Sex-specific chemical cues from immatures facilitate the evolution of mate guarding in *Heliconius* butterflies

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Competition for mates has substantial effects on sensory systems and often leads to the evolution of extraordinary mating behaviours in nature. The ability of males to find sexually immature females and associate with them until mating is a remarkable example. Although several aspects of such precopulatory mate guarding have been investigated, little is known about the mechanisms used by males to locate immature females and assess their maturity. These are not only key components of the origin and maintenance of this mating strategy, but are also necessary for inferring the level to which females cooperate and thus the incidence of sexual conflict. We investigated the cues involved in recognition of immature females in *Heliconius charithonia*, a butterfly that exhibits mate guarding by perching on pupae. We found that males recognized female pupae using sex-specific volatile monoterpenes produced by them towards the end of pupal development. Considering the presumed biosynthetic pathways of such compounds and the reproductive biology of *Heliconius*, we propose that these monoterpenes are coevolved signals and not just sex-specific cues exploited by males. Their maintenance, despite lack of female mate choice, may be explained by variation in cost that females pay with this male behaviour under heterogeneous ecological conditions.

**Keywords:** pre-copulatory mate guarding; pheromone; sexual conflict; *Heliconius*; butterflies; sexual selection

1. INTRODUCTION

Selective pressures for gaining access to sexually receptive females before other males have driven the evolution of a wide range of mate-searching and mating behaviours in animals (Emlen & Oring 1977; Parker 1978; Thornhill & Alcock 1983; Andersson & Iwasa 1996). One remarkable effect of such pressures is the ability of males to locate and establish permanent associations with sexually immature females (Parker 1974). Variations of such behaviour have evolved several times across vertebrate and invertebrate taxa and have been studied under names like ‘pre-copulatory mate guarding’ in aquatic crustaceans, salmon, frogs and ants (Ridley 1983; Jormalainen 1998; Foitzik et al. 2002; Morbey 2002), ‘pupal attendance’ in mosquitoes (Conner & Itagaki 1984), ‘cohabitation of males and juvenile females’ in spiders (Fahey & Elgar 1997), and ‘pupal mating’ in butterflies (Gilbert 1976). Most theoretical and empirical research has focused on the conditions under which this behaviour could evolve from a male’s fitness perspective (e.g. Parker 1974; Jormalainen 1998; Bel-Venner & Venner 2006), whereas little is known about the underlying mechanisms and the evolutionary origin of cues used by males to choose immatures likely to become sexually receptive females (Jormalainen 1998).

Identifying cues involved in such recognition is important to understand how this behaviour evolves and is maintained, especially given the potential for sexual conflict that it can generate (Jormalainen 1998; Parker 2006). Pre-copulatory mate guarding is a male time-investment strategy when fitness gained by spending time guarding an unreceptive female is higher than that gained by continuing searching for mates (Parker 1974). The benefit of this behaviour to males is clear: in populations with highly biased operational sex ratios and very dispersed receptive females, guarding increases their chances of mating (Parker 1974; Ridley 1983). In contrast, the benefits or costs of this strategy to females are unknown. In some species of crustaceans, females are able to resist males and decrease the duration of guarding or delay oviposition until a male they like is attached (Ridley 1983; Jormalainen 1998). In other cases, however, females do not have active mate choice as mating occurs with females not yet able to avoid copulation (e.g. spiders and butterflies; Gilbert 1976; Ridley 1983). Such coercive mating may impose direct or indirect fitness costs to females if they mate with low-quality males or if they experience increased mortality or decreased feeding capabilities. Understanding the cues that help males assess female maturity can give us hints about the levels of cooperation of females in this mating strategy, and thus the incidence of sexual conflict.

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Received 13 August 2009
Accepted 22 September 2009

Published online 1 This journal is © 2009 The Royal Society
2. MATERIAL AND METHODS

(a) Butterfly rearing

We studied a population of *H. charithonia* in a 22 × 10 × 10 m greenhouse in Brackenridge Field Laboratory at University of Texas, Austin. Evaporated cooled air flows across the facility’s habitat of trees, shrubs and vines so that butterflies experience approximately natural conditions. The population was started with adults collected in Austin in 2006 and was supplemented periodically with wild-caught individuals. Butterflies were allowed to breed freely but we controlled population size to prevent overuse of host plants (approx. 50 butterflies with a 2 : 1 male : female ratio). New individuals were permanently marked with a serial number written on their forewing. This, together with frequent census and listing of dead or removed butterflies, served to estimate their residence time in the greenhouse, which was used as an approximation for lifespan. Butterflies’ forewing lengths were also measured with a caliper to the closest 0.01 mm. Adults were fed *ad libitum* with sugar water solution (10%), and pollen and nectar from *Psiguria* spp., *Psychotria poeppigiana* and *Lantana camara* flowers.

Pupae are typically light tan but 24 h before eclosion their cuticles become translucent, revealing wings and adult body colours. However, males often sit and guard pupae before such colour change happens, and differences in colour or shape between sexes at this stage or earlier have not been detected (Estrada 2009). Therefore, it is unlikely that visual cues alone help males to estimate the sex of pupae or their proximity of eclosion. Because studies on pupal mating suggest that males recognize females only in the last part of the pupal stage (Deinert 2003), we classified as early pupae those from 1 to 7 days old, and as late pupae those whose cuticles were already translucent. We marked and monitored pupae daily until eclosion to determine whether they had been guarded by males.

(b) Chemical analysis

Pupal odours were characterized with two types of samples. First, cuticular chemicals from individual pupae were extracted for 5 min with 1.5 ml of methylene chloride at room temperature. The solvent was reduced to about 30 μl using a stream of nitrogen and then kept at −20°C until analysis. Extracts from four late male and six late female pupae were analysed. Second, we sampled volatiles released by pupae using solid-phase microextraction (SPME) with a 65 μm polydimethylsiloxane/divinylbenzene fibre (Supelco, Sigma-Aldrich Corporation). The SPME fibre was exposed for 5 h to a pupa inside a 4 ml glass vial with a plastic lid and septum, and kept at 24°C. Samples were analysed immediately by gas chromatography–mass spectrometry (GC–MS). Five late pupae and one young pupa of each sex were sampled. GC–MS of methylene chloride extracts and SPME were performed with a Hewlett-Packard model 5973 mass-selective detector connected to a Hewlett-Packard GC model 6890 using a BPX-5 fused silica capillary column (SGE, 25 m × 0.25 mm, 0.25 μm). Injection was performed in splitless mode (250°C injector temperature) with helium as the carrier gas (constant flow of 1 ml/min). The temperature programme started at 50°C, held for 1 min, and then rose to 320°C with a heating rate of 5°C min⁻¹. All compounds were identified by comparison of the mass spectra, gas chromatographic retention index and retention times with those of authentic reference samples. Chiral analyses were performed using a Supelco Beta-Dex 225 capillary column (30 m × 0.25 mm, 0.25 μm) in the same GC–MS equipment and a temperature programme starting at 50°C, held for 1 min and then rising to 200°C with a heating rate of 2°C min⁻¹. Quantifications of main volatiles identified by SPME were carried out using external standardization. We created a calibration curve for each compound by analysing the headspace of 1 μl of 1, 10, 100 or 1000 ng μl⁻¹ solutions dissolved in pentadecane. This hydrocarbon was used to simulate volatilization from the hydrophobic cuticle of pupal cases. Such head space samples were done under the same conditions as samples with pupae.

Gas chromatogram peaks from methylene chloride extracts were compared between male and female samples. We included in the analysis compounds present in at least two individuals with peak areas higher than 0.1 per cent of the total peak area. Such areas were converted into percentages, and, since they showed the relative abundance of compounds in a sample, they were then transformed.
according to the analysis of compositional data (Aitchison 1986; Liebig et al. 2000). First, we considered the absence of compounds as an artefact of the measuring process and applied the zero replacement technique by Fry et al. (2000). Zero percentages were replaced by \( \tau_i = \delta(M + 1)(N-M)/N^2 \), and non-zero ones by \( W_i \times \tau_i \), where \( W_i \) is the percentage of peak \( i \) (when \( W_i > 0 \)), \( \tau_i = \delta(M + 1)/N^2 \), \( M \) is the number of zeros in an individual sample, \( N \) the total number of peaks analysed, and \( \delta \) is the maximum rounding error (\( \delta = 0.0001 \)). Then percentage data were log-ratio transformed following Reyment’s (1989) formula \( Z_{i,j} = \log(X_{i,j}/g(X_j)) \), where, for individual \( j \), \( X_{i,j} \) is the peak area of compound \( i \), \( g(X_j) \) is the geometric mean of the area of all peaks and \( Z_{i,j} \) is the transformed area for peak \( i \). The number of peaks was reduced using principal component analysis (PCA) and extracted factors with eigenvalues higher than 1 were then compared between sexes using Hotelling’s \( T^2 \) test for multiple variables (STATISTICA, v. 8, 2007).

(c) Behavioural assays

Once positioned on pupae, males wait until eclosion and will leave only in the case of intense male–male competition or other disturbances. However, within a few minutes after departing, the same males typically return and perch again or compete with any new resident male. We took advantage of this behaviour to test potential compounds used by males to assess the sex of a focal pupa. Males perching on a pupa were gently disturbed and the pupa was then left either unmanipulated or painted with a test odour. We monitored whether males came back, and considered them to have accepted a pupa if they perched and guarded it again. Test runs in which males did not return within 10 min were excluded. The sex of each test pupa was determined after eclosion; thus only experiments done on female pupae were included in the analysis.

Chemicals used for painting pupae were racemic linalool (Fluka) and racemic cis- and trans-furanoid linalool oxide (2-methyl-2-vinyl-5-(1-hydroxy-1-methylthyl) tetrahydrofuran) synthesized following Reiter et al. (2003). The proportion of female pupae accepted after treatment with odours was compared with control pupae using a Fisher’s exact test.

3. RESULTS

Males repeatedly flew circuits of host and non-host plants, frequently inspecting hanging objects resembling pupae, presumably based on size, shape and colour (e.g. dead leaves or branches, empty pupal cases). Following the encounter with a pupa, males would fly very close for about 5 s, their antennae surrounding it. About 77 per cent of female pupae and 29 per cent of male pupae were guarded (\( \chi^2 \)-test, \( d.f. = 1 \), \( p < 0.001 \), \( n = 297 \) pupae, 1:1 sex ratio). Guarding started in most cases on the afternoon prior to the day of eclosion (71%), but occasionally males perched on pupae up to 10 days before, although for a short time (3 out of 102).

Less than half of the males that were at least 5 days old were observed guarding pupae (40%, \( n = 121 \)). Those males that guarded did it on average 1.8 times in their lifetime (\( \pm 1.41 \) s.d., \( min = 1 \), \( max = 11 \)). They were similar in size (41.36 and 40.78 mm mean wing length of guarding and non-guarding males, respectively, \( t \)-test, \( d.f. = 107, p = 0.26 \)), but lived on average about 10 days more than those never seen guarding pupae (26.35 and 17.59 days mean live span of guarding and non-guarding males, respectively, \( t \)-test, \( d.f. = 83, p < 0.001 \)). Although longevity could be correlated with competitive abilities for gaining access to pupae, we did not find a relation between guarding and age, as males as young as 5 days old perched often on pupae (17.34 days \( \pm 9.12 \) s.d. mean age of guarding males). Because observations resulted from periodic visits to the greenhouse, it is not known whether males never seen guarding had different searching strategies or had failed to win a position on pupae.

Methylene chloride extracts of male and female H. charithonia pupal cuticle contained similar compounds at comparable ratios. A PCA of 55 peaks produced seven principal components that explained 97 per cent of the variation. Scatter plots of the first three factors did not separate male from female samples and results from the Hotelling’s \( T^2 \) test also indicated lack of significant differences between sexes (\( T^2 = 102.461, F_{2,4} = 3.6593, p < 0.231 \)). The main compounds in the pupal cuticle were saturated straight chain alkanes with lengths between 23 and 33 carbon atoms preferentially odd numbered, 11,15,19-trimethylhentriacontane, octacosanal, 1-octacosanol and 2,5-dialkyl tetrahydrofuranes. Minor compounds included tricosene, octacosyl hexadecanoate and octadecanoate as well as additional alkenes, aldehydes and branched alkanes (see the electronic supplementary material).

Seven compounds were found in the head space of pupae when sampled with SPME (table 1). From those, only two appeared consistently in only one sex. The monoterpene linalool was exclusively found in late male pupae, while linalool oxide (furanoid) was released from late female pupae and found only in traces in early ones. Chiral analysis indicated that female pupae produced a racemic mixture of all four enantiomers of linalool oxide. During 5 h of sampling, late pupae released about 10 ng of such compounds (see the electronic supplementary material). The monoterpene plus linalool oxide acetate were the only compounds found almost exclusively in late pupae and thus could indicate the proximity to eclosion. Compounds detected in SPME and methylene chloride extracts were similar among pupae raised with different species of Passiflora host plant, thus suggesting that diet has little effect on pupal odours.

Bioassays to test sex-specific odours were performed in 20 female pupae for each of the following treatments: control (unmanipulated pupae), linalool oxide (female odour) and linalool (male odour). Following disturbance, males returned to hover in front of control pupae in 2.1 min on average (\( \pm 2.6 \) min s.d.), and accepted and perched on their original pupae after 2.5 min on average (\( \pm 3.2 \) s.d.). Adding the male-specific odour significantly decreased the chance that a given male would accept the female pupa again. In only 3 of 20 tests did males resume perching on the same female pupa upon application of the male odour. This is a significantly lower proportion than seen in controls (14 of 20; Fisher’s exact test, \( p = 0.001 \)). In contrast, the percentage of males accepting pupae perfumed with female odour
cyclohexyl
linalool oxide

Table 1. Volatiles released by pupae of *H. charithonia* identified using SPME.\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>early male pupae</th>
<th>late male pupae</th>
<th>early female pupae</th>
<th>late female pupae</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyclohexanol</td>
<td>923</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>(\alpha)-pinene</td>
<td>936</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>cyclohexyl acetate</td>
<td>1056</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>linalool oxide</td>
<td>1077</td>
<td>+++</td>
<td>trace</td>
<td>+</td>
</tr>
<tr>
<td>linalool</td>
<td>1107</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>linalool oxide acetate</td>
<td>1280</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>dihydroedulan I</td>
<td>1298</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^a\)Signals represent percentage of the area under the compound peak relative to the largest peak in the gas chromatogram.

\(^b\)RI: retention indexes.

+ = 1–10%.

+++ = 10–20%.

 ++++ = More than 20%.

(13 of 20) was similar to those accepting controls (Fisher’s exact test \(p = 1\)).

4. DISCUSSION

A key component in the origin and maintenance of pre-copulatory mate guarding is the ability of males to recognize immature conspecific females and estimate their maturity (Parker 1974; Jormalainen 1998). We found that males of *H. charithonia* are efficient in finding and guarding female pupae, which they recognize using simple sex-specific compounds released only at the end of pupal development. Males probably use a combination of olfactory and visual cues to find pupation sites (Estrada 2009). However, at a closer range, visual cues rather than long-range pheromones seem to be used to locate individual pupae within a zone of search primed by odour. This is indicated by the searching behaviour of males, which includes close inspection of pupae and similar objects, and the lack of attraction to concealed immatures (Estrada 2009). Morphology and coloration (including UV reflection) of male and female *Heliconius* pupae are similar (Estrada 2009). Therefore, once a pupa is discovered, males probably use mainly short-range olfactory cues to judge its maturity and sex.

Although males were efficient in recognizing female pupae, a high proportion of male pupae were also guarded (29%). Whether such mistakes were due to recognition failures or the release of wrong sex-specific cues is yet to be determined. Learning in the context of sexual behaviour has been demonstrated in insects (Dukas 2008), but we did not find any evidence of mistakes related to experience. For example, males that perched on male pupae were as old as those that perched in female pupae (average ages of 15.3 and 14.7 days, respectively, of males that guarded male or female pupae; \(t\)-test d.f. = 58, \(p = 0.76\)). Furthermore, for those that guarded both sexes in their lifetime, there was no indication that mistakes were made differentially by younger individuals (7 out of 15 males guarded male pupae first). On the other hand, strong motivation to mate in insect males is known to commonly drive failures in discrimination and courtship towards or mating attempts with males, heterospecifics or inappropriate objects (Thornhill & Alcock 1983). Alternatively, some male pupae could be releasing female odours. Female mimicry is a competitive mating strategy which serves to distract competitors from actual females (Field & Keller 1993; Steiner *et al*. 2005). In *Heliconius*, however, the cost to guarded male pupae (e.g. from harassment during eclosion; Gilbert 1984) is probably higher than the potential benefits of distracting competitors. Furthermore, although females can mate immediately after eclosing, males need 3–5 days to mature. These factors, together with the fact that this tropical species has continuous generations, make the prospect of female mimicry very unlikely.

The main compounds used in sex recognition by *H. charithonia* males were the monoterpenoids linalool and linalool oxide (furanoid) found in male and female pupae, respectively. They are common semiochemicals released by flowers or by leaves in response to insect feeding (Raguso & Pichersky 1999) and are known to elicit antennal electrophysiological responses in several insects, including *H. charithonia* and *Heliconius melpomene* (Andersson & Dobson 2003a; Bruce *et al*. 2005; C. Estrada & C. Rodriguez-Saona 2008, unpublished data). These compounds have also been found in scent mixtures of insects (El-Sayed 2008). Several male sexual pheromones of phytophagous insects are identical to plant chemicals. In most cases, such compounds are attractive to females in all contexts, while looking for either food, oviposition sites or partners, suggesting that males may mimic plant odours to increase the chance of attracting females (Landolt & Phillips 1997). In this case, however, volatiles have an opposite effect on male butterflies. Linalool and linalool oxide in combination with other floral scents and visual cues elicit feeding responses in *Heliconius* butterflies (Andersson & Dobson 2003b). During pupal mating, however, linalool, in concert with other cues, works to repel *H. charithonia* males from male pupae. Therefore, the attractive or repellent activity of linalool seems to be context-dependent, as it has been found for other pheromones in the genus (Schulz *et al*. 2008). This is in agreement with observations that in some species signal interaction, rather than compounds alone, are important to elicit insect behavioural responses (Bruce *et al*. 2005). Furthermore, in our greenhouse and field experiments (L. Mendoza-Cuenca, personal communication 2007) pupae artificially placed on host plants within one day of eclosion were rarely guarded by males even after being found. This suggests that previous location and subsequent monitoring of pupae could also be important factors in guarding decisions.

Other volatiles from extracts made with SPME varied slightly among samples, but such variation did not consistently correlate with sex. However, it is possible that other components in combination with the principal cues help males to discriminate among pupae. In particular, occurrence of dihydrosodulan preferentially in late male pupae is interesting as this compound is also part of *Passiflora* scents (Prestwich *et al*. 1976) and occurs in a pheromone that turns *H. charithonia* and congener females unattractive after mating (authors’ unpublished data 2006; Estrada 2009).
(a) Is there sexual conflict in Heliconius due to pupal mating?

During pupal mating, females are unable to refuse mating with guarding males as they could during regular courtship as adults. Yet selection on female choice with respect to male size and mating history is expected in butterflies because ejaculate size, and thus male-transferred nutrients and chemical defences, vary with these traits (Boggs & Gilbert 1979; Boggs 1981; Wiklund et al. 1998; Cardoso et al. 2009). Furthermore, the amount of such nuptial gifts is correlated with female fecundity and longevity (Wiklund et al. 1998, 2001). Therefore, the lack of pre-copulatory mate choice could potentially impose fitness costs on females and lead to sexual conflict. Whether there is such fitness reduction in Heliconius females is not clear. Several reasons suggest that a lack of active choice might not compromise female’s fitness. First, adults of Heliconius feed on pollen, obtaining amino acids that become as much as 80 per cent of their reproduction budget (e.g. Dunlap-Pianka et al. 1977). Heliconius charithonia females, in particular, are known to sharply increase pollen consumption after male-derived nutrients are depleted (Boggs 1990). Second, during pupal mating, males compete for a position on the pupa and for mating, and presumably only strong competitors with good searching abilities will mate (Deinert et al. 1994). Mating with winners of such competition might indirectly increase females’ fitness if these traits are inheritable and their sons are also good at competing for pupae. Gaining indirect benefits through sons might explain the persistence of traits that impose fitness costs to females (Cordero & Eberhard 2003), though such indirect benefits are weak relative to direct selection against harmful traits (Parker 2006). Furthermore, in species with mate guarding, larger males are more likely to evict smaller competitors (Elgar & Pierce 1988; Deinert et al. 1994; Bel-Venner & Venner 2006), and mating with bigger males might imply that relatively more nutrients and chemical defence are transferred to females (Boggs 1981; Cardoso et al. 2009).

On the other hand, female fitness could be reduced because of pupal mating if pollen supply is limited and mating with winning males does not guarantee better direct benefits to females. For example, although we did not find any evidence for the influence of size on guarding abilities, there were individuals that were very good competitors for pupae and that mated with several eclosing females. Similar patterns have been found in Jahnemus evagoras, the only butterfly outside Heliconius known to have pre-copulatory mate guarding (Elgar & Pierce 1988). In species where male mating success is highly skewed, male success and vigorous courtship displays are known to correlate with lower fertilization rates (Wärner et al. 1995; Drony 2003). In butterflies, this could also be true given that the size of the ejaculate transferred to females decrease after the male first mating (Boggs & Gilbert 1979; Wiklund et al. 1998). However, spermatophore size is not always affected by the time since last mating (Boggs 1981), and it is unknown whether Heliconius males in nature can ever mate as frequently as some individuals do in captivity. Pupal mating behaviour evolved once in the genus (Beltrán et al. 2007), and the difficulty of explaining its spread to other pollen-feeding Heliconius is perhaps highlighted by the lack of obvious benefits to females.

Understanding whether linalool and linalool oxide are coevolved signals produced to communicate pupal sex or, alternatively, whether they are mere consequences of sex differentiation adopted as cues by searching males, could give a hint to the level of cooperation of females in this mating strategy. Two observations support the idea that both compounds could be signals. First, these monoterpenes, together with linalool oxide acetate which appeared in both sexes, are biosynthetically closely related (Raguso & Pichersky 1999). Linalool is the precursor to linalool oxide. Whether or not this reaction is mediated enzymatically in pupae is unclear. Linalool oxide then can be easily esterified to form linalool oxide acetate. In an evolutionary perspective, given that pupae of both sexes can synthesize the last product of the pathway (linalool oxide acetate), it seems unlikely that accumulation of linalool or linalool oxide would occur if there were strong selection pressures for concealing the sex to searching males. Second, neither of these compounds have been found in extracts of abdominal glands or wings of H. charithonia adults (authors’ unpublished data 2006; Estrada 2009), suggesting they are not a part of courtship pheromones, as previously proposed (Gilbert 1976) and as is commonly found in mating systems where males locate pre-emergent females at eclosion sites (Thornhill & Alcock 1983; Steiner et al. 2005).

In summary, estimation of the relative fitness of females involved in pupal mating and those that mate as adults choosing among males will be essential in determining costs associated with this behaviour and the potential for sexual conflict (Chapman et al. 2003). Given the likely variation in net effects of pupal mating for female fitness due to factors such as pollen availability and population density, it is likely that a continuum from cooperation to conflict between the sexes exists as a result of temporal and spatial heterogeneity of these ecological conditions. Such a continuum would explain the maintenance of the sex-specific pupal compound, despite the potential cost associated with the lack of female direct mate choice. It would also predict the incidence of variations in quantity of linalool oxide produced by female pupae within and between populations as a result of diverse selection pressures for hiding sex. The fact that about a quarter of female pupae emerged without being guarded in our highly dense and male-biased greenhouse population suggests that such variation in production is possible. Different levels of female resistance to male guarding have been observed among populations of crustacean species (Jormalainen 1998). This implies that guarding generates intersexual conflict, but also suggests that, comparable to butterflies, the costs for females are variable and thus are the selection pressures to hide their forthcoming mating receptivity to males.

We thank Mary Owens, Angie Martinez, Jamsho, John and Grace Crutchfield, and Tony Alexander for help in the greenhouse facilities, and Samraat Pawar, Molly Cummings, Ulrich Mueller, Michael Singer, Erika Deinert and two anonymous reviewers for comments on the manuscript. This work was supported by the National Science Foundation and the Office of International Science and Engineering (DDIG 0608167), the Texas Academy of Sciences, Southwestern Association of Naturalists and
University of Texas at Austin graduate program in Ecology, Evolution and Behaviour. Greenhouse facilities were developed through NSF grants to L.E.G.

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