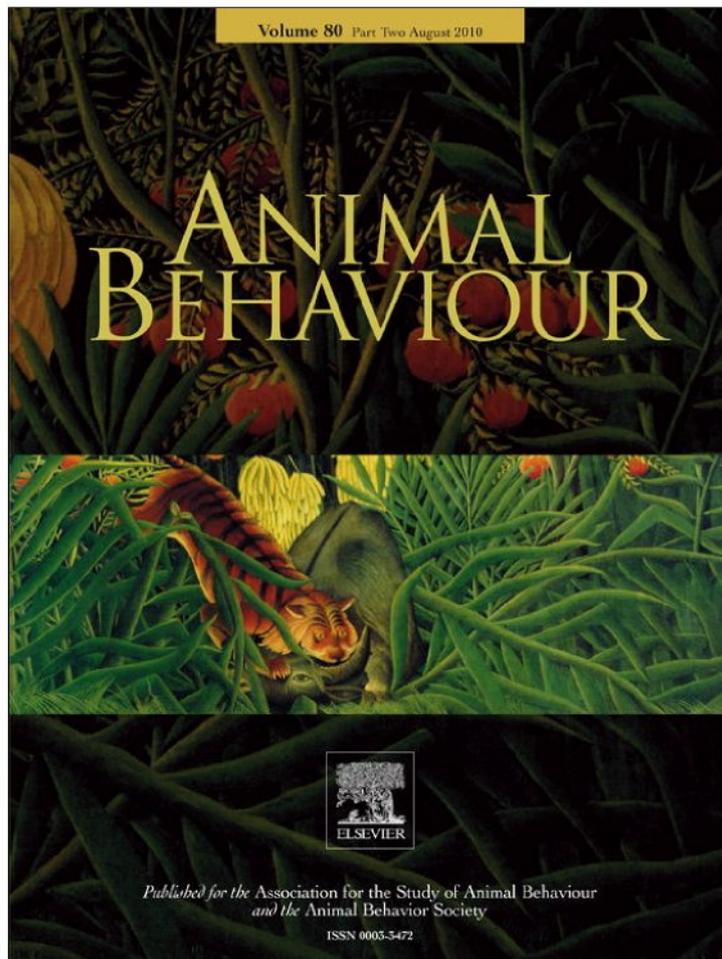


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Host plants and immatures as mate-searching cues in *Heliconius* butterflies

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The study of interactions between phytophagous insects and their host plants extends beyond understanding how insects deal with plant chemical defences. Sexual behaviour of these herbivores is integrated in several ways with host plants, as the latter influence timing and location of reproduction, and can provide clues for finding mates. Nevertheless, while numerous studies link butterfly evolution to host plant adaptations, the influence of plants on butterfly sexual behaviour has been little studied. We conducted experiments to determine the role of host plant cues in mate-searching behaviour of *Heliconius charithonia* butterflies. This species exhibits precopulatory mate guarding behaviour, wherein males find and perch on pupae, then copulate with eclosing females ('pupal mating'). We found that males (1) visited plants damaged by feeding larvae more often than they visited undamaged plants and (2) displayed searching behaviour around the plant and in front of larvae, suggesting that odours signal the location of potential partners (pupae). Although males were attracted to common plant odours released after tissue damage, plants damaged by heterospecific butterfly larvae were less attractive, indicating that species recognition can occur at early life stages. Overall, our results suggest that host plants influence mate-searching behaviour of *Heliconius*. This might also be true for other species of butterflies with more conventional mating strategies, potentially contributing to the diversification of this group of phytophagous insects.

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Sexual behaviours of phytophagous insects and their host plants are connected in numerous ways (Landolt & Phillips 1997; Cocroft et al. 2008). Plants can influence the timing and location of insect reproduction as several species of insects meet, court and mate on or close to their host plants (e.g. Feder et al. 1994; Thomas et al. 2003; Cocroft et al. 2008). Such plants are good encounter sites since females are likely to visit these sites to feed or oviposit. Moreover, for species whose females are sexually receptive shortly after eclosion, the probability of finding virgins around host plants can be high (Thornhill & Alcock 1983; Rutowski 1991). Mate searching by males can also be influenced by plant chemical and visual characteristics (Prokopy & Owens 1983; Landolt & Phillips 1997; Reddy & Guerrero 2004). In several orders of insects host plant volatiles released after larval or female feeding can be used as mate-finding cues (e.g. Ruther et al. 2000; Tooker et al. 2002; Linn et al. 2003; Ginzel & Hanks 2005) or might enhance attractiveness of aggregation or sex pheromones (e.g. Deng et al. 2004; Soroker et al. 2004; Yang et al. 2004).

As with other phytophagous insects, mate-locating behaviour in some species of butterflies is linked to larval host plants (Scott 1975; Rutowski 1991). In the latest review of such behaviour in butterflies Rutowski (1991) found that host plants influence mate searching in 10 of 44 species (9 genera, 4 families). These examples range from males choosing to perch or patrol in areas where host plants are abundant, to those that focus their attention on the plant themselves when seeking females, in some cases even before female eclosion (Borch & Schmid 1973; Gilbert 1976; Elgar & Pierce 1988). Several studies have shown that adaptation to new hosts plants is important for butterfly diversification (Ehrlich & Raven 1964; Braby & Trueman 2006; Weingartner et al. 2006; Peña & Wahlberg 2008). While this has traditionally been considered mainly a consequence of coevolution of larval feeding and plant chemical defences (Ehrlich & Raven 1964), it is possible that incorporating plant and plant habitat cues while searching for mates could also significantly influence the evolution of this group of insects (Gilbert 1978; Dennis & Shreeve 1988) as has been demonstrated in other phytophagous insects (Feder 1998; Funk 1998; Via 1999; Drès & Mallet 2002; Cocroft et al. 2008).

Here we investigate the importance of host plants in male-searching behaviour of *Heliconius* butterflies (Nymphalidae: Heliconiinae). In these insects, habitat and host species partitioning have been seen in groups of sympatric species whose larvae feed exclusively on plants in the genus *Passiflora* (Passifloraceae) (Benson

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1978; Gilbert 1991; Estrada & Jiggins 2002). Similar to other butterflies (Gilbert & Singer 1975), in some *Heliconius*, oviposition preferences rather than plant palatability to larvae alone (Smiley 1978) have shaped patterns of host plant use. This case of ecological monophagy has been explained in part by *Heliconius* male searching and mating behaviours (Gilbert 1978, 1991). Males in the genus establish home ranges where they periodically visit habitat patches with adult resources and approach host plants looking for mates (Ehrlich & Gilbert 1973; Brown & Benson 1977; Mallet & Gilbert 1995). Moreover, in about half of the *Heliconius* species males specialize in searching for immatures. Their mating strategy involves repeated visits to locations with pupae to monitor their development. At the end of the pupation period males identify a pupa's sex using chemical signals (Deinert 2003; Estrada et al. 2010), perch on female pupae, and wait until they are able to mate with the emerging adult (pupal mating) (Gilbert 1976; Deinert et al. 1994). The role of host plants seems to be central in finding conspecific pupae given that long-range pupal pheromones have not been found, and that pupae detached from host plants are less likely to be detected by males in insectaries (Estrada et al. 2010). Pupal-mating behaviour evolved once in *Heliconius* and appears to occur in all species within a monophyletic clade in the genus (18 spp.) (Beltrán et al. 2007).

We conducted experiments designed to test the hypothesis that host plants influence mate-locating behaviour in the pupal-mating species *Heliconius charithonia* (Linnaeus). We measured attraction of males to host plants with different treatments in a greenhouse population in order to answer the following four questions. (1) Are host plants or particular immature stages of *H. charithonia* on the plants attractive to males? (2) Which cues do males use to find future mating opportunities? (3) Are cues produced by host plants, immature *H. charithonia*, or both? (4) At what point in the process of locating future mating sites does species recognition occur?

METHODS

Butterfly and Plant Rearing

Bioassays were performed using *Heliconius charithonia vazquezae* from a captive population started with about 20 adults and larvae collected around Austin (TX, U.S.A.) and supplemented periodically with wild-caught individuals. Breeding populations were maintained in 4 × 6 m Lord and Burnham greenhouses at the University of Texas at Austin at about 32 °C and at high humidity where they breed freely using *Passiflora biflora* and *P. lutea*. Adults were fed ad libitum with sugar and honey water solution (10%) and with pollen and nectar from *Psiguria* spp., *Psychotria poeppigiana* and *Lantana camara* flowers.

Table 1
Experimental design used to find cues involved in discovery and recognition of mates in the pupal mating butterfly *Heliconius charithonia*

Experiment objective	Control	Treatment	N
To examine whether host plant or immature stages of <i>H. charithonia</i> are attractive to males	Undamaged host plant	Fifth-instar larvae on host plant	19
	Undamaged host plant	Prepupae on host plant	10
	Undamaged host plant	Young pupae on host plant	10
To examine the use of visual and chemical cues to find host plants	Exposed larvae on host plant (visual and olfactory cues from larvae and damaged tissue)	(1) Silicone larvae model on host plant (remove damaged tissue and larvae odours)	12
		(2) Concealed larvae on host plant (remove visual cues from larvae)	
To examine origin of cues	Concealed fifth-instar larvae allowed to eat (odours of larvae and host plant)	(1) Host plant material cut with scissors (remove odours from larvae)	15
		(2) Concealed fifth-instar larvae on host plant not allowed to eat (remove odours of damaged tissue)	
To test for species recognition cues during mate searching	Conspecific larvae	Heterospecific larvae (<i>Agraulis vanilla</i>)	15

White-coloured larvae pupate on or close to host plants and eclose as adults after 9–12 days depending on ambient temperature. Pupae are typically light brown, but the cuticle becomes translucent, and wings and adult body colours are revealed about 24 h before eclosion. The main host plant of *H. charithonia* in central Texas is *Passiflora lutea*, which is also used by other Heliconiini such as *Agraulis vanilla* and *Dryas iulia*. For the experiments, we collected *P. lutea* from natural populations along Waller Creek at the campus of the University of Texas in Austin, potted them and kept them isolated from female butterflies prior to the experiments.

Greenhouse Assays: General Methodology

We conducted tests to assess the role of larval host plants on mate-searching behaviour of *H. charithonia* males from May to August 2005–2007. We conducted simultaneous choice experiments using a 22 × 10 × 10 m subcompartment of a 22 × 22 × 10 m greenhouse at Brackenridge Field Laboratory at the University of Texas (Austin). Butterflies were kept in a semi-natural, heterogeneous habitat. In addition to host plants for the experimental butterflies, the greenhouse housed an array of tropical trees, understory monocots and large vines as well as *Psiguria* spp., *Psychotria poeppigiana*, *Cnidoculus multilobus* and *Lantana camara* that provided nectar and pollen. Temperature fluctuated daily from about 20 to 32 °C, and light/dark periods followed natural cycles, as the walls and roof of the greenhouse were made with clear polycarbonate panels. Exhaust fans extending the entire length of one side of the greenhouse drew outside air over an evaporative surface. This allowed for a gentle unidirectional flow of cool humid air perpendicular to the long axis of the greenhouse during warm parts of the day when butterflies are active.

To answer each of the four questions mentioned in the Introduction we performed 10–19 experimental replicates wherein a specific potential searching cue (treatment) was paired with a control. Experiments consisted of the introduction of *P. lutea* in the greenhouse with a resident population of *H. charithonia* males. Depending on the question, such plants were presented intact or had different types of damage (see below and Table 1).

Before each test, we introduced 13–15 unmated males to the greenhouse from our breeding population. These experimental males were allowed to habituate to the arena for about 20 h. Only males more than 5 days old were used. For each individual, forewing length was measured with a calliper to the closest 0.01 mm and wings were marked with a colour code using Sharpie® permanent markers so individual recognition was possible. Males were used no more than twice, but individuals were exchanged between different experiments. Thus, typically, males without previous experience in the experimental greenhouse, beyond the

habituation period, accounted for about half of the tested group. After tests, males were returned to the breeding populations. Butterflies (adult or immature) different to those used in the assays were excluded from the experimental arena during the entire period the trials were conducted.

We placed potted control and treatment *P. lutea* plants in the greenhouse at the start of each experiment. They were located in places where butterflies had not encountered a host plant previously, and at equivalent distances from pollen and nectar sources. Test *Passiflora* were placed more than 5 m apart from each other, and their position inside the greenhouse was changed between consecutive experiments to minimize the effect of plant location on the results. *Passiflora lutea* chosen from a pool of 20 plants were matched for size and rotated between tests as much as possible. We videotaped control and experimental plants for 5 h (0900–1600 hours) and registered the number and identity of males that approached the plant, the time they spent visiting, and the behaviours shown. We consider the plant to have been approached if a male diverted his flight path and flew in front of any part of the plant. Males attracted to plants showed three main characteristic behaviours: (1) they hovered in front of one restricted section of the plant, (2) they hovered near the immature or an experimentally damaged part of the plant, or (3) they flew up and down the plant and surroundings in a conspicuous 'searching' mode. In some cases, males displayed a combination of behaviours 2 and 3 in the same visit. Video cameras were set on tripods attached to step ladders such that the field of view included the whole focal plant plus a similar area around this. This allowed us to register the total number of butterflies that flew by the plant without approaching it. Such information was used to control for the effect of plant location and butterfly activity levels across tests. Videos were digitized using Windows Movie Maker version 5.1. Visiting time was measured to the closest second using this software.

Greenhouse Assays: Specific Tests

Table 1 summarizes the experiments conducted to answer the four study questions.

Experiment 1

Are host plants or particular immature stages on the plants attractive to males? We first examined whether males were searching for host plants alone, or whether they were attracted to a particular immature stage of *H. charithonia* present on the plant. For this set of experiments we used two plants. We paired undamaged *P. lutea* (control) with plants carrying (1) a fifth-instar larva, (2) prepupae or (3) a young pupa of *H. charithonia*. Prepupae are larvae at the end of fifth-instar that have ceased feeding and have changed coloration slightly before suspending themselves for pupation. To decrease the probability of confounding results from male attraction due to release of plant volatiles after tissue damage, plants used as undamaged plant treatments or those carrying prepupae or pupae had not been exposed to herbivory before the experiment. In contrast, plants with larvae were often reused for this treatment, although larvae were removed after the tests and plants were protected from herbivory for several days prior to the following experiment. The sex of immature *H. charithonia* used was determined after experiments, but was not considered in the analysis since males in another pupal-mating species are apparently unable to distinguish the sex of the pupae until few days before eclosion (Deinert 2003).

Experiment 2

Which cues do *H. charithonia* males use to find future mating opportunities? In the second set of experiments, we asked whether

male preference for plants carrying larvae (from experiment 1) is triggered by (1) olfactory cues from larvae alone or larval interaction with the plant, (2) visual cues from larvae, or (3) a combination of both (1) and (2). For each test we placed three *P. lutea* plants (each representing a separate treatment) in the greenhouse. The first plant had two *H. charithonia* fifth-instar larvae. We assumed that this situation provided both olfactory and visual cues from the plant and the larvae, and hence used it as a control to compare with data from the other two treatments (see below). Larvae were set on the tops of leaves, and although they typically moved little during experiments, there were occasions when they went below leaves and were not visible to us (and probably not visible to males) from above. The second plant had two models of a fifth-instar caterpillar. Silicon rubber models were made following Papaj & Newsom (2005) with larval setae imitated using pieces of black minutien (Austerlitz insect pins 0.15 mm). The rubber models were painted with water colours. We matched the dominating colour of the larval body with the one in the model using a spectrophotometer Ocean Optics Base 32, Version 1.03.0 (see Supplementary material, Fig. S1). Models were set in a wire and attached to plants on the tops of leaves. Plants from this treatment lacked odours from either larvae or damaged tissue when compared to the control. Finally, in the third treatment, two larvae were placed on one branch of the plant that was isolated inside a green net and hidden behind the foliage. Plants from this treatment thus provided olfactory cues (larvae were allowed to eat and thus damage the plant) but lacked visual cues from larvae when compared to the controls because odours escaped the net, but larvae could not be seen by males.

Experiment 3

Are cues produced by host plants, immature *H. charithonia*, or both? The third set of experiments attempted to find the source of attractive chemical compounds indicated by the previous experiments. Each test consisted of two treatment *P. lutea* plants and one control *P. lutea* plant. As a control, we used a plant with two larvae and a plant branch concealed inside a green net. The treatment had both larval and host plant odours because the larvae were allowed to eat. In one treatment, we cut a small section of one leaf, proportional to the amount typically consumed by two fifth-instar caterpillars, every hour using scissors. This treatment lacked odours from larvae or larva–plant interactions when compared with control plants. The other treatment was similar to the control except that larvae concealed inside the net did not have access to leaves and could not eat. Thus, this treatment lacked odours from plant-damaged tissue when compared to the control.

Experiment 4

At what point in the process of locating future mating sites does species recognition occur? The final set of experiments aimed to determine whether *H. charithonia* males use species-specific cues in their searching behaviour for immature *H. charithonia*. For each test, we placed two *P. lutea* plants, one with two fifth-instar larvae of *H. charithonia* and the other with two larvae of *Agraulis vanilla* (see Supplementary material, Fig. S2), which normally coexists and shares hosts with *H. charithonia* in nature.

Statistical Analysis

General activity levels and searching behaviours of *H. charithonia* males varied considerably within and among experiments. Some males were frequently seen searching around plants while others primarily showed patrolling behaviours and engaged in long male–male chases. Such variation was seen between individuals as well as between groups of males through time in these experiments. To control for this variability, we calculated an attraction

index, measured as the number of visits received by each plant divided by the total number of butterflies captured in the videos on that particular day. Results of the analyses with the index were more conservative but in all cases similar to those obtained with raw data from male visiting behaviour (see Supplementary material, Fig. S3). Proportions were arcsinesquare-root transformed and the mean indexes compared with a paired *t* test or a general linear model (GLM) in experiments with two and three plant treatments, respectively. Analyses with GLM were done with the index of attraction as the dependent variable, and treatment (control and experimental host plants) and test (replicates) as fixed factors, thus comparable to a two-way ANOVA without replication (Sokal & Rohlf 1969). The average time males spent visiting each plant was square-root transformed and analysed in the same way as the index of attraction. Post hoc pairwise comparisons were done with Tukey HSD test. Data were analysed using SPSS 16.0.1 (SPSS, Inc., Chicago, IL, U.S.A.) for Windows.

RESULTS

Within each group of males only a small proportion approached test plants (mean \pm SE = 0.25 ± 0.02), and often the same individuals visited them repeatedly throughout the duration of the trial (mean \pm SE number of visits by the same male = 2.78 ± 0.19 ; range 1–26). When data from all experiments were pooled together, males that approached plants had, on average, longer wing lengths than those that did not, although the difference was very small (mean \pm SE: approaching males: 43.48 ± 0.2 mm; other males: 42.33 ± 0.21 mm; *t* test: $t_{253} = 3.96$, $P < 0.001$). The probability of approaching test plants was independent of the previous experience males had inside the greenhouse, as indicated by the fact that a similar proportion of males used for the first and second trial responded to our treatments (chi-square test: $\chi^2_1 = 0.19$, $P = 0.66$).

Experiment 1

Males that flew close to experimental *P. lutea* were attracted more often and for longer periods to plants with larvae than they were to undamaged plants (controls) (Fig. 1, Table 2). No such preference was observed in treatments with nonfeeding immature stages (prepupae and pupae) (Fig. 1, Table 2). Males visited the same plant repeatedly during the course of a test, but, on average, more different individuals were attracted to plants with larvae than to plants with other immature stages or controls (chi-square tests: control versus plant with larvae: $\chi^2_1 = 13.69$, $P < 0.001$; control versus plant with prepupae: $\chi^2_1 = 0.48$, $P = 0.49$; control versus plant with pupae: $\chi^2_1 = 0.44$, $P = 0.51$; Fig. 1a). In a high percentage of visits to control plants (62%) and plants with prepupae (63%) and pupae (94%), males approached the foliage and left within a few seconds (versus 41% for plants with larvae). Plants with larvae more often elicited searching behaviour (47% visits), which consisted of males flying up and down the plant and the surroundings, than did control plants (37%), or plants with prepupae (22%) or pupae (24%). Males clearly recognized the presence of immatures as they hovered in front of them, often for several seconds. This behaviour was, however, more frequently seen in treatments with larvae (52%) than in treatments with prepupae (26%) or pupae (12%).

Experiment 2

In the second set of experiments we investigated whether host plants with larvae caused arrestment of males due to (1) olfactory cues from larvae (or their interaction with the plant), (2) visual cues from larvae, or (3) a combination of both. Plants with silicone larva models, lacking odours from larvae or damaged tissue, elicited

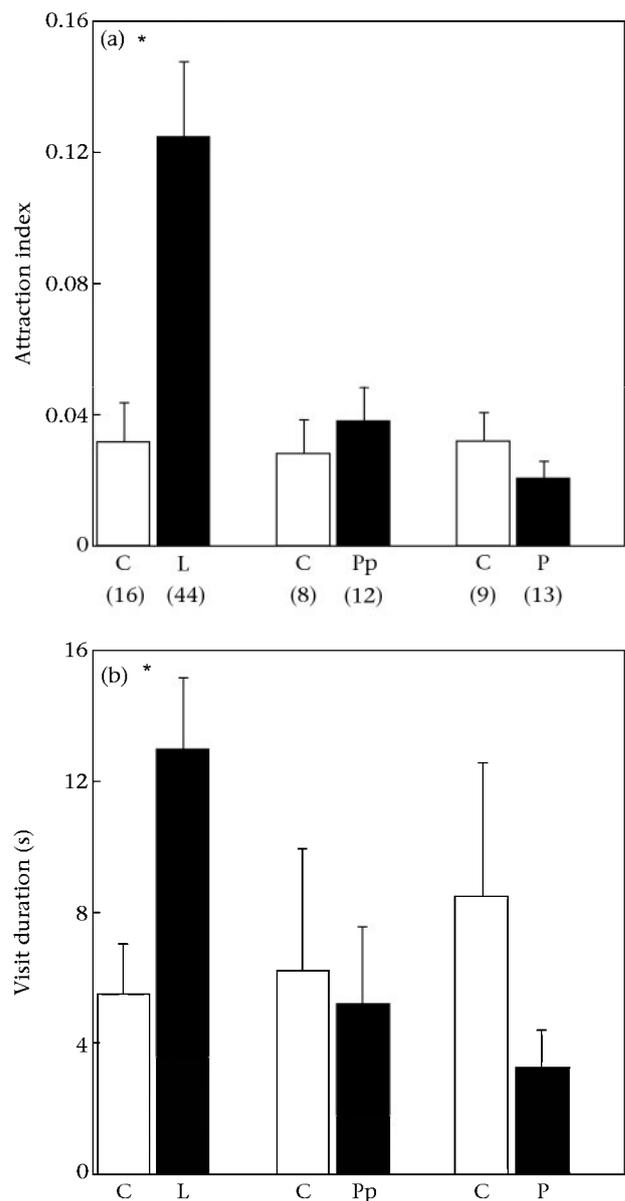


Figure 1. (a) Attraction index and (b) average visit duration of *Heliconius charithonia* males to *Passiflora lutea* plants in experiment 1 (mean \pm SE, $N = 19$ for experiments with larvae, $N = 10$ for experiments with prepupae and pupae). Treatments included undamaged *P. lutea* (C = controls) paired to plants with fifth-instar larvae (L), prepupae (Pp) or pupae (P). Asterisks (*) indicate significant differences ($P < 0.05$, paired *t* test; Table 2). Overall number of different males that visited plants in each treatment is given in parentheses in (a).

fewer and shorter visits compared to plants with live larvae, regardless of whether they were visible to or concealed from males (Fig. 2, Table 3). Significant differences were found in the relative number of visits between the three treatments, although Tukey post hoc pairwise comparisons were not significant (control versus visual cues: $P = 0.06$; control versus olfactory cues: $P = 0.95$). On the other hand, post hoc comparisons showed a significant difference in average visit duration between control plants and plants with silicone models ($P = 0.02$) but not between control plants and plants with concealed larvae ($P = 0.997$), suggesting that olfactory cues alone can attract males flying close by. In fact, most visitors of plants with exposed (control) and hidden larvae, presumably due to presence of attractive odours, showed searching behaviours

Table 2
Results of paired *t* test for experiment 1

	Control vs larvae		Control vs prepupae		Control vs pupae	
	t_{18}	P^*	t_9	P^*	t_9	P^*
A	5.197	<0.001	0.695	0.505	0.532	0.607
B	3.474	0.003	0.253	0.806	1.259	0.240

Treatments included undamaged *Passiflora lutea* (control) paired with plants carrying fifth-instar larvae, prepupae or pupae of *H. charithonia*. A: average attraction index, B: average visit duration.

* Reported *P* values are for two-tailed *t* test. Values in bold represent significant effects.

around the plant (72%), while fewer males searched when only visual cues from the silicone larva models were present (24%). As in experiment 1, males hovered in front of live larvae in most visits (69%), and at least in some cases, in front of models (35%). Although we matched the base coloration of the model to that of live animals, the former lacked ultraviolet (UV) marks present in some fifth-instar *H. charithonia* (Estrada 2009). Given that in some visits males hovered in front of our models, it is possible that the lack of UV signals might have accounted for the lower attraction of this treatment to males. More individual males were attracted to plants with live larvae than to plants with the silicone model, although the differences were not significant (chi-square tests: control versus plants with model: $\chi^2_1 = 2.77$, $P = 0.09$; control versus plants with concealed larvae: $\chi^2_1 = 0.6$, $P = 0.44$; Fig. 2a).

Experiment 3

Here we asked whether olfactory cues that caused arrestment in males were produced by the plant, the larvae or a product of their interaction. Male responses towards plants that were damaged with scissors, or exposed to herbivory or nonfeeding larvae did not differ significantly (Fig. 3, Table 4). While duration of visits to undamaged plants with nonfeeding larvae were slightly shorter, we found no significant difference in the relative number or duration of visits, or in the number of different males attracted to plants across treatments (chi-square tests: control versus plants cut with scissors: $\chi^2_1 = 1.12$, $P = 0.29$; control versus plants with larvae: $\chi^2_1 = 0.42$, $P = 0.52$; Fig. 3a). In about half of the visits to each of the three treatments, males showed the characteristic searching behaviour around the plant. Cues that attracted males appear to be released by both plants with damaged tissue and larvae.

Experiment 4

When we exposed males to plants with *H. charithonia* or *A. vanilla* larvae, they were more likely to visit and spend time searching plants that had conspecific larvae (paired *t* test: average attraction index: $t_{14} = 2.31$, $P = 0.04$; Fig. 4a), although the duration of visits did not differ significantly between treatments ($t_{14} = 1.55$, $P = 0.15$; Fig. 4b). Similarly, almost twice the number of different males responded to plants with conspecifics than to plants with *A. vanilla* (chi-square test: $\chi^2_1 = 5.96$, $P = 0.02$; Fig. 4a). However, once attracted to the plant, males in both treatments displayed a comparable high proportion of searching behaviour up and down the foliage (62%).

DISCUSSION

Our results show that host plants are important in mate-locating behaviour of *Heliconius* butterflies. These experiments do not allow us to establish how males narrow their search to suitable host plant habitats (Papaj & Prokopy 1989; Bell 1990), but suggest that once males are there, cues from the plant help them find

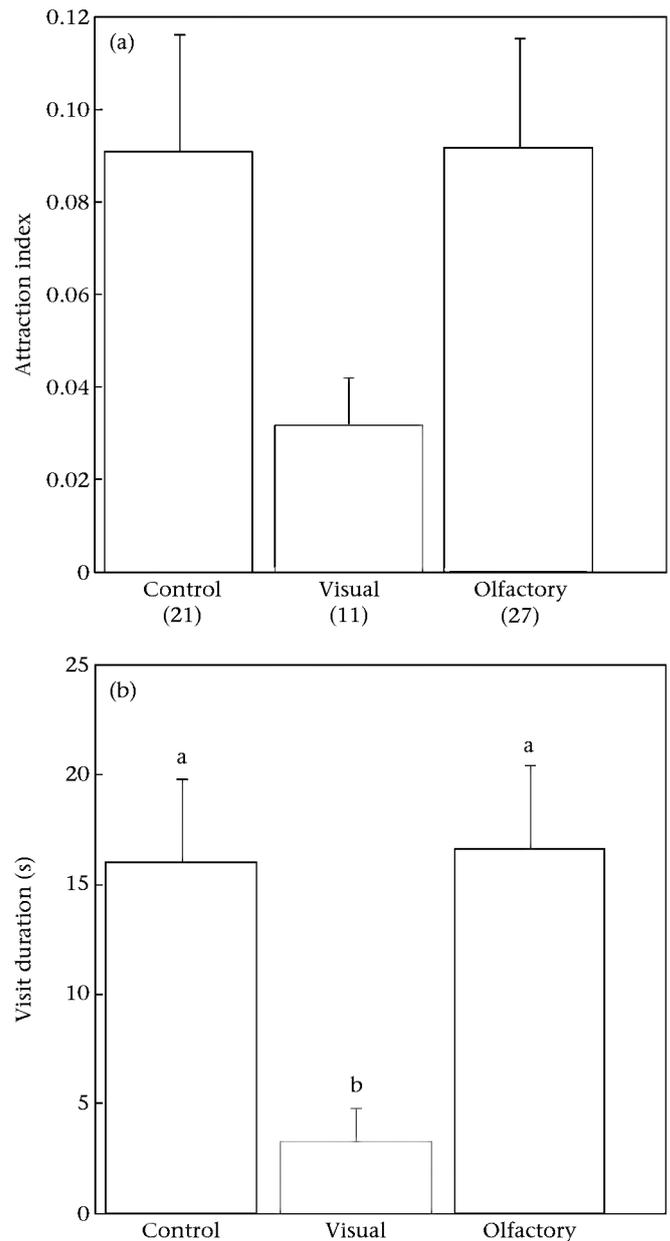


Figure 2. (a) Attraction index and (b) average visit duration of *Heliconius charithonia* males to *Passiflora lutea* plants in experiment 2 (mean \pm SE, $N = 12$). Treatments included plants with larvae (control), silicone model larvae (visual) and concealed larvae (olfactory). Bars labelled with different letters were significantly different ($P < 0.05$, ANOVA, Tukey HSD post hoc analysis; Table 3). Overall number of different males that visited plants in each treatment is given in parentheses.

potential mates. Although undamaged plants were visited occasionally, particularly when new shoots were exposed, visits and searches around plants increased by about threefold in the presence of chemical cues released by larvae or damaged tissue. This increase in the number of approaches was the compounded effect of more individual males being attracted, and more visits performed by those males. This suggests that odours probably not only signal the potential to find pupae, but could also trigger learning of a plant's location for future visits.

Before perching and guarding pupae, males typically monitor pupae periodically to assess their developmental state (Deinert 2003). Hence learning pupal location is a key factor in this mating strategy. There is compelling evidence of learning in insects

Table 3
Results of two-way ANOVA without replications for experiment 2

	Source	df	MS	F	P*
A	Tests	11	0.036	1.480	0.208
	Treatments	2	0.083	3.446	0.050
	Error	22	0.024		
B	Tests	11	4.525	1.515	0.196
	Treatments	2	19.801	6.631	0.006
	Error	22	2.986		

Treatments included *Passiflora lutea* plants carrying larvae (control), silicone model larvae and concealed larvae. A: average attraction index, B: average visit duration.

* Reported *P* values are for two-tailed *F* test. Values in bold represent significant effects.

(Papaj & Prokopy 1989; Dukas 2008), including butterflies (e.g. Rausher 1978; Weiss 1997; Weiss & Papaj 2003). *Heliconius*, in particular, probably have a sophisticated spatial memory. Their ability to remember locations is suggested by the establishment of site-specific nocturnal roosting (Turner 1971; Mallet & Gilbert 1995) and regular patterns of visits to adult resources (Gilbert 1991). This behaviour might also allow the incorporation of host plants themselves into periodic patrols until meristems are suitable for oviposition (Ehrlich & Gilbert 1973) or until mature pupae on those plants are found and ready to be guarded. Experiments in natural habitats (Mendoza-Cuenca & Macías-Ordóñez 2010) and observation in our insectaries have shown that pupae are rarely found and females eclose unguarded when pupae are artificially suspended on *Passiflora* only a few days prior to eclosion. This is not surprising, given that pupae have cryptic coloration and lack long-range attraction pheromones (Estrada et al. 2010). In contrast, bright coloration and plant volatiles released by feeding activity of larvae make the larvae more conspicuous than pupae to searching males. In fact, in only 3 of 20 tests plants with prepupae or pupae (15%) males hovered near the immature, in contrast to 68% when larvae were present. That the number of visits towards plants with prepupae and pupae increased about threefold when this behaviour was observed suggests that these immature stages are also interesting to searching males but are more difficult to find than larvae.

Mate searching, species recognition and mate choice in butterflies commonly involve the use of wing colour patterns (e.g. Wiernasz 1995; Jiggins et al. 2004; Papke et al. 2007). However, like *Heliconius*, several species of butterflies also utilize host plants and host plant habitats to find mates (Scott 1975; Rutowski 1991; Wiklund 2003). While not generally recognized as a mate-searching strategy, the ability of males to find plants is well known in other contexts among Lepidoptera. For example, males of certain butterflies and moths harvest alkaloids from withered plant tissue to use as defences and precursors for pheromones (Pliske et al. 1976; Weller et al. 1999), respond to female sex pheromones more effectively in the presence of plant volatiles (Emelianov et al. 2001; Deng et al. 2004; Yang et al. 2004), or choose nectar sources using visual and chemical floral cues (Weiss 1997; Andersson & Dobson 2003; Cunningham et al. 2006). Plants release a wide range of organic volatiles as a result of their interaction with herbivores (Paré & Tumlinson 1999). Results from our experiments here and chemical analysis (C. Estrada & C. Rodriguez-Saona, unpublished data) provide evidence of the potential role of green-leaf volatiles in *Heliconius* mate-searching behaviour. Green-leaf volatiles are six-carbon alcohols, aldehydes and acetates commonly released at low rates from intact plants and at larger amounts soon after tissue damage (Visser et al. 1979; Turlings et al. 1995). Such patterns of emission agree with the behaviours found in our tests. First, males showed higher visiting rates to plants with some damaged tissue than they did to undamaged plants. Second, males

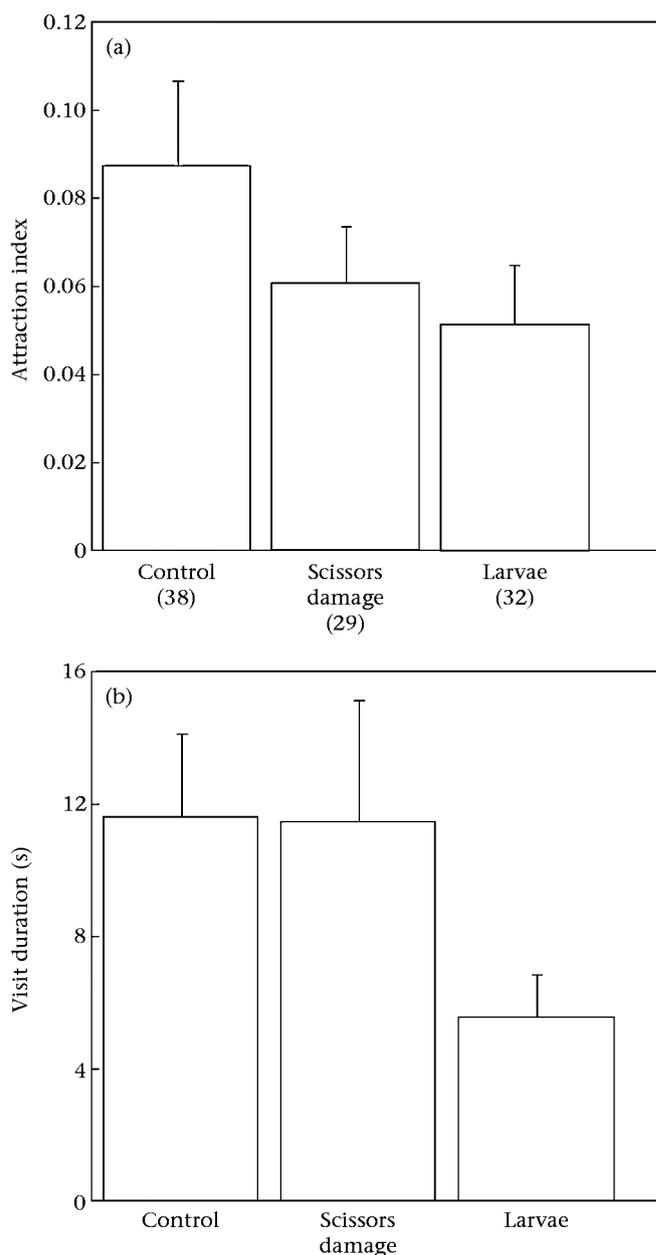


Figure 3. (a) Attraction index and (b) average visit duration of *Heliconius charithonia* males to *Passiflora lutea* plants in experiment 3 (mean \pm SE, $N = 15$). Treatments included olfactory cues from larvae feeding on host plant (control), damaged tissue using scissors, and larvae. Overall number of different males that visited plants in each treatment is given in parentheses.

were equally attracted to plants damaged with scissors and to plants with caterpillars. Odours from caterpillars also caused arrestment in males towards host plants. Preliminary chemical analysis indicates that the volatiles released by *Heliconius* larvae are similar to some of the volatiles emitted by the plant, albeit in much lower quantities (C. Estrada & C. Rodriguez-Saona, unpublished data). Whether larvae typically release such volatiles or they are produced only under stress (e.g. starvation and confinement in the net) is still an open question. Although females often land on or taste plants when choosing oviposition sites (Rausher 1978), males do not, suggesting that compounds with lower volatility perceived by contact might not be involved in male mate searching. Finally, although green-leaf volatiles are more likely to be involved in mate

Table 4
Results of two-way ANOVA without replications for experiment 3

	Source	df	MS	F	P*
A	Tests	14	0.034	2.391	0.024
	Treatments	2	0.015	1.095	0.348
	Error	28	0.014		
B	Tests	14	4.460	2.057	0.051
	Treatments	2	3.603	1.662	0.208
	Error	22	2.168		

Treatments included olfactory cues from larvae feeding on the host plant, *Passiflora lutea* (control), damaged tissue using scissors, and larvae. A: average attraction index, B: average visit duration.

* Reported P values are for two-tailed F test. Value in bold represent a significant effect.

searching of these butterflies, the role of additional cues such as herbivore-induced plant volatiles (e.g. terpenoids and aromatic compounds, Paré & Tumlinson 1999) cannot be ruled out.

If males respond to ubiquitous chemicals released after tissue damage, it might be expected that any herbivore feeding on their host plant could trigger similar reactions. Although some exceptions exist, different herbivores cause only minor variations in the volatile blend produced by a species of plant (Turlings et al. 1995; Geervliet et al. 1997). Results from experiments with plants carrying heterospecific larvae are thus intriguing, as we found that males were in general less attracted to plants with *Agraulis vanillae* than they were to plants with conspecific larvae. Consequently, it is possible that specificity when searching for mates in these butterflies is due to cues independent of the host plant. Larval coloration and odours are likely candidates. Males clearly recognized the presence of immatures of either species and hovered over them for several seconds, even in the case of the silicone models. Once attracted by host odours, the presence of heterospecific larvae might fail to elicit learning of plant location or fail to trigger frequent visits to the area. No evidence for major differences in volatiles released by larvae of *H. charithonia* and *A. vanillae* has been found (C. Estrada & C. Rodriguez-Saona, unpublished data). In contrast, late-instar caterpillars in the Heliconiini are brightly coloured, with diverse patterns occurring across species, particularly in those that exhibit pupal mating (Brown 1981; Mallet & Gilbert 1995). Such colorations probably function to advertise larval toxicity to predators, and, although few cases of mimetic convergence have been proposed, they could also provide species-specific information to searching males (Brown & Benson 1977). Signals that have evolved in a defence context are often also used in intraspecific signalling (Summers et al. 1999; Weller et al. 1999; Jiggins et al. 2001). For example, females of the pipevine swallowtail butterfly, cued by conspecific larval warning colorations, avoid oviposition on occupied plants, which probably reduces competition for their offspring (Papaj & Newsom 2005).

Similar to other *Heliconius*, local populations of *H. charithonia* are often monophagous, although they use more than 20 *Passiflora* species throughout their distribution, which range from Peru to the southern United States (Benson et al. 1975; Beccaloni et al. 2008). Their host plants belong to different subgenera (Plectostemma and Granadilla) (Yockteng & Nadot 2004), and show a wide diversity of leaf shapes. Although our experiments imply that plant volatiles play a key role in male mate-searching behaviour, the mechanisms that allow males to track female oviposition preferences along their geographical range remain poorly understood. Such mechanisms could include the coevolution of male and female plant-searching strategies, or the ability of males to learn characteristics from plant species carrying conspecific immatures (Papaj & Prokopy 1989).

Overall, our results show that plant volatiles released by larval feeding attract males and trigger searching of immatures. Although

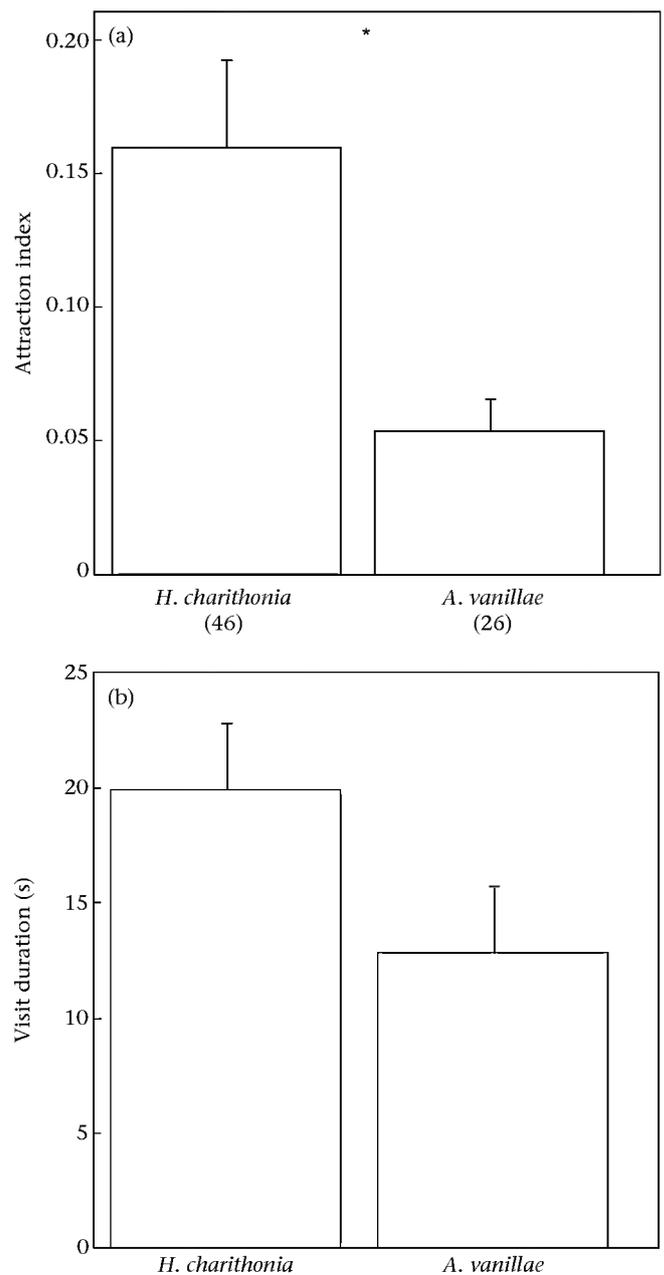


Figure 4. (a) Standardized number of visits and (b) average visit duration of *Heliconius charithonia* males to *Passiflora lutea* plants in experiment 4 (mean \pm SE, $N = 15$). Treatments included plants with *H. charithonia* and plants with *Agraulis vanillae* larvae. Asterisks (*) indicate significant differences ($P < 0.05$, paired t test). Overall number of different males that visited plants in each treatment is given in parentheses.

the influence of host plants in the sexual behaviour of other phytophagous insects is well established (Landolt & Phillips 1997), ours is the first study that has investigated their importance in butterflies. Adaptation allowing shifts to new larval hosts has been widely accepted as an important driver in butterfly diversification (Ehrlich & Raven 1964; Braby & Trueman 2006; Mullen 2006; Weingartner et al. 2006; Wheat et al. 2007; Peña & Wahlberg 2008). Measuring to what extent host plants are used as mate-searching cues is then relevant to understanding how such evolutionary processes have been facilitated by this interaction. Host plant fidelity, or the tendency of phytophagous insects to mate and oviposit in their host plants, has facilitated ecological speciation in

this group (Gilbert 1978; Feder 1998; Schluter 2001; Drès & Mallet 2002; Cocroft et al. 2008), and could have led to the diversification of at least three genera of lycaenid butterflies (Pratt 1994; Nice & Shapiro 2001; Nice et al. 2002; Forister 2005; Gompert et al. 2006). Thus, much remains to be done to assess the incidence and impact of the use of plant cues in male mate-searching behaviour in butterflies, and evaluate its importance to their evolution and diversification.

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Supplementary Material

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