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SEXUAL SELECTION DRIVES THE EVOLUTION OF ANTIAPHRODISIAC PHEROMONES IN BUTTERFLIES

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Competition for mates has resulted in sophisticated mechanisms of male control over female reproduction. Antiaphrodisiacs are pheromones transferred from males to females during mating that reduce attractiveness of females to subsequent courting males. Antiaphrodisiacs generally help unreceptive females reduce male harassment. However, lack of control over pheromone release by females and male control over the amount transferred provides males an opportunity to use antiaphrodisiacs to delay remating by females that have returned to a receptive state. We propose a model for the evolution of antiaphrodisiacs under the influence of intrasexual selection, and determine whether changes in this signal in 11 species of *Heliconius* butterflies are consistent with two predictions of the model. First, we find that as predicted, male-contributed chemical mixtures are complex and highly variable across species, with limited phylogenetic signal. Second, differences in rates of evolution in pheromone composition between two major clades of *Heliconius* are as expected: the clade with a greater potential for male–male competition (polyandrous) shows a faster rate of divergence than the one with typically monoandrous mating system. Taken together, our results provide evidence that for females, antiaphrodisiacs can be both honest signals of receptivity (helping reduce harassment) and chastity belts (a male-imposed reduction in remating).

KEY WORDS: **Female mating receptivity, *Heliconius*, male-male competition, male control on female reproduction, sexual conflict, signal evolution.**

Male control over female mating frequency is common in nature and involves remarkable morphological, behavioral, and physiological adaptations aimed at manipulating female receptivity or discouraging advances by other males (e.g., Parker 1970, Simmons 2001). Common mechanisms include the transfer of seminal fluid proteins, donation of nuptial gifts, formation of mating plugs, and mate guarding (Thornhill and Alcock 1983; Simmons 2001). The evolution of such strategies is the result of selection on males to reduce sperm competition when females mate repeatedly within a breeding period. Females often obtain direct benefits from multiple matings (Arnqvist and Nilsson 2000). Thus a conflict can arise if male traits reduce female remating below what is optimal for the latter leading to male–female antagonistic coevolution (Arnqvist and Rowe 2005).

Antiaphrodisiacs are chemical signals transferred during mating by males to females that temporarily reduce female attractiveness to subsequent courting males. Described first in the beetle *Tenebrio molitor* (Happ 1969), they have since been found in a wide range of taxa, including *Drosophila melanogaster,* garter snakes, and several species of bees and butterflies (Gilbert 1976; Ross and Crews 1977; Scott 1986; Andersson et al. 2000; Ayasse et al. 2001). Some authors have regarded these male-donated pheromones as nuptial gifts because they help unreceptive

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females repel unwanted males (Thornhill and Alcock 1983). The notion that these pheromones have little role in controlling remating has largely arisen because decreased attractiveness appears to be synchronized with inherently low female sexual receptivity. The assumption that reduced receptivity follows mating is based mainly on a few taxa where females themselves produce additional chemicals to repel males, or are physically incapable of remating immediately (Scott 1986; Ayasse et al. 2001).

Recent studies have challenged the view that donor males and mated females always have a common interest in the repelling of courting males. Antiaphrodisiacs are typically emitted passively during courtship displays. Such apparent lack of female control over the release of this pheromone, as well as the male's ability to modify the amount transferred according to the potential for sperm competition, suggests that the presence of antiaphrodisiacs in a mated female may not always be a honest signal of her receptivity to mate, and can in fact become an instrument of male control over female remating (Andersson et al. 2000; 2003; 2004). In this article, we propose a model for the evolution of antiaphrodisiac pheromones under the influence of male–male competition expected if there is a male–female conflict. We also analyze the chemical composition of antiaphrodisiacs in 11 species of *Heliconius* butterflies (Lepidoptera: Nymphalidae) to establish whether the evolution of this signal is consistent with two key predictions of the proposed model.

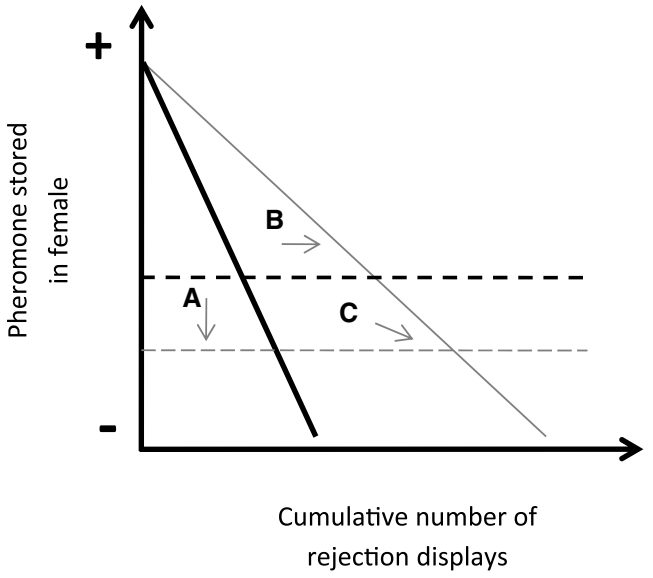

Figure 1. A model for antiaphrodisiac pheromone evolution. After mating, the quantity of pheromone decreases as a function of the number of times it is used during adoption of the rejection display. The initial quantity transferred during mating varies according to the donor male's quality or mating history. Horizontal dashed lines represent the threshold quantities below which males perceive the female as likely to accept mating. A decrease in the threshold (increased signal delectability and effectiveness) (A), increase in the number of displays necessary to reach the threshold quantity (decreased signal volatility) (B), or both (more potent, slowly diffusing signal) (C) represent ways to make the antiaphrodisiac more efficient and thus to increase the reproductive success of the donor male.

A Model of Antiaphrodisiac Evolution

We develop the following model based upon the mating biology of butterflies. However, similar processes can drive chemical changes in antiaphrodisiac signals in other taxa. In butterflies, with a few exceptions, mating is preceded by courtship (Wiklund 2003). Mated and unmated females typically respond to courtship by adopting a refusal posture that consists of raising the abdomen and releasing scents toward hovering males (Wiklund 2003). Male courtship toward virgin females can be sustained for a long time, but male displays toward mated females are often terminated quickly upon to the release of antiaphrodisiacs during the display (Gilbert 1976; Forsberg and Wiklund 1989; Andersson et al. 2000; Schulz et al. 2008). The amount of pheromone released varies considerably, depending on the number of times she has adopted the rejection posture since mating (Fig. 1; Andersson et al. 2004). Males are sensitive to this variation, the amount of pheromone being a key factor influencing courtship persistence (Andersson et al. 2004). Following copulation, butterfly females are unreceptive to remating either for some time or permanently (refractory period) due in part to male-donated seminal proteins and nonfertile sperm (Wiklund et al. 2001; 2003; Wedell 2005). Consequently, recently mated females are not only less attractive to males because of antiaphrodisiacs, but also sexually unreceptive because of their refractory period. Therefore, at that moment, antiaphrodisiac pheromones represent a honest signal of female receptivity and both sexes benefit when females are freed from time and energetically consuming male harassment (Forsberg and Wiklund 1989; Andersson et al. 2000; Bateman et al. 2006). Likewise, when females are unreceptive, males that respond to these pheromones and quickly end unproductive courtships also benefit because forced copulation in butterflies is rarely an option (Forsberg and Wiklund 1989). The presence of such male-donated pheromones may nevertheless create a conflict because in the long run, male and female reproductive interests differ as females gain from multiple mating (Boggs and Gilbert 1979; Boggs 1990; Arnqvist and Nilsson 2000; Wiklund et al. 2001), but are unable to voluntarily control the release of the signal that make them unattractive. An involuntary release of this pheromone is proposed since: (1) the refuse posture is adopted by virgin as well as mated females as part of a first response to male courtship, and (2) mated females display such posture and release antiaphrodisiacs

when approached by other females in enclosed populations without males (Andersson et al. 2004).

We propose that the evolution of antiaphrodisiac pheromones in butterflies could be in part driven by male–male competition. If antiaphrodisiac pheromones were always a honest signal of females' receptivity to mate, selection for change in the signal would not be expected because both donor males (the signaler) and subsequent courting males (the receivers) will benefit by short courtships. In contrast, if antiaphrodisiacs are occasionally present in sexually receptive females (or depleted in still unreceptive females), there is a conflict of interest between the signaler and the receiver (other males) resulting in selection for changes in the pheromone and the receiver's ability to assess females' receptivity (Arak and Enquist 1995). In Lepidoptera, last-male copulations have considerable sperm precedence in egg fertilization (Boggs 1979; Bissoondath and Wiklund 1997; Solensky and Oberhauser 2009). Thus the potential for intrasexual selection might arise under circumstances in which ignoring this signal and persisting courting mated females could offer a slim chance for copulation. Our model proposes that antiaphrodisiac pheromones could evolve due to selection on donor males to overcome resistance from subsequent males that challenge the antiaphrodisiac with persistent courtship. We propose a model for such pheromone evolution (Fig. 1). Assuming first, that the maximum amount of antiaphrodisiacs that can be stored or transferred between sexes has been reached, and second, that there is a threshold amount of compounds required for male perception and response, selection will favor males that transfer antiaphrodisiacs which (1) lower the threshold that subsequent males perceive as signals to discontinue courtship (Fig. 1A), (2) remain effective at repelling courtship (above thresholds) through more female rejection displays (Fig. 1B), or, (3) both (Fig. 1C). If antiaphrodisiacs evolve primarily as a result of male–male competition, we predict that rapid changes of this pheromone would likely remove any phylogenetic signal when their chemical composition is analyzed across species (Symonds and Elgar 2008). We also predict that such changes should happen faster between species with polyandrous than between species with monoandrous mating systems as the likelihood of sperm competition and thus pressure to delay female remating in the former is stronger (Arak and Enquist 1995; Arnqvist 1998).

We analyze the evolution of antiaphrodisiac composition across species of *Heliconius* and evaluate the degree to which changes in this signal agree with the model predictions. In this genus of butterflies, interclade differences in mating systems and male mate-searching strategies result in different degrees of intrasexual selection (Fig. 2). Females in the so-called "pupal mating" clade seldom mate more than once (Boggs 1979). Furthermore, although males in this clade search for and court eclosed virgin females (as some emerge before male discovery of pupae), they frequently seek pupae that they then guard with the goal of mating with eclosing females (Gilbert 1976; Deinert et al. 1994; Mendoza-Cuenca and Macías-Ordóñez 2005; Estrada et al. 2010). In contrast, females in a sister clade are polyandrous and males search for and court only eclosed females (nonpupal mating clade). Rate of courtship between males and previously mated females is very rare in species of the pupal-mating clade but very frequent in the nonpupal mating species (L. E. Gilbert, pers. obs.). These observations support the key assumption that in *Heliconius* courtship pressure on mated females is lower in the pupal mating than in the nonpupal mating clade.

Chemicals that *Heliconius* males transfer to females consist of a few dominant volatile compounds along with a matrix of less-volatile esters (Schulz et al. 2007, 2008). We have shown previously that major volatile components repel courting males whereas the heavier ones (the matrix) regulate the evaporation rate of the volatile pheromone during female rejection displays (Schulz et al. 2008; Estrada 2009). This division of functions in mixtures allows us to assess whether variation in the chemical composition of antiaphrodisiacs reflects changes targeted to: (1) reduce male threshold responses (volatile compounds), (2) the efficiency of pheromone dispersion (compounds in the matrix) or (3) both (Fig. 1). Although extensive work has been done on identifying mechanisms involved in reduction of female remating (Simmons 2001), our comparative analysis is one of the few studies of the divergence of such traits, and the selective forces influencing their evolution.

Materials and Methods

STUDY SYSTEM

Heliconius is an extraordinarily diverse genus of long-lived, toxic butterflies distributed from northern Argentina to southern United States (Brown 1981). They exhibit intra and interspecific color pattern diversity that together with habitat preferences and flight characteristics, often converge among species within the genus to form remarkable examples of mimicry (e.g., Brown 1981; Mallet and Gilbert 1995; Srygley and Ellington 1999; Estrada and Jiggins 2002). Antiaphrodisiac compounds are produced in glands located inside two chitinized claspers in the last abdominal segment in males (Eltringham 1925; Gilbert 1976; Schulz et al. 2008). Females of *Heliconius* and related butterflies possess a specialized abdominal gland that docks with the male's clasper gland at mating to receive and store transferred compounds whereas the male transfers the spermatophore to the bursa copulatrix (Eltringham 1925) (Supporting information).

We analyzed the chemical composition of abdominal glands of 10 *Heliconius* species (*H. cydno, H. pachinus, H. numata, H. charithonia, H. hewitsoni, H. hecale, H. ismenius, H. erato, H. sapho,* and *H. sara*) and combined the data with previously

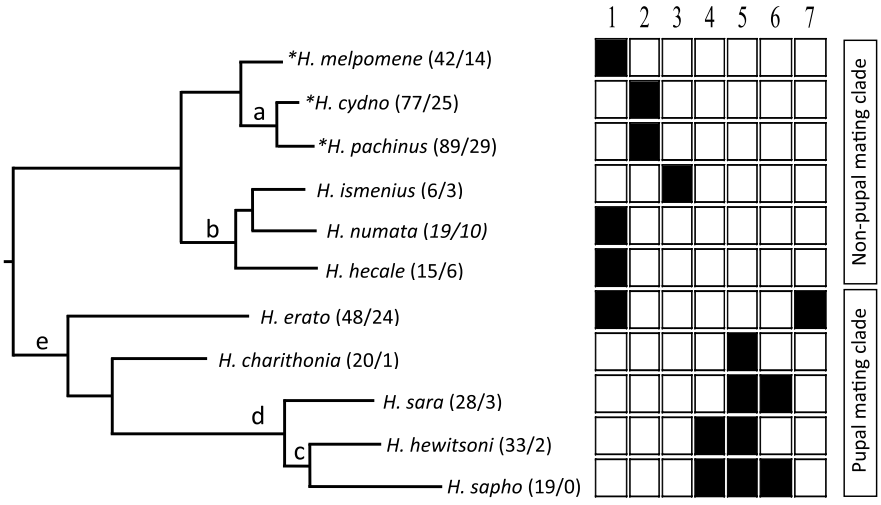

Figure 2. Antiaphrodisiac composition of 11 *Heliconius* species mapped on to their phylogeny. Species relationships and branch lengths are inferred from combined mitochondrial and nuclear data and are modified from Beltrán et al. (2007). The total number and number of unique chemical compounds detected in male glands of each species are in parentheses. Nodes in the phylogeny marked with letters a to e also appeared in trees built from similarities of pheromone composition (these letters are referenced in Table 1). The presence (black square) of major volatile compounds across species is shown next to the tree where 1 = (*E*)-β-ocimene, 2 = hexyl isopentanoate, 3 = (*E*)-α-ionone, 4 = dihydro-β-ionone, 5 = benzyl salicylate, 6 = (Z)-3-hexenyl decadienoate, 7 = esters of (*E*) 2,3-dihydrofarnesenic acid. Asterisk indicates species whose information about chemical composition of abdominal blends has been partly reported earlier (Schulz et al. 2007, 2008).

published information on an 11th species, *H. melpomene* (Schulz et al. 2008) (Fig. 2). Abdominal glands of *H. numata* from Ecuador were obtained from specimens donated by the Cockrell Butterfly Center, Houston Museum of Natural Science. Individuals from other species were collected in the wild or extracted from captive populations maintained at the Brackenridge Field Laboratory and Patterson building at the University of Texas, Austin. These stocks originated from butterflies collected in Corcovado, Osa Peninsula, and La Selva Biological Station (Costa Rica), or in the case of *H. charithonia,* from butterflies collected around Austin, Texas (United States). Butterflies were reared on their *Passiflora* host plants and had access to sucrose and honey solutions (10%), and flowers of *Gurania* spp., *Psiguria* spp., *Psychotria poeppigiana,* and *Lantana camara* all sources of nectar and pollen in nature. Although compounds or precursors of compounds included in pheromones can be sequestered by butterflies from their larval host plants (Nishida 2002), it is known that *Heliconius* males have the capacity to synthesize antiaphrodisiacs de novo with apparently little effect of the species of larval and adult food resources used (Schulz et al. 2008).

CHEMICAL ANALYSIS

Glands from mated and virgin females and claspers from males were dissected from freshly killed butterflies, and placed individually in vials with approximately 100 μl of pentane. The lower tip of the abdomen was also dissected and analyzed to identify compounds found in tissues surrounding the gland. Samples were kept at −70°C until analyzed. Three to six butterflies that were more than five-day old were examined individually for each sex and species.

Pentane extracts were analyzed with gas chromatography-mass spectrometry (GC-MS) with a Hewlett-Packard model 5973 mass selective detector connected to a Hewlett-Packard model 6890 gas chromatograph using a BPX5 fused silica capillary column (SGE, 30 m × 0.25 mm, 0.25-μm-thick film). Injection was in splitless mode (250°C injector temperature) with helium as the carrier gas (constant flow of 1 mL/min). The temperature program started at 50°C, was held for 1 min, and then rose to 320°C with a heating rate of 5°C/min. Compounds were identified by comparison of the mass spectra and retention times with those of authentic reference samples as well as analyses of mass spectral fragmentation patterns. Further details of the identification of *Heliconius* gland constituents can be found in Yildizhan (2009).

Males transfer most of the compounds from their abdominal glands into females during mating (Schulz et al. 2008). However, the chemical content of females' glands varied substantially, probably due the time elapsed since mating, and the use of the pheromone while adopting the rejection posture (Andersson et al. 2004). Thus, data from females were used only to test whether chemical transference happened in the same way as described for *H. melpomene* (Schulz et al. 2008). Analysis of the interspecific variation in gland content was performed based only on data from

males. Some intraspecific variation in such composition, particularly in minor compounds, also occurred among males. Thus, compounds used in the analysis were restricted to those that appeared in more than one individual male or mated female of the species, and were not found in the surrounding tissue of the gland, or in virgin females. Components were scored as present or absent in the 11 species of *Heliconius*.

Analysis of Chemical Composition of Abdominal Glands

Two indices were used to estimate the degree of phylogenetic signal in the evolution of abdominal pheromone blends and their individual components (Symonds and Elgar 2004; Symonds and Wertheim 2005): the consistency $CI = m/s$ (Kluge and Farris 1969), and the retention index $RI = (g - s)/(g - m)$ (Farris 1989), where s is the number of character state changes along the phylogeny (steps) based on parsimony, m the minimum possible steps (no homoplasy), and g the maximum possible number of changes in any tree (maximum homoplasy). Both indices range between zero and one with values closer to one indicating higher degrees of fit of characters to the phylogeny. Compounds present in only one species (autapomorphies) were excluded from both analyses as they by default have minimum number of steps (s) and tend to inflate the *CI* of the blend (Sanderson and Donoghue 1989). Both indices were calculated using PAUP* 4.0b10 (Swofford 2002). A randomization test was also used to estimate whether *CI* and *RI* differed from measurements obtained from a random distribution of characters (Maddison and Slatkin 1991). Using MESQUITE (Batch Architect Package, reshuffle states within characters) by Maddison and Maddison (2008), null distributions of *CI* and *RI* were calculated from 2000 matrices created by shuffling data of presence/absence of chemical compounds while keeping the phylogeny constant. Two-tailed *Z*-tests were then used to compare the null distribution with values inferred from our data. The characters were mapped in a phylogeny inferred by mitochondrial (*Co* and *16S*) and nuclear data (*Ef1*α, *dpp*, *ap* and *wg*) (Beltrán et al. 2007) pruned to contain only the species studied (Fig. 2, Supporting information). If antiaphrodisiac evolution has been mainly driven by intrasexual selection, we expect lower values of *CI* and *RI*, particularly for analysis including only species in the nonpupal mating clade.

Phylogenetic analysis with chemicals as characters was used to determine in which clades abdominal blends have evolved congruently with a molecular phylogeny of the group. Bootstrap analysis on trees constructed by maximum parsimony (MP) using heuristic search with TBR swapping algorithm were performed with 2000 replicates and confidence level of 50 using PAUP* 4.0b10 (Swofford 2002). Bootstrap values of the resulting consensus tree were then mapped on the molecular phylogeny to compare species associations found in both trees. If the chemical composition of abdominal glands changed gradually through time, suggesting little pressure for changes in the signal, finding consensus trees that closely resemble the inferred phylogeny of the group with high branch supports is expected. In contrast, if male–male competition has accelerated the evolution of the chemical blend, a poor match of trees is expected.

With MP two species either sharing or lacking particular compounds could be clustered together if either gains or losses of compounds happened in their closest common ancestor. This analysis can indicate whether the overall evolution of the blend mirrors the phylogenetic history of the species. However, as compounds in a blend produce a scent, it is reasonable to assume that the more compounds are shared between two blends the more similar they are when perceived by a receiver. Divergence of abdominal chemical composition among the 11 species was also calculated using the Jaccard-Tanimoto similarity coefficient (J) (Willett et al. 1998). This coefficient is calculated as a proportion of compounds shared between chemical blends of the two species relative to the total number of compounds in both blends. Similarity was then converted to distance ($1 - J$), and the resulting matrix correlated with a matrix of patristic distances calculated from the branch length of the molecular-based phylogeny using PATRISTIC (Fourment and Gibbs 2006). We used the Pearson's correlation and a Mantel test with 2000 permutations to calculate the significance levels of the correlation. Mantel tests were performed using R 2.7.1. (Vegan package 1.15–1, R Development Core Team, 2008). Results (not shown) of similar analysis using squared Euclidian Distance, another commonly used measure of chemical differences among species, were comparable to those obtained with the Jaccard coefficient.

Separate analyses were performed for all chemical compounds and for different sets of data based on the degree of volatility. Two divisions of the whole dataset were performed. In the first (A), all chemicals were divided into two groups (high/low volatility A), classifying compounds with molecular weights below 300 g/mol as volatiles (Bradbury and Vehrencamp 1998). In the second division (high/low volatility B), we classified as volatiles those chemicals with lower molecular weight than weight of compounds dominating virgin female glands, which are odorless for us (ca. 270 g/mol). We expected that if the evolution of this pheromone has been targeted toward reduction of the threshold amount needed to repel subsequent males (Fig. 1A), then only volatile components of the blend would show evidence of male–male competition (e.g., poor phylogenetic signal). By contrast, such poor phylogenetic signal found in the less-volatile portion of the mixture (matrix), considered responsible for controlling volatile component evaporation rates, provides evidence for

evolution to increase the number of rejection displays before repellant effects of the antiaphrodisiac is lost (Fig. 1B).

Several chemicals present in abdominal glands are biosynthetically related, and thus their joint occurrence in a single blend may not represent independent traits. Bias would occur in our analysis if a family of chemicals appears in two species that have independently acquired a new compound of that family (Schulz et al. 1993; Symonds and Elgar 2004). In an attempt to correct for this effect, compounds were also combined in chemical classes and biosynthetically related groups, and the new data of presence and absence of these groups analyzed as explained above. Groups of known or assumed biosynthetic relationships are given in Supporting information.

Results

The content of abdominal glands from *Heliconius* males varied considerably among species, both in composition and number of compounds (Fig. 2, Supporting information). We identified a total 211 compounds, more than 50% being found exclusively in one species. Blend composition ranged from a few compounds, as in *H. ismenius* (6) to over 75 compounds as in *H. cydno* and *H. pachinus*. The chemical mixture of the species studied included esters (41%), lactones (26%), and terpenes (15%), the remainder being alcohols, ketones, and aromatic compounds. With the exception of *H. ismenius*, which lacks heavy esters and lactones, male secretions had a similar broad composition across species, consisting of few major volatile compounds imbedded in a less-volatile matrix made up mainly of esters of common fatty acids. A complete antiaphrodisiac composition for each species is given in the Supporting information.

Both indices used to estimate whether blends have evolved in congruence with the phylogeny indicate that the phylogenetic signal in antiaphrodisiac blends was higher than expected by chance (all Z-tests, df $= 1999$, $P < 0.001$) (Table 1A). However, the correspondence is not very strong as *CI* and *RI* are near 0.5, half the maximum possible value. Similar results were obtained when chemical data were divided according to volatility that suggests that similar modes of evolution probably govern signal and matrix compounds. Grouping compounds according to their biosynthetic relationships only slightly decreased the phylogenetic signal of abdominal blends, indicating that the interdependence of chemical data did not bias our analyses of the entire dataset toward any particular result (Table 1A). Nearly half of the compounds that form abdominal blends and are present in more than one species showed high degrees of congruence with the phylogeny (e.g., $RI > 0.7$, Supporting information). Among those, half are lactones and esters found exclusively in the closely related pair *H. cydno* and *H. pachinus*. Values of *CI* and *RI* of blends not including those 25 chemicals are comparable with values obtained from the complete dataset, and significantly different from random ($CI = RI = 0.49$, mean $\pm$ SD of random matrixes, $CI = 0.377 \pm 0.01$, $RI = 0.16 \pm 0.03$, Z-tests, df $= 1999$, $P < 0.001$). This suggests that the congruence with the phylogeny for the complete dataset is not because of the high chemical similarity between these two species alone. When we considered only species within each of the two major clades (pupal/nonpupal mating clades), calculated *CI* and *RI* values did not matched our expectations.

Table 1. Measures of congruency of chemical blends from abdominal glands of *Heliconius* species with their molecular phylogeny. Columns represent different sets of data used for analysis with the number of characters included indicated in parenthesis. (A) Values of consistency and retention indices (*CI*, *RI*) obtained from chemical data (top) are compared with the mean (± SD) of 2000 randomized replicates (bottom). Congruency with the phylogeny was significantly higher than in a random distribution of chemicals across species with $P < 0.0001$ for all datasets (Z test with two tails). (B) Bootstrap support of species association using compounds as characters from 2000 trees obtained with maximum parsimony. Small letters represent clades marked in the pruned molecular-based phylogeny of the group (Fig. 2), with bootstrap values higher than 5%. A dash represents a value lower than 5%.

	All compounds (93)	High volatility A(67)	High volatility B(55)	Low volatility A(26)	Low volatility B(38)	Chemical groups (34)
A						
CI	0.567	0.573	0.534	0.553	0.623	0.486
	0.397±0.007	0.405±0.008	0.399±0.009	0.378±0.013	0.396±0.011	0.348±0.011
RI	0.567	0.558	0.5	0.588	0.662	0.542
	0.139±0.026	0.129±0.03	0.138±0.034	0.16±0.047	0.144±0.001	0.186±0.041
B						
a	100	100	99	98.7	100	99.3
b	67.5	12.3	6.3	73.5	74.3	84.7
c	45.9	55.7	53.5	–	–	9.1
d	80.2	59.2	58.5	58	56.8	72.3
e	–	13.9	11.2	–	–	–

Values of *CI* and *RI* were higher for the nonpupal mating clade ($CI = 0.82, RI = 0.79$) than in the pupal mating clade ($CI = 0.79$, $RI = 0.5$), and in both cases higher than those obtained from a random distribution of compounds across species (mean ± SD $CI = 0.54 \pm 0.01$ and 0.7 ± 0.02, and $RI = 0.22 \pm 0.05$ and 0.2 ± 0.08 for nonpupal and pupal mating clades, respectively, *Z*-tests, df = 1999, $P < 0.001$).

Despite the phylogenetic signal of chemical blends found with the consistency and retention indices, not all species associations (clades) were recovered when chemicals were used as characters and analyzed with maximum parsimony. Only nodes marked with letters a to e in the pruned phylogenetic tree (Fig. 2) had bootstrap support higher than 5% (Table 1B). Species associations obtained from these analyses show the highest support for the pair *H. cydno–H. pachinus* (clade a), which remained high regardless of the dataset used. Although with lower bootstrap support, the clade that include *H. sapho*, *H. hewitsoni*, and *H. sara* (clade d) also emerged from trees derived from all sets of chemical data. Support for other species associations was also found with particular groups of chemicals. For example, *H. sapho* and *H. hewitsoni* (clade c) clustered in about half of the trees only when volatile compounds were taken into consideration. Likewise, the high support that appeared for the clade that comprise *H. ismenius*, *H. hecale,* and *H. numata* (clade b) was produced by changes in chemical composition of the less-volatile compounds, but dropped dramatically when only volatiles were included. Consensus trees are shown in the Supporting information.

Chemical distances calculated with the Jaccard index were relatively high, with an average close to the maximum value (0.98 ± 0.10) (Fig. 3A). Even the species with the most similar blend, *H. sapho* and *H. hewitsoni,* had a distance of about 0.5. Divergence in composition of blends between species was weakly correlated with their phylogenetic distances when all pairs of species were considered (Pearson's correlation $R = 0.34$, Mantel test $P = 0.02$). Correlations were, however, much higher when only pairs of species within the pupal (Pearson's correlation $R = 0.8$, Mantel test $P = 0.005$) or the nonpupal mating clade were included (Pearson's correlation $r = 0.82$, $P = 0.013$). The rate of chemical composition change in the two major clades of *Heliconius* differs in the direction expected under the influence of sexual selection, given their contrasting mating systems (Fig. 3). Chemical composition has diverged faster among species with higher female remating rates (nonpupal mating clade) than among those in which females remate much less frequently (pupal mating clade). At any given level of genetic distance, pairs of species that belong to the pupal mating clade (squares) have pheromones more similar to each other than pairs of species in the nonpupal mating clade (triangles) (Fig. 3A). Identical results were obtained using sets of data separated by chemical volatility. Figure 3B, C shows this pattern for the first division of compounds

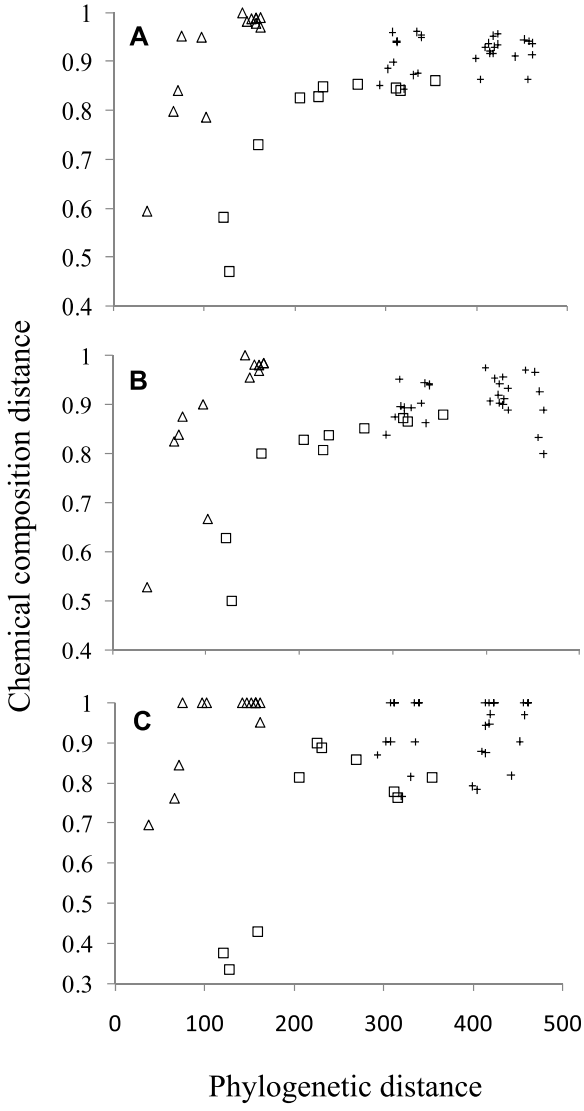

Figure 3. Divergence in chemical composition of blend from abdominal glands and phylogenetic distance between pairs of *Heliconius* species. Squares represent pairs of species that belong to the pupal mating clade; triangles, pairs of species in the nonpupal mating clade; and pluses, pairs of species that belong to two different clades. (A) Chemical distance using all compounds, (B) distance using highly volatile compounds with the first volatility division (A), and (C) distance using low volatile compounds with the first volatility division (A).

(high/low volatility A), but identical results were obtained for the second division of data (B), shown in Supporting information.

Although it is possible that the whole mixture of chemicals serve as a signal to courting males, individual major volatile compounds seem to be enough to reduce the attractiveness of mated females in greenhouse bioassays. For example, (*E*)-β-ocimene, hexyl isopentanoate and benzyl salicylate, are compounds known to trigger rejection of females by courting males in *H. melpomene, H. cydno, H.* and *charithonia,* respectively (Schulz et al. 2008;

Estrada 2009). A similar pattern of the evolution of antiaphrodisiacs in both major clades of *Heliconius* is apparent when considering only those major compounds within the more volatile region of the gas chromatograms (Fig. 2). Such compounds have diverged between sister species *H. ismenius* and *H. numata*, as well as the closely related *H. melpomene* and pair *H. cydno–H. pachinus,* all in the nonpupal mating clade. In contrast major compounds appear to be conserved across more divergent species of the pupal mating clade (*H. charithonia*, *H. sapho*, *H. hewitsoni*, and *H. sara*).

Discussion

Our results support the idea that the evolution of antiaphrodisiac pheromones in butterflies is in part driven by intrasexual selection. Analysis of the composition of this chemical signal in *Heliconius* was consistent with our predictions of (1) rapid changes in the chemical composition of antiaphrodisiacs (Symonds and Elgar 2008), and (2) faster rates of change among species with higher potential of sperm competition (Arnqvist 1998). First, we show that antiaphrodisiac pheromones in *Heliconius* are complex mixtures and highly diverse. Both composition and number of compounds along the whole range of volatility analyzed varied substantially among species. Although some compounds were exclusively found in particular clades, most appeared scattered across species, suggesting that gains and losses of compounds throughout the evolutionary history of the genus have been common. Consequently, indices used to estimate the congruence of chemical changes with the phylogeny although significantly higher than random, were generally low. Similarly, consensus trees constructed with maximum parsimony that grouped species based on their chemical similarity only recovered some of the phylogenetic clades and often with low bootstrap support. The only exception was the pair *H. cydno–H. pachinus* which has 48 compounds in common, 25 of them exclusive, and was clustered together more than 98% of the time. This is not unexpected as these are completely interfertile species that appear to have diverged very recently (about 500,000 years ago), and exist as allopatric populations (Kronforst et al. 2006; Beltrán et al. 2007). Overall, these results suggest that divergence of antiaphrodisiac components across species is higher than that expected if gradual changes over evolutionary time have governed this pheromone's evolution (Symonds and Elgar 2008).

Second, there has not been a consistent rate of evolution in the chemical composition of antiaphrodisiacs across all species in the genus. As expected in the case of lineages that differ in the potential for sperm competition (Arnqvist 1998), more gradual shifts in composition were detected among species in the clade with mostly monoandrous (pupal mating), than in the clade with polyandrous mating system (nonpupal mating). With the exception of results of *CI* and *RI* indices, this difference was found in all analyses. Divergence in chemical composition among pairs of species measured with the Jaccard index accumulated faster with the increase of phylogenetic distance in the nonpupal than in the pupal mating clade. Similarly, consensus trees tracked phylogenetic relationships of the group more closely in the pupal than in the nonpupal mating clade. Recently diverged species (Beltrán et al. 2007), such as *H. melpomene* and *H. cydno* or the sister species *H. ismenius* and *H. numata* in the later clade were never clustered together in consensus trees. Furthermore, support for the cluster of *H. numata*, *H. hecale,* and *H. ismenius* was due mostly to the likely loss of multiple compounds in their closest common ancestor and not to the chemical similarity of their blends. These three species have only few compounds in their glands, with few commonalities between *H. hecale* and *H. numata* and only one compound with *H. ismenius*.

Variation in signals that reduce female attractiveness is not exclusive to *Heliconius* but has also been found among other butterfly species (Andersson et al. 2000, 2003) and *Drosophila* strains (Scott and Jackson 1988). Although comparisons of antiaphrodisiacs among species with contrasting mating systems have not previously been performed, similar studies exist for another well-known mechanism of male sperm competition, and are consistent with our finding of antiaphrodisiacs evolution in *Heliconius*. Seminal fluid proteins transferred to females in the ejaculate are known to reduce females' receptivity to mate, and promote the formation of mating plugs, along with many other effects on female and male reproductive success (Chapman and Davies 2004). Other studies have found extremely rapid evolution of genes coding for these proteins both within and among *Drosophila* species (Chapman 2001; Haerty et al. 2007). Although the contribution of different sources of selection both in a sexual or nonsexual context has not been identified (Simmons 2001; Haerty et al. 2007), such genes have evolved more rapidly in species with higher remating rate, and presumably higher potential for sperm competition (Wagstaff and Begun 2005, 2007).

Currently, there is no direct evidence for a key assumption of the model of antiaphrodisiac evolution we propose: that more complex mixtures or novel elements make antiaphrodisiacs more effective in repelling males or controlling volatile evaporation rates. However, there is compelling theoretical and empirical evidence that the intensity of conflict between sender and receiver influences the evolution of complex signals (Arak and Enquist 1995). Data here and elsewhere indicate that our model for antiaphrodisiac evolution driven by intrasexual selection is highly tenable. First, the complex matrix of less-volatile esters that characterize blends from abdominal glands of *Heliconius* (and related genera) (Ross et al. 2001; Schulz et al. 2007; 2008, and this article) has apparently evolved in a similar mode as the more volatile portions of the blend, which is most probably better detected by males

and conveying information. This is unexpected unless similar selection pressures are driving changes in all parts of the mixture. Whether adding compounds to the matrix is necessary to improve its potential to regulate evaporation of volatiles requires further investigation. Second, behavioral test with *H. cydno* has shown that males in captivity significantly reduce courtship time toward virgin females painted with some of the main volatile compounds found in other *Heliconius* species (Estrada 2009). Although this result clearly indicates that males can sense and respond to odors that are not present in their own abdominal scents, whether it is due to a natural predisposition to reject novel odors or to biases against ancestral compounds remains an open question.

Several factors known to affect female sexual receptivity in butterflies are controlled by males (e.g., Wedell 2005). For example, males transfer, at mating, seminal fluids with substances that apparently suppress receptivity in females (Walters and Harrison 2010). Similarly, males pack into their spermatophores substantial amounts of nonfertile sperm (apyrene) that extend the duration of females' refractory periods (Wedell 2005). Our results, which are consistent with a model of antiaphrodisiac evolution driven by intrasexual selection, support the hypothesis that antiaphrodisiacs can also become a mechanism of male control over female remating (Andersson et al. 2004). Male–male competition select for males that extend the period females are unreceptive (e.g., by selecting on seminal proteins) and also extend the period females are found unattractive by other males (e.g., by transferring more efficient antiaphrodisiac pheromones). Males that transfer pheromones that last throughout more female rejection displays have the advantages of (1) decreasing time and energy-consuming male harassment for longer time when females are still unreceptive, and (2) reducing the chances of females remating if they have become receptive again. While in the former case both male and females benefit from the presence of the signal, in the latter only donor males do. It is difficult to experimentally assess whether mated females are receptive to mate (Andersson et al. 2004). However the assumption that female attractiveness and sexual receptivity not always match seems both logical and consistent with what we know of the biology of this system. While attractiveness of mated females is controlled by pheromones that are spent in proportion to the number of courtship attempts by males (Andersson et al. 2004), sexual receptivity in mated females is influenced by factors (e.g., stored sperm, seminal proteins) whose effects decrease as a function of oviposition rate or time since mating (Wedell 2005). In butterflies, the posture adopted when antiaphrodisiacs are released is part of the typical female response to male courtship displays. Whether females can sense the presence of this pheromone in courting males or during their own display, or whether mechanisms of female control over the release of the pheromone have evolved are questions worthy of further study.

Factors other than sexual selection could also select for chemical variation in blends of the abdominal glands of *Heliconius* but none are expected to generate the observed differences in rates of evolution between clades. First, besides antiaphrodisiac effect, there are other potential functions of these male-contributed compounds. For instance, they could provide the female with defensive compounds that advertise their toxicity to predators, reinforcing warning colorations (Eltringham 1925). It may be that the evolution of the storage and display organ in Heliconiinae females evolved for this protective function. Consequently, if odors, together with coloration, are used for signaling predators, a convergence among mimetic species in the chemical signal would be expected because multimodal signaling might be more effective in educating local predators (Moore and Brown 1989; Moore et al. 1990; Rowe and Guilford 1999; Jetz et al. 2001; Siddall and Marples 2008). Similarity of chemical composition of abdominal glands between co-mimic species was not found in *Heliconius* (Estrada 2009).

Odors released by abdominal glands could also provide additional signals for species recognition. Because *Heliconius* butterflies exist as communities of up to nine coexisting species, many of which are known to hybridize, males and females will share an interest in reducing heterospecific rematings (Gilbert 2003; Mallet et al. 2007; Kronforst 2008). Although selection for reproductive isolation is generally assumed to act on precourtship behavior in *Heliconius*, components of the abdominal glands could contribute to species recognition of females by males when present in mated females, or recognition of males by females if the latter can perceive components of the pheromone when present in males. In this case, rapid changes in the pheromone composition as those observed among species could also be expected, particularly among nonpupal mating species where male courtship pressure continues through adult lives of females. Although a potential selective pressure that could reinforce the between-clade pattern due to sexual selection that we documented, the potential of interspecific hybridization is relatively insignificant in this regard. This is because color pattern and local microhabitat separation of species pairs capable of mating generally holds in local communities (e.g., Gilbert 2003).

Finally, selection pressure for divergence in abdominal gland might arise if natural enemies include the detection of those signals as part of their foraging strategies. Eavesdropping on sexual or aggregation signals is widespread in nature, and several changes in signals and signaling behaviors have been suggested to be adaptation to escape detection by predators and parasitoids (Stowe et al. 1995; Zuk and Kolluru 1998; Cardé and Haynes 2004; Raffa et al. 2007). In particular, the antiaphrodisiac pheromone of the butterfly *Pieris brassicae* not only attracts the egg parasitoid *Trichogramma brassicae*, but also triggers phytochemical changes in this butterfly's host plant that attract females of this

parasitic wasp (Fatouros et al. 2005, 2008). The extent to which antiaphrodisiacs are exploited by parasitoids in *Heliconius* is unknown. However, if it occurs, high egg mortality by these natural enemies could be a strong selective force driving the divergence of the pheromone. In this case, we could also expect rapid divergence in the chemical composition of these signals, but not at different rates between lineages with different mating systems.

Analysis of variations in chemical composition of pheromones in a phylogenetic context provides the opportunity to explore possible mechanisms promoting the evolution of these signals, as has been done for other sensory modalities (Ryan and Rand 1995; McCracken and Sheldon 1997; Sullivan et al. 2000; Päckert et al. 2003; Ord and Martins 2006). Although our results with *Heliconius* are consistent across analyses and datasets, the complex structure of blends and variation of abdominal scents among *Heliconius* warrants more research before the causes of variation can be fully understood. Here, we show results obtained from one-third of the species in the genus, have included many chemicals in the analyses whose functions have not been firmly established, and have ignored the quantitative information of blend components. Each of these limitations suggests potential sources of error that could obscure real patterns of evolution among portions of chemical blends involved in communication. Nonetheless, our results show conclusively that shifts in the chemical composition of antiaphrodisiacs among *Heliconius* have happened fast, particularly in species with a higher potential for sperm competition. This suggest a role of sexual selection in the evolution of such signals, which is important because it provides evidence that antiaphrodisiacs can become a mechanism for male-imposed reduction in female mating choice.

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Supporting Information

The following supporting information is available for this article:

Figure S1. Female (A) and male (B) abdominal glands in *Heliconius charithonia*.

Figure S2. Phylogeny of *Heliconius* inferred from combined mitochondrial (*Co* and *16S*) and nuclear data (*Ef1*α, *dpp*, *ap* and *wg*) modified from Beltrán et al. (2007).

Figure S3. Consensus trees obtained from bootstrap analysis with 2000 replicates on trees constructed by maximum parsimony using heuristic search with TBR swapping algorithm.

Figure S4. Divergence in chemical composition of blend from abdominal glands and phenotypic distance between pair of *Heliconius* species.

Table S1. Chemical composition of blends from male abdominal glands of 11 species of *Heliconius*.

Supporting Information may be found in the online version of this article.

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