *H. erato* males to distinguish conspecific female models disappeared when paper or hexane-washed models were used. These results suggest that in these experiments, *H. erato* but not *H. melpomene* males were using additional cues, presumably chemical, in the initial recognition of conspecifics. The difference between species might be a result of the *H. erato* mating system. Pupal mating males strongly depend on chemical cues in order to localize pupae and determine their sex and developmental state (Deinert, 2003). It is possible that a chemical recognition system might also function in distinguishing adult females in flight, as suggested by our experiments.

The use of multiple signals is common in animal communication and several hypotheses have been proposed to explain the evolution of different cues used at various stages of the courtship display (Candolin, 2003; Hebets & Papaj, 2005). Multiple signals might provide receivers with different types of information about potential mates (multiple messages), or give the same information, increasing the accuracy of the assessment of species and quality (Backup signal) (Johnstone, 1996; Hankison & Morris, 2003). The relative importance of chemical and visual signals in mate choice varies among butterflies. Although brightness of UV reflectance was a better predictor than pheromones of male mating success in *Colias eurytheme* (Pieridae) (Papke *et al.*, 2007), both kind of signals where equally important in choices made by female *Bicyclus anynana* (Nymphalidae: Satyrinae) (Costanzo & Monteiro, 2007). Our experiments did not specifically tested the relative importance of visual and chemical signals but suggest that pheromones are necessary for mate recognition and mating in *Heliconius*. First, species-specific pheromones, and other signals, likely different between co-mimics, override initial attraction

and recognition by wing colouration, discouraging mating. Similarly, high levels of hybridization are found where geographic races come into contact in the wild (Mallet, 1993). This suggests that despite some degree of premating isolation because of colour pattern preferences, the presence of species-specific signals are enough for mate recognition in the absence of precise visual cues. Finally, courtship displays toward wing models, in this and similar studies, were infrequent and short, and males never tried to copulate with a model (Crane, 1955; Jiggins et al., 2004). This could be explained, in part, by the lack of additional signals. Our data thus support earlier observations that in butterflies visual cues play a major role in initial attraction between potential mates, whereas visual, pheromonal, and additional signals and behaviours are used during courtship to assess mate quality (reviewed by Scott, 1973; Silberglied, 1977).

Colour vision in Heliconius and other species of butterflies has been extensively studied (Swihart, 1967; Briscoe & Chittka, 2001; Hsu et al., 2001; Stavenga, 2002; Zaccardi et al., 2006). Visual acuity is low, so it is perhaps not surprising that we find no evidence that co-mimics can distinguish one another in flight using minor wing pattern differences (Rutowski & Kimball, 2000). The colour of the patches seems to be the more important cue driving male preferences (Kronforst et al., 2006), and species have evolved visual systems that maximize the reception in those ranges of the spectrum that match those colours (Swihart, 1967). Such spectral tuning is probably because of filtering pigments (Stavenga, 2002; Zaccardi et al., 2006) rather than to modification in the amino acid sequence of the opsin protein sensitive to long wavelength region (yellow to red) (Hsu et al., 2001). We found general patterns in the degree of attraction of some colours to males comparable to those found in H. melpomene (Jiggins et al., 2004). For example here, as in H. melpomene trials, wide red bands in the forewing (e.g. H. e. petiverana and H. e. hydara) were attractive to males of all races, except for the iridescent blue co-mimics H. e. cyrbia and H. m. cythera (Jiggins et al., 2004). It is possible that the latter races were using polarized light for communication in a manner similar to the iridescent blue butterfly H. cydno chioneus (Sweeney et al., 2003). Our results thus suggest that co-mimic races have converged in their preferences for particular cues present on their wings (e.g. polarized light, red bands, UV reflectance).

The evolutionary consequences of a conflict between species recognition and mimicry are unknown. Identification errors likely persist in nature because the benefits of mimicry strongly outweigh the costs. Nonetheless, interspecific confusion generated by mimicry is expected to lead to selection for alternative recognition systems, preferably not detectable by predators that could serve as species-specific mate recognition. Indeed, it has been suggested that closely related mimetic butterflies may rely more on olfactory than visual cues for sexual attraction (Poulton, 1907; Boppré, 1978; Vane-Wright & Boppré, 1993). There is evidence for highly developed chemical signalling in mimetic butterflies. For example, male Danaini (Nymphalidae: Danainae) have complex and often species-specific abdominal hairpencil structures and aphrodisiac pheromones that are used during courtship (Boppré, 1984; Boppré & Vane-Wright, 1989). Similarly, Ithomiini (Nymphalidae: Danainae) males sequester pyrrolizine alkaloids from plants and use them for defence and as precursors of pheromones (Brown, 1984; Schulz et al., 2004). Lactones released from hairpencil structures are known to serve as chemical signals that allow recognition in intra and interspecific male-male and male-female courtship pursuits, which happen frequently among individuals belonging to the same mimicry ring (Pliske, 1975). The opportunity for interspecific attraction is high since these butterflies have temporal and spatial associations between species that belong to the same mimicry ring, due perhaps to host plant spatial distribution (Medina et al., 1996; Beccaloni, 1997; Devries et al., 1999; Willmott & Mallet, 2004).

Heliconius butterflies, although often involved in mimicry rings, do not have complex male scent organs like those found in Danainae. Nevertheless, there is an intriguing pattern whereby intrageneric mimicry is commonly found between species in different clades, normally involving one member of the 'pupal mating' clade and one of the 'nonpupal mating' clade (Gilbert, 1991; Beltrán et al., 2007). Perhaps the costs of interspecific attraction override the benefits of mimicry when closely related species resemble one another, thus reducing the occurrence of such mimicry in nature. Interspecific hybridization in Heliconius occasionally happens in the wild but the rates of hybridization are negatively correlated with the degree of genetic divergence (Mallet et al., 2007). Therefore, it seems possible that mimicry evolves more readily between distantly related species where hybridization is unlikely (Mallet et al., 2007), reducing the costs of interspecific attraction to a waste of energy but not gametes.

In summary, although the role of visual communication and colour pattern in species recognition is well established in butterflies (Brower, 1959; Silberglied & Taylor, 1973; Shapiro, 1983; Wiernasz, 1989; Cook et al., 1994; Jiggins et al., 2001), this is the first study that has shown mutual sexual attraction between con-generic mimetic species. Our results do not begin to measure the actual or relative cost of this confusion to wild butterflies but imply that such a cost exists. Given the exact and repeated mimetic convergence between the species studied, any cost of convergence is presumably heavily outweighed by mimetic advantage. Nonetheless, this phenomenon could alter the dynamics of Müllerian mimicry. Number-dependent mimicry theory suggests that once patterns have reached certain abundance, selection might be relatively weak (the 'plateau' of Mallet & Joron, 1999). Thus, in this region the cost to mimicry

imposed by mating confusion might outweigh the selection pressure for convergence. Such a cost might therefore contribute to an explanation of apparently stable diversity in mimetic patterns.

Many organisms besides the Lepidoptera are known to be involved in visual mimicry. For example, mimicry occurs in other arthropods such as spiders, hoverflies, and lycid beetles (Linsley et al., 1961; Oliveira, 1988; Howarth et al., 2004), and vertebrates such as poison dart frogs, fish, birds and snakes (Dumbacher & Fleischer, 2001; Symula et al., 2001; Caley & Schluter, 2003; Moland et al., 2005). At least some of these groups are also known to use visual cues for species recognition and in sexual selection (Saetre & Slagsvold, 1996; Andersson & Amundsen, 1997; Seehausen & van Alphen, 1998; Summers et al., 1999; Couldridge & Alexander, 2002; Siddiqi et al., 2004). Even though in most cases individuals rely in multiple signals to choose a partner (Candolin, 2003; Hebets & Papaj, 2005), it might be a general phenomenon that close phenotypic resemblances could impose a cost to mimicry because of mistaken identity during courtship.

Acknowledgments

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Supplementary Material

The following supplementary material is available for this article:

Table S1 Summary of pairwise tests done between *H. erato* geographic races and between co-mimic races of *H. melpomene* and *H. erato*.

Table S2 Reflectance spectra of colour patterns in wing and paper models.

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