Beyond magic traits: Multimodal mating cues in *Heliconius* butterflies

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Species coexistence involves the evolution of reproductive barriers opposing gene flow. *Heliconius* butterflies display colorful patterns affecting mate choice and survival through warning signaling and mimicry. These patterns are called "magic traits" for speciation because divergent natural selection may promote mimicry shifts in pattern whose role as mating cue facilitates reproductive isolation. By contrast, between comimetic species, natural selection promotes pattern convergence. We addressed whether visual convergence interferes with reproductive isolation by testing for sexual isolation between two closely related species with similar patterns, *H. timareta thelxinoe* and *H. melpomene amaryllis.* Experiments with models confirmed visual attraction based on wing phenotype, leading to indiscriminate approach. Nevertheless, mate choice experiments showed assortative mating. Monitoring male behavior toward live females revealed asymmetry in male preference, *H. melpomene* males courting both species equally while *H. timareta* males strongly preferred conspecifics. Experiments with hybrid males suggested an important genetic component for such asymmetry. Behavioral observations support a key role for short-distance cues in determining male choice in *H. timareta*. Scents extracts from wings and genitalia revealed interspecific divergence in chemical signatures, and hybrid female scent composition was significantly associated with courtship intensity by *H. timareta* males, providing candidate chemical mating cues involved in sexual isolation.

KEY WORDS: Lepidoptera, pheromones, reproductive isolation, sex, signaling/courtship, speciation.

Stable coexistence of sibling species following speciation is documented from various lineages and in diverse ecological situations (Bolnick and Fitzpatrick 2007; Fitzpatrick et al. 2008; Sobel et al. 2009). A critical question in evolutionary biology is how closely related taxa evolve and maintain reproductive isolation without geographical barriers. A large number of studies have investigated the mechanisms preventing hybridization (The Marie Curie Speciation Network 2012; Sobel and Chen 2014) and have shown that a major barrier to gene flow in animals is behavioral premating isolation based on sexual preferences (Coyne and Orr 2004; Hohenlohe and Arnold 2010; Safran et al. 2013). Such sexual isolation relies on divergence in mating cues and mating preferences, triggering species-specific sexual communication. Signaling traits are generally under intraspecific sexual selection, but also evolve within a specific environment, implying that natural selection can

also influence mating traits (Maan and Seehausen 2011). A key question is then to know in which cases the interaction between the ecological context and sexual communication can facilitate, or constrain, reproductive isolation.

In cases of ecological speciation, an association between niche specialization and sexual traits may favor the maintenance of reproductive isolation. For instance, in cichlid fishes, females tend to prefer the conspicuous males but different visual sensitivities are favored by natural selection in different light environments. Therefore, taxa that separated recently along an ecological gradient in water depth show different mating preferences associated with a divergence of male nuptial coloration (Seehausen et al. 2008b; Maan et al. 2010). *Heliconius* butterflies are another classical example demonstrating how natural selection on mating traits can enhance sexual isolation. In those butterflies,

sexual communication relies on visual cues and males preferentially court females exhibiting their own color pattern (Jiggins et al. 2001; Kronforst et al. 2006; Merrill et al. 2011). Wing color pattern has been called a "magic trait" because this feature is not only a mating cue whose variation triggers assortative mating, but also acts as a signal of toxicity to predators whose variation can be under disruptive selection for alternative Müllerian mimicry associations with local species (Kapan 2001; Merrill et al. 2012). In cases where speciation is associated with joining different mimetic communities, strong disruptive natural selection on wing pattern also causes divergence of wing pattern as a mating cue, facilitating reproductive isolation. For its dual role, the wing patterns of *Heliconius* were taken as an example of a "magic trait," whose changes trigger ecological speciation.

However, when speciation is not associated with a shift in mimicry coloration, natural selection may on the contrary prevent character displacement of this mating cue, and a conflict between natural selection and mate choice can generate costs for sexual communication. For instance, in the two distantly related comimics, *H. melpomene* and *H. erato,* which cannot hybridize but share the same color pattern, males spend a considerable amount of time approaching heterospecific females (Estrada and Jiggins 2008). When species are closely related and can hybridize, interference in mating signals may generate costs as well as reduce reproductive isolation. This has been suggested for cichlid fishes (Seehausen et al. 2008a), which show higher levels of hybridization in disturbed areas of Lake Victoria. Among other factors, it is likely that the turbidity of water interferes with the perception of nuptial colors and with mate choice (Seehausen 2009). Within the *Heliconius* butterfly radiation, recent studies uncovered cases of sister species, *H. timareta* and *H. melpomene,* which coexist in sympatry and display a very similar wing color pattern (Giraldo et al. 2008; Mérot et al. 2013). In this case, the "magic trait" does not diverge between species and cannot contribute to reproductive isolation, providing an opportunity to investigate the consequences of convergent natural selection on a mating cue.

The closely related species *H. melpomene* and *H. timareta* bear the same color pattern and can hybridize in the wild, with a limited frequency of hybrids of 1% or 2% in the area of sympatry (Mérot et al. 2013). Mimicry between recently diverged species is somewhat unusual in the *Heliconius* clade because visual recognition plays the major role in reproductive isolation, contributing to 75% of total reproductive isolation between *H. melpomene* and *H. cydno* (Jiggins 2008) and 89% between *H. erato* and *H. himera* (Muñoz et al. 2010). The maintenance of relatively strong reproductive isolation between the comimics *H. melpomene* and *H. timareta* then challenges the general observation that reproductive isolation in *Heliconius* is largely maintained by one barrier and one "magic trait," that is, male choice based on color pattern recognition. Two alternative predictions arise: either sexual isolation is low but total reproductive isolation is maintained by other isolating mechanisms, such as postmating isolation, or sexual isolation is high but is determined by other cues than color pattern.

Most studies have focused on a single-trait preference, yet a large variety of sensory modalities can be involved in sexual communication. Across animal taxa, courtship patterns include complex signals combining chemical, visual, auditory, and tactile cues (Candolin 2003; Chenoweth and Blows 2006). In insects, reproduction and sexual communication frequently involve chemical cues, such as long-range attracting pheromones for moths or short-range contact substances such as cuticular hydrocarbons (Symonds and Elgar 2008; Smadja and Butlin 2009). Divergence in chemical profiles can be a key component of species isolation. This role is well known in moths where closely related or incipient species usually display different pheromones that elicit species-specific mate choice (Frérot and Foster 1991; Thomas et al. 2003; Pelozuelo et al. 2004; Wicker-Thomas 2011; Saveer et al. 2014). Cuticular hydrocarbon specificity also contributes to isolation in *Drosophila* (Mas and Jallon 2005; Wicker-Thomas 2011). In butterflies, sexual communication through visual cues has received much attention but chemical cues should not be ignored because both males and females also emit volatile and nonvolatile compounds (Vane-Wright and Boppre 1993). They are involved in intraspecific communication, informing on the mating status of females (antiaphrodisiac role; Schulz et al. 2008; Estrada et al. 2011) or the quality or age of males (Nieberding et al. 2008, 2012). However, their role in interspecific sexual isolation in butterflies is poorly known. Some studies suggests that female pheromones can trigger male courtship at close range (Magnus 1958; Vane-Wright and Boppre 1993), that females may respond differently to heterospecific male pheromones (Vane-Wright and Boppre 1993), and that male sex pheromones may display rapid divergence between sympatric species (in *Bicyclus;* Bacquet et al. 2015).

In pairs of *Heliconius* species that differ on color pattern, the total level of premating sexual isolation observed in experimental conditions is higher (97%) than isolation due to male-driven color choice only (75%) (Jiggins 2008), suggesting nonvisual cues such as chemical scents must be involved in interspecific male choice However, untangling the multidimensionality of mate choice is difficult when variations in color pattern and other traits or behaviors are correlated.

In this study, we investigate a case of convergent natural selection on a mating cue by examining sexual isolation in the hybridizing pair of species *H. timareta thelxinoe* and *H. melpomene amaryllis*, which display the same wing pattern. Their mutual resemblance allows us to evaluate the role of visual versus nonvisual modalities of mate choice in sexual isolation, using individuals spanning the range of hybrid genotypes. Our results suggest that male courtship preferences rely on a

quantitative genetic control and are asymmetric between species. Male behavioral decisions demonstrate their ability to evaluate, at close range, species identity and hybrid component. Our chemical analyses allowed characterizing differences in chemical signature between the two species and their hybrids, and associate female mating success with chemical composition.

Material and Methods

SPECIES STUDIED AND EXPERIMENTAL POPULATION

Heliconius timareta thelxinoe (further called *H. timareta* or "T") and *H. melpomene amaryllis* (further called *H. melpomene* or "M") are two comimetic species coexisting in the Amazonian foothills of the Peruvian Andes in San Martín (Mérot et al. 2013). *Heliconius melpomene* is widespread and occurs from sea level to 1600 m of altitude, whereas *H. timareta* is less frequent and mostly found between 1000 and 1600 m of altitude.

About 15 mated females of each species were collected in their area of sympatry (Fig. S1) to found a stock population that was used for experiments and crosses. We obtained F1 hybrids in both directions (T × M and M × T, mother is given first) and backcross hybrids toward each species ("BT," backcross toward timareta and "BM," backcross toward melpomene). Larvae were reared in plastic containers and fed ad libitum on *Passiflora* sp. All butterflies were kept in large stock insectaries (3.5 × 2.5 × 2.5 m) in Peru, with plants, flowers, and access to sources of sugar and pollen, keeping them in conditions as close as possible to natural conditions. All behavioral experiments were performed in experimental insectaries under the same natural conditions preferably on sunny days. They took place from June to November 2012 and from May to October 2013.

NO-CHOICE MATING EXPERIMENT

To investigate reproductive isolation encompassing the entire courtship and mating process, we simulated a natural situation by a no-choice experiment in which males meet a single female. In large stock insectaries, a virgin female was released singly during 48 h in the presence of 10 mature males, either 10 *H. melpomene* or 10 *H. timareta*. Mating was checked every hour between 8 a.m. and 6 p.m. To ensure that no unobserved mating had occurred, male spermatophore presence in the genital tracts of the female was controlled in the evening of the first and second day and at the end of the experiment. For each experiment, a group of males was randomly composed from a stock of 20–50 mature males. The stock was continuously renewed as new mature males became available. A total of over 572 males composed the stock over the experiments. Experiments were performed along two independent field seasons (2012 and 2013) and the sessions were conducted on different days assuring that most trials were done with a largely renewed group of males. In addition, the identifi-

cation of males involved in mating excluded a disproportionate effect of a few males on our results. Females were never reused.

We analyzed the probability of mating and calculated confidence interval as described in Naisbit et al. (2001). Briefly, the distribution of getting m_{ij} realized mating out of N_{ij} trials between males of species i and a female of species j follows a binomial law B (P_{ij}, N_{ij}). The probability P_{ij} of mating can be calculated by maximizing the $\log_e$-likelihood expression given by

$$ML(P_{ij}) = m_{ij}\log_e(P_{ij}) + (N_{ij} - m_{ij})\log_e(1 - P_{ij}).$$

Therefore the value of the probability P_{ij} corresponds to the value that gives $ML'(P_{ij}) = 0$ (ML' being the derivative of ML) and can be calculated as $P_{ij}^* = \frac{m_{ij}}{N_{ij}}$. The 95% confidence intervals are equivalent to the parameter value that decreases the maximum value of ML by two units (Edwards 1972).

To test whether the probabilities of mating were significantly different between experimental combinations, we fitted the likelihood model with different hypotheses on parameters, going from a null model with $P_{MM} = P_{TM} = P_{TT} = P_{MT}$ (cf. Table 1) to the full model with different parameter values. The best model was selected with a likelihood ratio test with $G = 2\Delta ML$, which asymptotically follows a χ^2-distribution (Edwards 1972).

WING MODEL EXPERIMENTS

To investigate the importance of visual cues in mate location and approach by males, we performed experiments where males were presented with female wing models. Models were made with real female wings dissected and washed with hexane to remove volatile compounds and cuticular hydrocarbons. In large insectaries, groups of five to 15 males of one species were exposed simultaneously to female models of both species, fixed on a flexible wire on lightweight handle, for sessions of 10 min, swapping their position on the presentation device after 5 min. Experiments were performed under sunny conditions between 9 a.m. and 4 p.m. Total number of approaches and courtships were recorded for each female model. An approach was recorded when a male flew close (<10 cm) and toward the model and a courtship was recorded when the male initiated a stationary flight over the model using a hovering movement typical of courtship behavior seen with real females, although this behavior never lasted as long as toward virgin females. We performed 18 sessions with *H. timareta* males and 20 sessions with *H. melpomene* males with different subsets of males, constituted randomly from the "stock" pool as explained for the no-choice experiment. The different sessions do not represent independent replicates but rather provide a level of support for male preference. Besides, the high renewal rate of tested groups and the repeatability of the results ensure that the observations do not result from few individuals with extreme behavior. No significant differences of preference was observed

Table 1. Probabilities of mating in the no-choice experiments.

Males	Female	Mating	No mating	Probability of mating
H. melpomene	*M*	17	2	$P_{MM} = P_{TT} = 0.912$ [0.784–0.978]
H. melpomene	*T*	3	18	$P_{TM} = P_{MT} = 0.132$ [0.058–0.243]
H. timareta	*T*	14	1	$P_{TT} = P_{MM} = 0.912$ [0.784–0.978]
H. timareta	*M*	4	28	$P_{MT} = P_{TM} = 0.132$ [0.058–0.243]

between small (5–8), medium (9–12), or larger (13–15) groups of males (M: $P = 0.09$; T: $P = 0.41$). Wing models were changed when damaged.

We analyzed data as described above, following Jiggins et al. (2001), and obtained a probability $P_{i,T}$ of a male of species i approaching/courting a *timareta* model rather than a *melpomene* model by maximizing the $\log_e$-likelihood expression given by

$$ML(P_{i,T}) = \sum_j (T_{ij}\log_e(P_{i,T}) + M_{ij}\log_e(1 - P_{i,T})),$$

where j is the experimental session, T_{ij} the total number of approaches/courtships toward the *timareta* model within this session, and M_{ij} the total number of approaches/courtships toward the *melpomene* model. A probability of 1 means full preference for the *H. timareta* model, whereas a probability of 0 corresponds to full preference for *H. melpomene*. Confidence intervals were estimated as described above and we compared the parameters for the two species with a null model with random mating ($P_{i,T} = 0.5$) using a likelihood-ratio test as described before.

LIVE VIRGIN FEMALES EXPERIMENTS
Male preference
To investigate male choice and sexual interactions at close range, we monitored male behavior in experiments with live virgin females. In experimental cages of $2 \times 1 \times 2$ m, three to five mature males (>8 days old) were marked on the wing patch so as to be individually identified when flying or courting. Experiments were performed under sunny conditions between 9 a.m. and 4 p.m., using males of *H. timareta, H. melpomene,* F1, BT, and BM.

Males were presented a young virgin female of one species (*H. timareta*) for 10 min and then a young virgin female of the other species (*H. melpomene*) for 10 min again. Females were matched by age and were under five days old. Each male was monitored individually and repeated the experiment at least five times with different female pairs, randomizing the presentation order and on a different day.

To standardize behavior quantification between experiments, each 10-min session was divided into 20 time periods of 30 sec, in which we recorded, for each identified male, the occurrence (1) or the absence (0) of the following behavioral events: "approach," "courtship," "abdomen bending," and "mating" (Figs. 1, S2). Multiple successive identical behaviors by the same male during
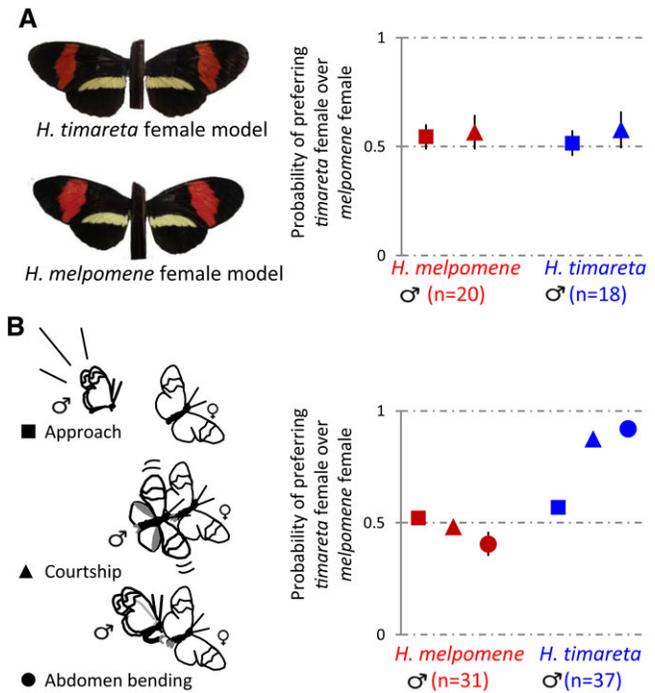

Figure 1. Male mating behavior and preference. (A) Probability (P_{iT}) of males to perform an approach (square) or a courtship (triangle) toward a *H. timareta* model rather than a *H. melpomene* model. (B) Probability (P_{iT}) of males to perform a mating behavior toward a live virgin *H. timareta* female rather than a *H. melpomene* female. Error bars show maximum-likelihood support limits. Drawings offer a schematic vision of the behaviors monitored. The body or wing areas shown in gray correspond to part of the body that potentially expose volatile compounds (the overlapping part of the wing, the claspers for the male, and the genital gland of the female).

the same interval were not counted multiply, but as a single event. Approach was defined as flying toward the female and getting very close (<10 cm). Following an approach, a male can then fly away or initiate courtship. Courtship involves a stationary, hovering flight above the female with small swaying movements. If the courtship lasted more than one 30-sec time lag, it was counted as present in each time interval until it stopped. If the male bended his abdomen while courting, as an attempt to mate, an additional record of "abdomen bending" was noted in the time interval. Mating was recorded when it occurred, but male and

female were separated gently and quickly. This does not affect subsequent behavior (Jiggins et al. 2001).

Male preferences were analyzed as described for the color pattern experiment. For each individual male and each genotype group (M, T, F1, BM, BT), we calculated a probability $P_{i,T}$ of approaching/courting/bending abdomen toward a *timareta* female rather than a *melpomene* female. Confidence intervals were estimated as described above and we compared parameters for each genotype group (M, T, F1, BM, BT) with a probability of 0.5 (no preference) using a likelihood-ratio test as described before.

Hybrid female courtship success

Gametes recombine unlinked parental traits, so the association of traits involved in sexual recognition (size, pattern, hue, mating acceptance, chemical compounds, and so on) can be broken in second-generation hybrids. This may be used to untangle the role of distinct female traits in male choice, so we evaluated courtship behavior toward hybrid females. Each hybrid female was presented with eight to 12 pure males of each species (*H. timareta, H. melpomene*), following the same protocol as described above, although experimental time was reduced to 5 min (ten 30-sec intervals) to assess the behavior of more males.

Variations in the mean number of courtships toward hybrid and pure females were tested using a two-way ANOVA and pairwise *t*-test with a correction (Benjamini and Hochberg 1995). Hybrid female attractiveness was defined as the mean number of courtships received by the female per 5-min experiment. Based on trials with *H. timareta* males (*H. melpomene* males were not choosy; see results), we defined two hybrid female groups: attractive females obtained as many courtships as pure *timareta* females (i.e., ≥2.5 mean courtships/experiment with *H. timareta* males); unattractive females obtained a number of courtships comparable to pure *melpomene* females (i.e., <2.5 mean courtships/experiment with *H. timareta* males).

Several potential mating cues may be associated with genotype, and were quantified in the hybrid females: chemical signature (Fig. S11), wing size (Fig. S9), color pattern (Fig. S8), and hue (Fig. S7). The chemical matrix was reduced to the compounds exhibiting significant differences between the parental species (*indval* and Kruskal–Wallis test, reported in Table S1). The association between each trait and hybrid female success (with *H. timareta* males and with *H. melpomene* males) was tested using per multivariate analysis of variance (MANOVA) and Mantel tests.

FEMALE CHOICE: MATING REALIZATION WITH PERFUMED MALES

To evaluate the use of male chemical cues in female choice, a virgin *H. melpomene* female was introduced during one day in an experimental cage of $2 \times 1 \times 2$ m with two *H. melpomene* males. The first male was "perfumed" by spreading a hexanic extract of *H. melpomene* male wings and claspers onto its wings and claspers, whereas the second male was "perfumed" with extracts from *H. timareta* males. Mating was checked every hour. When mating occurred, we reported the successful male and the experiment stopped. Results were analyzed with a chi-square test compared to a null expectation of random mating.

PHEROMONE ANALYSES

Chemical analyses of wings and genitalia

Mature male (>8 days old) claspers and hindwing androconial parts, and young (<5 days old) virgin female abdominal tip and hindwing friction zones (gray area in Fig. 1B) were dissected and extracted individually in 200 µl of hexane, containing 100 ng/µl of dodecane as an internal standard.

The composition of each extract was analyzed by gas chromatography coupled to mass spectrometry (GC/MS) using a Bruker Scion 436-GC linked to a Bruker Scion SQdetector. The fused silica capillary column (30 m × 0.32 mm i.d.) was coated with Rxi®-5SilMS (0.25 µm film thickness, Restek), the column temperature was programmed from 50°C to 300°C at 8°C/min and carrier gas was helium N60 at constant flow of 2 ml/min. Each extract was injected in the split/splitless injector at 250°C (0.5 µl via Combipal automatic injection). Mass spectra were recorded in the electron impact mode at 70 eV. Some extracts were reanalyzed under the same conditions in chemical ionization mode (150 eV), with methane as reactant gas, which gives the molecular weight of the compounds. Kovats' retention indices (RIs) were computed using n-alkanes from C10 to C24 that were eluted under the same conditions as the samples (external standards). Compounds were identified according to their RI, molecular weight, and mass spectra compared with the laboratory and with NIST 2011 libraries. Mass spectra of unidentified compounds are shown in Supporting Information (Fig. S17).

The amount of each compound was determined by integrating the area under each peak corrected by the area of the internal standard. A data matrix with relative amounts of all compounds for each individual was computed and aligned using GCAligner 1.0 (Dellicour and Lecocq 2013). The accuracy of the alignment was checked with original chromatograms.

Statistical analyses of variation in chemical blend

To detect variations in chemical composition between sexes and species/genotypes, we used the multivariate matrix describing the chemical signature of each individual, in which each line represents a sample with its relative amount in each chemical compound. Wings and genitalia were analyzed separately. We analyzed differences between sexes, between pure species, between genotypes (M, T, F1, BM, BT), and between attractive and unattractive hybrid females.

First, to visualize compositional differences between groups, we performed a nonmetric multidimensional scaling (NMDS) ordination. All NMDS plots were based on a Bray–Curtis similarity matrix and two or three dimensions, following the conventional cutoff at <0.15 for the stress value, using the function metaMDS in the package Vegan (Oksanen et al. 2013). The visualization was completed by an unscaled PCA (Principal Component Analysis) whose detailed methods and results are given in Supporting Information (Fig. S12-S13).

Second, differences in chemical signature between groups were tested using permutation-based MANOVA (per-MANOVA) using Bray–Curtis similarity matrix and 1000 permutations in the package Vegan (Oksanen et al. 2013). Homogeneity of variance was checked by performing a multivariate analysis of the homogeneity of group dispersion.

Third, we compared pure species to investigate which compounds are associated with species divergence (within each sex and body part). A nonparametric Kruskal–Wallis test was run with the relative area of each compound as variable and species ID as a factor. Following Heuskin et al. (2014) and Lecocq et al. (2013), we used the *indval* index, usually used for ecological data as an estimator of the association between species abundance and an ecological factor. Here, this index describes the association between a compound and a group (species or sex). It was computed only on pure species, using the *indval* function of the *labdv* package (Roberts 2013). We applied the same analysis between the groups of attractive females and unattractive hybrid females to test which compounds may be associated with *H. timareta* male courtship behavior. All analyses were performed with R (R Core Team 2014).

Results

BEHAVIORAL SEXUAL ISOLATION

No-choice mating experiments: Full male–female interaction

Eighty-seven no-choice tests were performed between *H. timareta* and *H. melpomene*. The probability of positive assortative mating was very high (Table 1), and over 90% of the trials involving conspecifics led to successful mating. On the contrary, mating rarely occurred within two days when a heterospecific female was introduced in the male cage, (4/28 for *timareta* males and 3/18 for *melpomene* males). Heterospecific mating had a low probability in both directions of crosses (Table 1) and in the best model, P_{TM} does not differ significantly from P_{MT} ($P = 0.66$).

Color pattern model experiments

When presented with wing models, males did not show significant preference for conspecific or heterospecific models, neither for the approach behavior ($P_{m,T} =0.52$; $P_{t,T} =0.55$, Fig. 1A) nor for short courtship events ($P_{m,T} =0.52$; $P_{t,T} =0.58$; Fig. 1A).

Male behavior toward pure virgin females

Heliconius timareta and *H. melpomene* males approached equally conspecific and heterospecific virgin females (Fig. 1B). The probability of approaching a *timareta* female rather than a *melpomene* female did not significantly differ from 0.5 for *H. melpomene* males ($P_{m,T} =0.54$; $P = 0.42$) and was significantly slightly higher than 0.5 for *H. timareta* males ($P_{t,T} =0.57$; $P < 0.01$). However, when considering *H. timareta* males individually (Fig. S3A), most of them did not show a significant preference for approaching *H. timareta* females. The same result was observed for all categories of hybrids males and for *H. melpomene* males individually (Fig. S3A).

Heliconius timareta males displayed a highly significant courtship preference toward conspecific virgin females ($P_{t,T} =0.89$, Fig. 1B). The same preference is observed for the abdomen bending behavior ($P_{t,T} =0.92$, Fig. 1B), which was more frequent toward conspecific *H. timareta* than toward *H. melpomene*. When approaching *H. melpomene* females closely, *H. timareta* males would fly away within a few seconds, before the female reaction could be observed. By contrast, *H. timareta* males would start a prolonged hovering courtship with most *H. timareta* females. Courtship would sometimes last a few minutes and included several mating attempt where the male would bend the abdomen and open his claspers around the female. Variability among males was observed but all *H. timareta* males displayed a significant preference for *H. timareta* females (Fig. 2).

On the contrary, *H. melpomene* males showed no significant differences in courtship probability toward conspecific or heterospecific virgin females ($P_{m,T} =0.51$, Fig. 1B, $P = 0.14$). For the abdomen bending behavior, males tended to attempt more matings toward conspecific virgins than toward *H. timareta* ($P_{m,T} =0.40$; Fig. 1B) but this was very variable among individuals and may be due to an interaction with female behavior (Fig. S3B).

Males resulting from backcross toward *H. timareta* (BT) also showed strong and significant preference for *H. timareta* females, both for courtship ($P_{bt,T} =0.77$; Fig. 2) and abdomen bending behavior ($P_{bt,T} =0.86$; Fig. S3B). F1 males from the T × M crosses tended to prefer *H. timareta* females ($P_{t \times m,T} = 0.67$ for courtship and $P_{t \times m,T} = 0.89$ for abdomen bending; Figs. 2, S3B). However, all T × M males tested here came from the same family and their consistent behavior might to some extent reflect their relatedness. F1 males from M × T crosses and BM behaved like *H. melpomene* males and courted both kinds of virgin female equally ($P_{m \times t,T} = 0.58$; $P_{bm,T} = 0.55$; Fig. 2).

Conspecific matings are a rare event in a 10-min trial and heterospecific matings were even rarer. With *H. timareta* females, only four matings were observed out of 1001 experiments, three with a conspecific *H. timareta* male, and one with *H. melpomene*
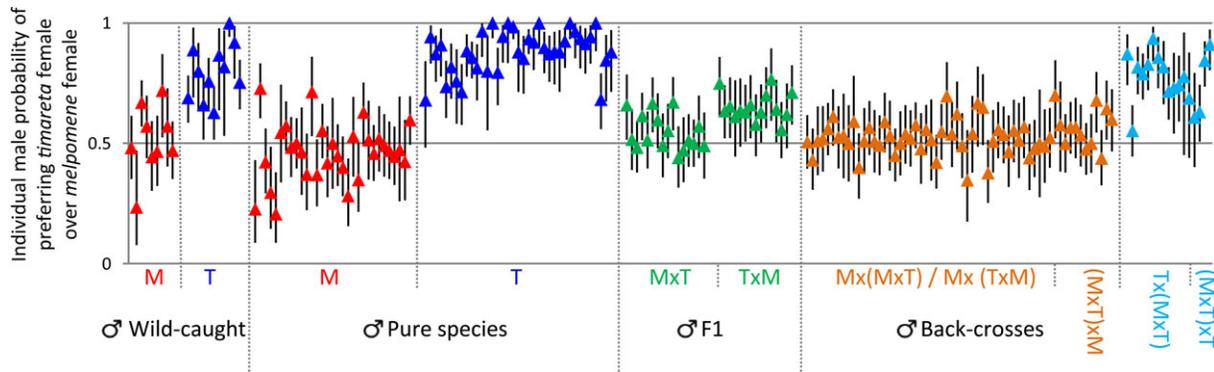
Figure 2. Individual courtship preference. Probability, for each male, to court a *H. timareta* female rather than a *H. melpomene* female. Error bars show maximum-likelihood support limits.

male. *Heliconius melpomene* females would mate more frequently because we observed 116 matings of 1001 experiments, 49 with *H. melpomene*, 13 with F1, 53 with BM, and one with BT males.

SPECIES DIVERGENCE IN CHEMICAL SIGNATURE
Chemical signature of abdominal tips

Chemical signature was significantly different between species (per-MANOVA: DF = 1, $F = 4.2$, $P < 0.001$, Fig. 3A) and between sexes (per-MANOVA: DF = 1, $F = 29.6$, $P < 0.001$, Fig. 3A). On the NMDS (stress value = 0.12) based on the composition of genitalia extracts, individual chemical signature clusters by sex on the first axis and by species on the second and third axis.

Female genital chemical signature was significantly different between *H. melpomene* and *H. timareta* (per-MANOVA DF = 1, $F = 2.7$, $P = 0.016$; Fig. 3A). Two compounds were exclusively found in *H. timareta* (alkane RI = 2338, *C4* RI = 2471). Other products were consistently more abundant in one species and the *indval* index identified 12 compounds significantly associated with species difference (Table S1).

Male genital glands display a significantly different chemical composition between *H. timareta* and *H. melpomene* (per-MANOVA DF = 1, $F = 4.5$, $P < 0.001$). Interspecific variations are displayed on the NMDS analysis (stress = 0.12, Figs. 3A, S14) and PCA (Fig. S12). The *indval* index identified 37 compounds that display significant interspecific variations in males (Table S1). In *H. melpomene* male genital bouquet, the most abundant product is a highly volatile compound, ocimen, not detected at all in *H. timareta*. In *H. timareta*, male genital bouquet is dominated by a blend of esters and fatty acids (Fig. S12 and Table S1).

Hybrid males and females occupy an intermediate position in the multivariate chemical space and usually displayed a combination of scents from both parental species, with BT being more similar to *H. timareta* and BM to *H. melpomene* (Figs. S11, S12, S14, Table S1).
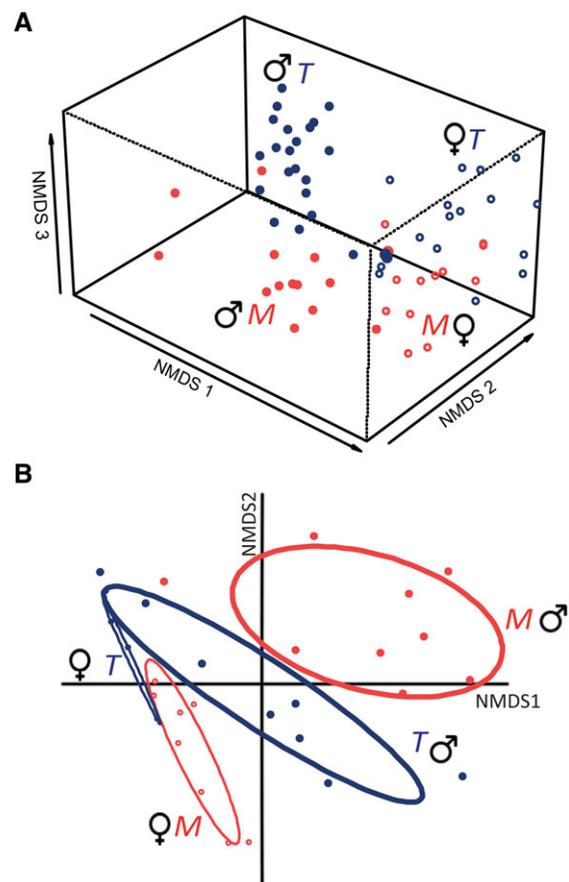

Figure 3. Male and female chemical signature. nMDS ordination plot based on Bray–Curtis distances calculated on the chemical composition of (A) abdominal tip extracts (stress value = 0.12) and (B) wing extracts (stress value = 0.11). Full dots represent males and circles represent females.

Chemical signature of wings

Wing chemical signature was significantly different between sexes (per-MANOVA: DF = 1, $F = 6.01$, $P < 0.001$) and sexes and species are separate in the NMDS analysis on wing composition (stress value = 0.11, Fig. 3B).
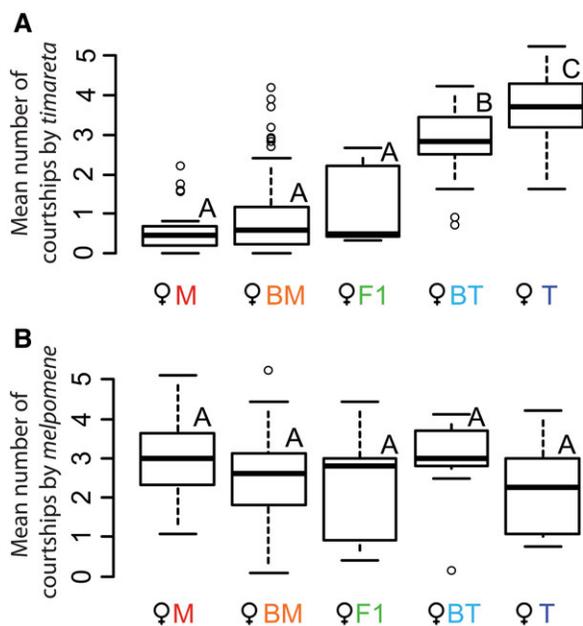
Figure 4. Mating success of hybrid females. Mean number of courtships in a 5-min trial directed toward a female by *H. timareta* males (A) and *H. melpomene* males (B). The box is drawn between the quartiles, with a thick line at the median value. Whiskers indicate the range of the data with outliers shown as circles. Groups marked with the same letter are not significantly different.

For female wings, despite a low sample size (only three samples for *H. timareta*), our analysis suggest differences in chemical signature between *H. melpomene* and *H. timareta* (per-MANOVA DF = 1, $F = 3.3$, $P = 0.019$, Table S1).

For male wings, the NMDS analysis (stress = 0.11, Figs. 3B, S13) and PCA (Fig. S12) revealed consistent and significant divergence in the chemical composition between *H. timareta* and *H. melpomene* (per-MANOVA DF = 1, $F = 4.6$, $P = 0.002$). *Heliconius melpomene* male wing extracts were strongly dominated by the octadecanal, which is 100–1000 times more abundant than other chemical compounds and not found in any extract of *H. timareta* male wings, nor in females. Hybrid male wing occupy an intermediate position in the multivariate chemical space with BT being more similar to *H. timareta*, whereas F1 and BM exhibit much octadecanal like *H. melpomene* (Fig. S13, Table S1).

THE USE OF CHEMICAL CUES IN MATING BEHAVIOR
Male behavior toward hybrid virgin females
The preference expressed by *H. timareta* males was also observed in the experiments with hybrid females. Females from backcrosses with *H. melpomene* and F1 females obtain significantly less courtships than pure *H. timareta* females (*t*-test, $P < 0.001$, Fig. 4A). Females from backcrosses with *H. timareta* (BT) obtained a higher number of courtships (and abdomen bending) from *H. timareta* males compared to BM, M, and F1 females

($P < 0.001$) but slightly inferior to courtship toward pure *H. timareta* females ($P = 0.002$, Fig. 4A). However, those results hide great interindividual variability in the number of courtships received by hybrid female (Figs. 5A, S15). Some hybrid females were approached by *H. timareta* males but males mostly flew away without attempting courtship, like they do with *H. melpomene* females, while other hybrid females received numerous courtship like *H. timareta* females.

The number of courtships received by *H. timareta* males appears significantly associated with the composition of the hybrid female chemical signature (Mantel test: $z = 1442$, $P = 0.023$; Per-MANOVA: $F = 3.1$, $P < 0.01$). Attractive and unattractive females display a significantly different chemical signature (Per-MANOVA: $F = 4.56$, $P < 0.001$) with some compounds significantly associated with success difference (Fig. 5B). By contrast, other potential mating cues such as wing size, pattern, or hue, which display subtle interspecific and genotypic quantitative variations (Figs. S7–S9), do not display any significant association with hybrid female success (Table S2, Fig. S16).

All hybrid females (F1, BM, BT) and control (M) were approached and courted equally by *H. melpomene* males (Fig. 4B). No association was expected nor found between the number of courtships received by *H. melpomene* males and any of the potential mating cues in hybrid females (chemical signature, wing size, pattern, and hue, Table S2).

Female choice: Mating realization with perfumed males
Mating between *H. melpomene* virgin female and a "perfumed" *H. melpomene* males was achieved significantly more often with the control male, perfumed with *H. melpomene* extract (12 control matings out of 16 trials) than with the experimental *H. timareta* perfumed male (four out of 16; χ^2 test, $P = 0.045$).

Discussion
By evaluating sexual behavior in the case of two comimetic sibling species, *H. timareta/H. melpomene,* we aimed to investigate how convergence on a similar color pattern would affect premating reproductive isolation and to assess the relevance of visual and nonvisual cues in sexual behavior of mimetic butterflies.

Although visual resemblance results in frequent interactions between *H. timareta* and *H. melpomene*, strong assortative mating was observed. Our experiments show that sexual isolation is partially explained by *H. timareta* male preference for conspecific females. *Heliconius timareta* male behavior suggests that nonvisual female cues, perceived at short range, might be the key of premating isolation. Analysis of chemical signature and experiments with hybrid females confirmed that chemical cues may be a species-specific medium of sexual communication triggering male preference.
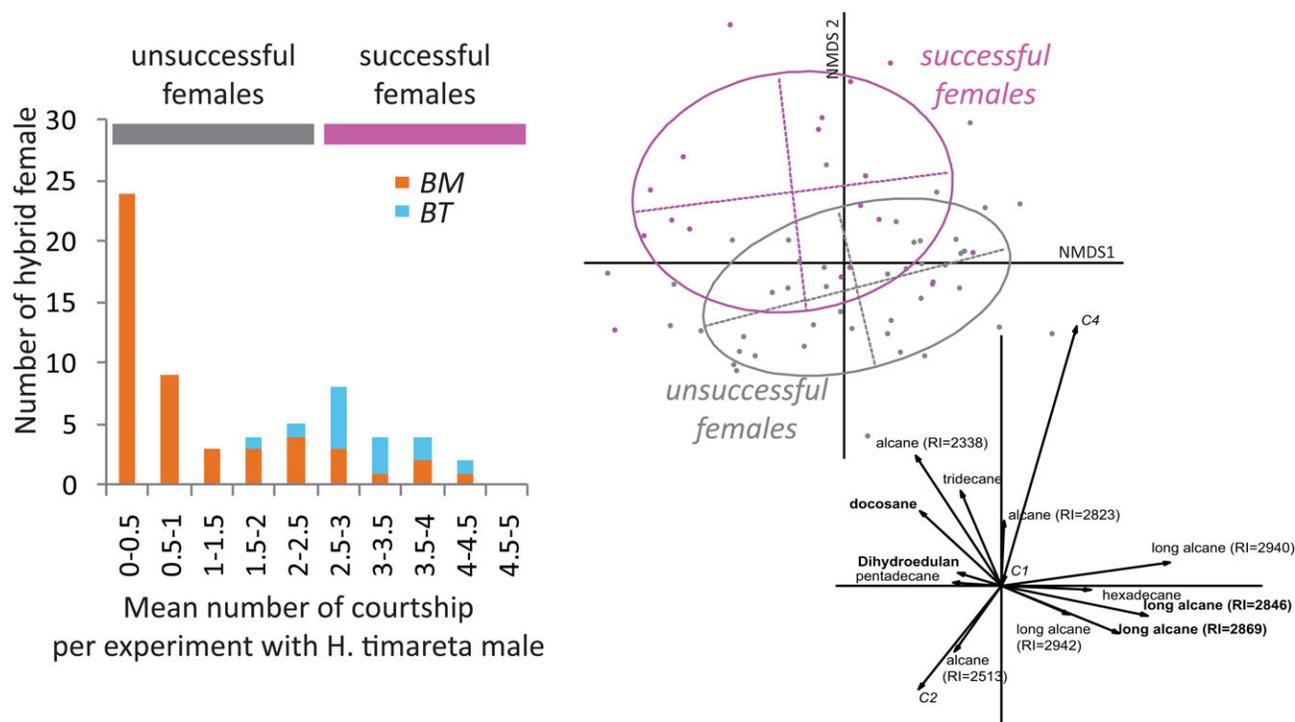
Figure 5. Female hybrid success and association with chemical signature. (A) Distribution of the mean number of courtships received by a hybrid female from one *H. timareta* male in a 5-min experiment and definition of the two groups: attractive and unattractive females. (B) nMDS ordination plot in two dimensions based on Bray–Curtis distances calculated on the chemical composition of abdominal tip extracts of the hybrid virgin females involved in the experiment (stress = 0.15). The directions of variation at each compound within the nMDS are represented by arrows on the second plot. Compounds in bold with a star display significant variations between the two groups with the *indval* index/Kruskal–Wallis test.

MALE PREFERENCE ON FEMALE VISUAL CUES VERSUS NONVISUAL CUES

Premating isolation appears also enhanced by female behavior given the numerous courtships addressed by *H. melpomene* males to heterospecific females without mating achievement. Such female preference also involves chemical cues. Interestingly, male preference appears asymmetric between the two species and heritable with multiple additive contributions in the different generation of hybrids.

Approach based on long-distance vision by *H. timareta* males was similar toward live virgin females of both species, as well as with hexane-washed models. However subsequent behavior differed radically, engaging in courtship with *H. timareta* females while flying away from *H. melpomene* females. Those results suggest that *H. timareta* males use visual color pattern cues to locate potential mates, but the decision of courtship is based on species-specific short-range cues from virgin females.

Even at close range, the two species exhibit very few visual differences: they differ slightly on size and display minute differences in the extent of the color patches, the hue of the ventral patches, and wing shape. However, no preference was observed on hexane-washed visual models even for close-range courtship behavior. Besides, no correlations were found between any of those traits and the number of courtships by *H. timareta* males toward variable hybrid females.

Close range discrimination by males had been described between two other comimics, the distantly related *H. erato* and *H. melpomene* (Estrada and Jiggins 2008). *Heliconius erato* males were more likely to court its own species when presented with real female wings but not when presented with wings washed with hexane, which removes most chemical compounds (Estrada and Jiggins 2008). Altogether with our observations that males sometimes engage courtship through a black net, this suggests that male preference is likely associated with the perception of chemical compounds present on or diffusing from the body or the wings of the female. Indeed, the approach-and-leave male behavior, observed on *H. timareta* male with heterospecific females, is rather reminiscent of behavior previously described when males investigate another male or a mated female and associated with the perception of antiaphrodisiac pheromones (Schulz et al. 2008).

Male attraction by species-specific female pheromones is well known from moths but little is known on female pheromone in butterflies. Work on *Argynnis paphia* by Magnus (1958)

suggested, like our results on *Heliconius,* that males are first attracted by color pattern, and that receptive females would then release a close range pheromone necessary to initiate courtship. In *Heliconius,* it was previously thought that an honest cue of a receptive virgin female was the absence of an antiaphrodisiac chemical compounds, transferred by male on first mating (Schulz et al. 2008; Estrada et al. 2011). However, in behavioral experiments, young virgin females are consistently more courted than older virgins (Klein and de Araujo 2010; Fig. S5), suggesting that virgin females also display attractive pheromones during the first days of their adult stage (or repulsive scents later).

In *H. timareta* and *H. melpomene,* female chemical signatures includes possible candidates for sexual signaling at short distances and several high molecular weight hydrocarbons, which are consistently different between species. Cuticular hydrocarbons are frequently involved in tactile sexual recognition in insect. In another butterfly species, *Bicyclus anynana,* the composition of cuticular compounds in females differs between body parts and covaries with age (Heuskin et al. 2014). Our experiments with hybrid females show that the intensity of *H. timareta* male courtship is correlated with chemical composition of the presented female, supporting that male courtship decision relies on particular and species-divergent female chemical cues.

Together with the recent results on cuticular compounds in *B. anynana* (Heuskin et al. 2014), as well as former studies on *Argynnis,* and longstanding observations of mating behavior at short range, our study calls for assessing the relevance of female chemical cues for intraspecific and interspecific sexual interactions not only in moths but also in butterflies.

FEMALE CHOICE ON MALE VISUAL AND NONVISUAL CUES

Although *H. melpomene* males attempt mating with *H. timareta* females as much as with conspecifics, heterospecific mating are not as frequent as conspecific mating, suggesting that female choice is critical for mating achievement and species isolation. In other butterfly species, *Leptidea reali* and *L. sinapis,* males court both conspecific and heterospecific females but a rejection behavior prevents heterospecific mating (Friberg et al. 2008). Female butterflies can prevent mating by flying away or by adopting a rejection posture. They accept mating by alighting, motionless, and wings closed. *Heliconius* females have been proposed to be unable to express a choice because courtship usually occurs soon after emergence, while their wings are still soft. This is certainly true in the pupal-mating clade in which male guard pupae and mate literally upon female emergence when females cannot reject (Gilbert 1976). However, whether this also stands for the nonpupal-mating *H. melpomene/H. timareta* has not been properly tested. In a preliminary experiment in which females were released in a male cage shortly after emergence, we noted that females received few courtships during the first hours. Males were seen courting intensely and attempting mating only from after about one day, a time when females were able to express acceptance or rejection. In addition, *H. melpomene* females and BM and F1 hybrids were frequently seen accepting to mate with *H. melpomene* males, whereas *H. timareta* females were seen mostly rejecting mating attempts.

In butterflies, intra- or interspecific sexual selection by females is generally based on elements of male wing pattern such as UV reflectance (Silberglied and Taylor 1973; Papke et al. 2007) and/or male sex pheromones (Silberglied and Taylor 1978; Vane-Wright and Boppre 1993; Andersson et al. 2007; Nieberding et al. 2008). In the case of *H. timareta/H. melpomene,* female choice may use some visual cues seen on the ventral sides of male wings during courtship. Indeed, male ventral patches (but not dorsal patches) display greater color differences between species than within species, due to a higher UV reflectance in *H. timareta* (Fig. S6).

Male courtship behavior in butterflies generally involves prolonged hovering over the female antenna, which favor the release of male sex pheromones directly on female sensory organs (Vane-Wright and Boppre 1993). Our experiments on female preference support the implication of male scent in female choice because males perfumed with heterospecific extracts obtained less matings than control males. In addition, we found strong divergence in chemical composition of genital glands and androconial wing patches between male of *H. melpomene* and *H. timareta.* Octadecanal, the most abundant compound found on the wings, is a lepidoptera pheromone-like compound (El-Sayed 2014) and only present in *H. melpomene.* Similarly, the most abundant compounds found in genital extracts (esters in *H. timareta* and ocimen in *H. melpomene*) are strikingly divergent between species and likely to be volatile compounds. Some genital male compounds have a demonstrated antiaphrodisiac role when transferred to female (Gilbert 1976; Schulz et al. 2008). However, interspecific variation in *Heliconius* male chemical blends and rapid shifts in multiple-mating clades led to the suggestion that male scents could also be subject to intraspecific sexual selection by female choice as well as mediate species recognition (Estrada et al. 2011). Male sex pheromones are generally honest indicators of the quality and age of the male (Johansson and Jones 2007), which could favor the evolution of female scent perception and preference.

BEYOND MAGIC TRAIT, THE DIMENSIONALITY OF SEXUAL ISOLATION

This study of two hybridizing species sharing their wing patterns improves our understanding of the modalities of reproductive isolation in *Heliconius* by going beyond the scenario of premating isolation through "magic trait" (wing pattern) divergence

previously described in *H. cydno* and *H. melpomene.* Premating sexual isolation appears as a multidimensional and multimodal process, involving both male and female choice and both visual and chemical communication. It is likely underpinned by a multilocus genetic architecture because hybrid preference (Fig. S4), female success (Fig. S15), or chemical signature (Figs. S11–S14) show gradual changes associated with the hybrid fraction of genome, as expected if hybridization reshuffled the multiple genes in a combinatorial and largely additive way. This contrasts with premating isolation due to color pattern divergence, which relies on a relatively simple genetic background dominated by major-effect genes with clear dominance in hybrids (Naisbit et al. 2003), and on visual male preference physically linked to color pattern loci (Kronforst et al. 2006; Merrill et al. 2010).

The coupling or the absence of association between isolating barriers has different implications regarding the modalities of speciation. Pleiotropic effects of color pattern variation and the genetic association between mating cue and preference favors early divergence and the maintenance of reproductive barriers in the face of gene flow (Smadja and Butlin 2011; Seehausen et al. 2014). Alternatively, the accumulation of multifarious barriers may be a factor of stability for reproductive isolation in the long term (Nosil et al. 2009). For instance, in the case of secondary contact or if gene flow homogenize certain traits (for instance traits involved in mate choice), reproductive isolation would be more easily maintained if other uncorrelated barriers are already present.

The most recent studies on the clade *cydno/timareta-melpomene* suggests that early divergence between *H. melpomene* and the *cydno/timareta* clade (1.5 My ago) likely occurred with limited migration, in allopatry or parapatry, followed by a recent increase in contact and gene flow (Martin et al. 2015). This recent period would have seen adaptive introgression of mimetic pattern between *H. timareta* and *H. melpomene,* while *H. cydno* and *H. melpomene* would have maintained or evolved a shift in color pattern. Under such a scenario, it is unclear whether chemical isolation evolved before or after color pattern divergence, through drift and intraspecific sexual selection during a period of low migration or after increased secondary contact. One can hypothesize that chemical divergence has been reinforced in mimetic pairs, especially if adaptive introgression has recently homogenized visual mating cues. However, total premating isolation between *H. cydno* and *H. melpomene* is higher that isolation due to visual male choice only, suggesting that chemical communication and female choice should also be involved in this pair (Jiggins 2008). Comparing the prevalence and the importance of divergence in visual versus chemical cues in other pairs of sister species will be the next step for understanding the evolution of reproductive barrier and to what extent the "magic trait" has been relevant for speciation.

ASYMMETRY IN MATE PREFERENCE, SEXUAL INTERFERENCE, AND COMPETITION

An intriguing result is the asymmetry of male preference between the two species: *H. timareta* males (and probably females) show a strong preference for conspecifics, whereas *melpomene* males court both species equally. *Heliconius melpomene* is a widespread abundant species whereas *H. timareta* has a limited range, restricted to higher altitudes. This observation that the choosiest morph/species of a pair is the rarest or the one with the most restricted range has also been reported in *Ischnura* damselfies (Sanchez-Guillen et al. 2012), *Sceloporus undulates* lizards (Hardwick et al. 2013), *Nasonia* jewel wasp (Bordenstein et al. 2000), and *Laticuada* sea snakes (Shine et al. 2002). Such asymmetric strength of preference might reflect differences in the risks and costs incurred to either species by heterospecific mating. Ecological speciation by adaptation to local conditions is necessarily associated with strong preferences for local mates, while the coexisting population of the widespread species would undergo continuous gene flow from allopatric populations where mate choice is not selected. Alternatively, the spatial distribution could also reflect the strength of premating competition. Indeed, theoretical model and empirical studies show that reproductive interference from a nonexclusive species could exclude the choosiest species or limit its range (Kuno 1992).

Conclusion

In butterflies and in *Heliconius,* behavioral premating isolation between closely related species has often been shown to be associated with male or female preference for conspecific wing colors (Silberglied and Taylor 1978; Jiggins et al. 2001; Papke et al. 2007). Our study on comimetic species shows that males indeed use color pattern to locate females, which induces approaches toward heterospecific females. However, despite this interference, premating isolation remained high because *H. timareta* males display strong preference for conspecifics females, presumably based on species differences in chemical signature. Our results also suggest a relevant role of female choice in premating reproductive isolation. We demonstrate interspecific differences in chemical signature that provide cues on sex and species and are likely involved in partner choice at both intra- and interspecific level. This highlights the exciting role of chemical cues in speciation, sometimes hidden in butterflies or *Heliconius,* and calls for further exploration of the multiple behaviors and cues involved of premating isolation and their sequence of evolution along the process of speciation.

DATA ARCHIVING

The doi for our data is http://dx.doi.org/10.5061/dryad.26qd7.

LITERATURE CITED

Andersson, J., A. K. Borg-Karlson, N. Vongvanich, and C. Wiklund. 2007. Male sex pheromone release and female mate choice in a butterfly. J. Exp. Biol. 210:964–970.

Bacquet, P. M. B., O. Brattström, H. L. Wang, C. E. Allen, C. Löfstedt, P. M. Brakefield, C. M. Nieberding. 2015. Selection on male sex pheromone composition contributes to butterfly reproductive isolation. Proc. R. Soc. B-Biol. Sci. 282:20142734.

Benjamini, Y., and Y. Hochberg. 1995. Controlling the false discovery rate—a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B (Stat. Methodol.) 57:289–300.

Bolnick, D. I., and B. M. Fitzpatrick. 2007. Sympatric speciation: models and empirical evidence. Annu. Rev. Ecol. Evol. Syst. 38:459–487.

Bordenstein, S. R., M. D. Drapeau, and J. H. Werren. 2000. Intraspecific variation in sexual isolation in the jewel wasp Nasonia. Evolution 54:567–573.

Candolin, U. 2003. The use of multiple cues in mate choice. Biol. Rev. Camb. Philos. Soc. 78:575–595.

Chenoweth, S. F., and M. W. Blows. 2006. Dissecting the complex genetic basis of mate choice. Nat. Rev. Genet. 7:681–692.

Coyne, J. A., and H. A. Orr. 2004. Speciation. Sinauer Associates, Sunderland, MA.

Dellicour, S., and T. Lecocq. 2013. GCALIGNER 1.0: an alignment program to compute a multiple sample comparison data matrix from large eco-chemical datasets obtained by GC. J. Sep. Sci. 36:3206–3209.

Edwards, A. W. F. 1972. Likelihood. Cambridge Univ. Press, Cambridge.

El-Sayed, A. 2014. The pherobase: database of pheromones and semiochemicals. Available at http://www.pherobase.com.

Estrada, C., and C. D. Jiggins. 2008. Interspecific sexual attraction because of convergence in warning colouratoin: is there a conflict between natural and sexual selection in mimetic species? J. Evol. Biol. 21:749–760.

Estrada, C., S. Schulz, S. Yildizhan, and L. E. Gilbert. 2011. Sexual selection drives the evolution of antiaphrodisiac pheromones in butterflies. Evolution 65:2843–2854.

Fitzpatrick, B. M., J. A. Fordyce, and S. Gavrilets. 2008. What, if anything, is sympatric speciation? J. Evol. Biol. 21:1452–1459.

Frérot, B., and S. Foster. 1991. Sex pheromone evidence for two distinct taxa within Graphania mutans (Walker). J. Chem. Ecol. 17:2077–2093.

Friberg, M., N. Vongvanich, A.-K. Borg-Karlson, D. J. Kemp, S. Merilaita, and C. Wiklund. 2008. Female mate choice determines reproductive isolation between sympatric butterflies. Behav. Ecol. Sociobiol. 62:873–886.

Gilbert, L. E. 1976. Postmating female odor in Heliconius butterflies—male contributed anti-aphrodisiac. Science 193:419–420.

Giraldo, N., C. Salazar, C. D. Jiggins, E. Birmingham, and M. Linares. 2008. Two sisters in the same dress: Heliconius cryptic species. BMC Evol. Biol. 8:324.

Hardwick, K. M., J. M. Robertson, and E. B. Rosenblum. 2013. Asymmetrical mate preference in recently adapted White Sands and black lava populations of Sceloporus undulatus. Curr. Zool. 59:20–30.

Heuskin, S., M. Vanderplanck, P. Bacquet, M.-J. Holveck, M. Kaltenpoth, T. Engl, C. Pels, C. Taverne, G. Lognay, and C. M. Nieberding. 2014. The composition of cuticular compounds indicates body parts, sex and age in the model butterfly Bicyclus anynana (Lepidoptera). Front. Ecol. Evol. 2:37.

Hohenlohe, P. A., and S. J. Arnold. 2010. Dimensionality of mate choice, sexual isolation, and speciation. Proc. Natl. Acad. Sci. USA 107:16583–16588.

Jiggins, C. D. 2008. Ecological speciation in mimetic butterflies. Bioscience 58:541–548.

Jiggins, C. D., R. E. Naisbit, R. L. Coe, and J. Mallet. 2001. Reproductive isolation caused by colour pattern mimicry. Nature 411:302–305.

Johansson, B. G., and T. M. Jones. 2007. The role of chemical communication in mate choice. Biol. Rev. 82:265–289.

Kapan, D. D. 2001. Three-butterfly system provides a field test of müllerian mimicry. Nature 409:338–340.

Klein, A. L., and A. M. de Araujo. 2010. Courtship behavior of Heliconius erato phyllis (Lepidoptera, Nymphalidae) towards virgin and mated females: conflict between attraction and repulsion signals? J. Ethol. 28:409–420.

Kronforst, M. R., L. G. Toung, D. D. Kapan, C. MCNeely, R. J. O'Neill, and L. E. Gilbert. 2006. Linkage of butterfly mate preference and wing color preference cue at the genomic location of wingless. Proc. Natl. Acad. Sci. USA 103:6575–6580.

Kuno, E. 1992. Competitive-exclusion through reproductive interference. Res. Popul. Ecol. 34:275–284.

Lecocq, T., Dellicour, S., Michez, D., Lhomme, P., Vanderplanck, M., Valterová, I., . . . & Rasmont, P. (2013). Scent of a break-up: phylogeography and reproductive trait divergences in the red-tailed bumblebee (Bombus lapidarius). BMC evolutionary biology, 13:263.

Maan, M. E., and O. Seehausen. 2011. Ecology, sexual selection and speciation. Ecol. Lett. 14:591–602.

Maan, M. E., O. Seehausen, and J. J. M. Van Alphen. 2010. Female mating preferences and male coloration covary with water transparency in a Lake Victoria cichlid fish. Biol. J. Linn. Soc. 99:398–406.

Magnus, D. B. E. 1958. Experimentelle Untersuchungen zur Bionomie und Ethologie des Kaisermantels Argynnis paphia. Über optische Auslöser von Anfliegereaktionen und ihre Bedeutung für das Sichfinden der Geschlechter. Z. Tierpsychol. 15:397–426.

Martin, S. H., A. Eriksson, K. M. Kozak, A. Manica, and C. D. Jiggins. 2015. Speciation in Heliconius butterflies: minimal contact followed by millions of generations of hybridisation. bioRxiv, 015800.

Mas, F., and J. M. Jallon. 2005. Sexual isolation and cuticular hydrocarbon differences between Drosophila santomea and Drosophila yakuba. J. Chem. Ecol. 31:2747–2752.

Mérot, C., J. Mavarez, A. Evin, K. K. Dasmahapatra, J. Mallet, G. Lamas, and M. Joron. 2013. Genetic differentiation without mimicry shift in a pair of hybridizing Heliconius species (Lepidoptera: Nymphalidae). Biol. J. Linn. Soc. 109:830–847.

Merrill, R. M., B. V. Schooten, J. A. Scott, and C. D. Jiggins. 2010. Pervasive genetic associations between traits causing reproductive isolation in Heliconius butterflies. Proc. R. Soc. B Biol. Sci. 278:511–518.

Merrill, R. M., Z. Gompert, L. M. Dembeck, M. R. Kronforst, W. O. McMillan, and C. D. Jiggins. 2011. Mate preference across the speciation continuum in a clade of mimetic butterflies. Evolution 65:1489–1500.

Merrill, R. M., R. W. R. Wallbank, V. Bull, P. C. A. Salazar, J. Mallet, M. Stevens, and C. D. Jiggins. 2012. Disruptive ecological selection on a mating cue. Proc. R. Soc. B Biol. Sci. 279:4907–4913.

Muñoz, A. G., C. Salazar, J. Castaño, C. D. Jiggins, and M. Linares. 2010. Multiple sources of reproductive isolation in a bimodal butterfly hybrid zone. J. Evol. Biol. 23:1312–1320.

Naisbit, R. E., C. D. Jiggins, and J. Mallet. 2001. Disruptive sexual selection against hybrids contribute to speciation between *Heliconius cydno* and *Heliconius melpomene*. Proc. R. Soc. B Biol. Sci. 268:1849–1854.

Naisbit, R. E., C. D. Jiggins, and J. Mallet. 2003. Mimicry: developmental genes that contribute to speciation. Evol. Dev. 5:269–280.

Nieberding, C. M., H. de Vos, M. V. Schneider, J. M. Lassance, N. Estramil, J. Andersson, J. Bang, E. Hedenstrom, C. Lofstedt, and P. M. Brakefield. 2008. The male sex pheromone of the butterfly *Bicyclus anynana*: towards an evolutionary analysis. PLoS One 3:12.

Nieberding, C. M., K. Fisher, M. Saastamoinen, C. E. Allen, E. A. Wallin, E. Hedenstrom, and P. M. Brakefield. 2012. Cracking the olfactory code of a butterfly: the scent of ageing. Ecol. Lett. 15:415–424.

Nosil, P., L. J. Harmon, and O. Seehausen. 2009. Ecological explanations for (incomplete) speciation. Trends Ecol. Evol. 24:145–156.

Oksanen, J., F. Blanchet, R. Kindt, P. Legendre, P. Minchin, and R. O'Hara. 2013. vegan: community ecology package. R package version 2.1-27/r2451.

Papke, R. S., D. J. Kemp, and R. L. Rutowski. 2007. Multimodal signalling: structural ultraviolet reflectance predicts male mating success better than pheromones in the butterfly *Colias eurytheme* L. (Pieridae). Anim. Behav. 73:47–54.

Pelozuelo, L., C. Malosse, G. Genestier, H. Guenego, and B. Frerot. 2004. Host-plant specialization in pheromone strains of the European corn borer *Ostrinia nubilalis* in France. J. Chem. Ecol. 30:335–352.

R Core Team. 2014. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

Roberts, D. W. 2013. labdsv: ordination and multivariate analysis for ecology. R package version 1.6-1. Available at http://CRAN.R-project.org/package=labdsv.

Safran, R. J., E. S. C. Scordato, L. B. Symes, R. L. Rodriguez, and T. C. Mendelson. 2013. Contributions of natural and sexual selection to the evolution of premating reproductive isolation: a research agenda. Trends Ecol. Evol. 28:643–650.

Sanchez-Guillen, R. A., M. Wullenreuther, and A. C. Rivera. 2012. Strong asymmetry in the relative strengths of prezygotic and postzygotic barriers between two damselfly sister species. Evolution 66:690–707.

Saveer, A. M., P. G. Becher, G. Birgersson, B. S. Hansson, P. Witzgall, and M. Bengtsson. 2014. Mate recognition and reproductive isolation in the sibling species *Spodoptera littoralis* and *Spodoptera litura*. Front. Ecol. Evol. 2:18.

Schulz, S., C. Estrada, S. Yildizhan, M. Boppre, and L. E. Gilbert. 2008. An antiaphrodisiac in *Heliconius melpomene* butterflies. J. Chem. Ecol. 34:82–93.

Seehausen, O. 2009. Progressive levels of trait divergence along a 'speciation transect' in the Lake Victoria cichlid fish Pundamilia. Pp. 155–176 *in* R. Butlin, J. R. Bridle, D. Schluter, eds. Speciation and patterns of diversity. New York: Cambridge Univeristy Press, New York.

Seehausen, O., G. Takimoto, D. Roy, and J. Jokela. 2008a. Speciation reversal and biodiversity dynamics with hybridization in changing environments. Mol. Ecol. 17:30–44.

Seehausen, O., Y. Terai, I. S. Magalhaes, K. L. Carleton, H. D. J. Mrosso, R. Miyagi, I. van der Sluijs, M. V. Schneider, M. E. Maan, H. Tachida, et al. 2008b. Speciation through sensory drive in cichlid fish. Nature 455:620-U623.

Seehausen, O., R. K. Butlin, I. Keller, C. E. Wagner, J. W. Boughman, P. A. Hohenlohe, C. L. Peichel, G.-P. Saetre, C. Bank, A. Brannstrom, et al. 2014. Genomics and the origin of species. Nat. Rev. Genet. 15:176–192.

Shine, R., R. N. Reed, S. Shetty, M. Lemaster, and R. T. Mason. 2002. Reproductive isolating mechanisms between two sympatric sibling species of sea snakes. Evolution 56:1655–1662.

Silberglied, R. E., and O. R. Taylor. 1973. Ultraviolet differences between sulfur butterflies, *Colias eurytheme* and *C. philodice*, and a possible isolating mechanism. Nature 241:406–408.

———. 1978. Ultraviolet reflection and its behavioral role in courtship of sulfur butterflies *Colias eurytheme* and *C. philodice* (Lepidoptera, Pieridae). Behav. Ecol. Sociobiol. 3:203–243.

Smadja, C., and R. K. Butlin. 2009. On the scent of speciation: the chemosensory system and its role in premating isolation. Heredity 102:77–97.

———. 2011. A framework for comparing processes of speciation in the presence of gene flow. Mol. Ecol. 20:5123–5140.

Sobel, J. M., and G. F. Chen. 2014. Unification of methods for estimating the strength of reproductive isolation. Evolution 68:1511–1522.

Sobel, J. M., G. F. Chen, L. R. Watt, and D. W. Schemske. 2009. The Biology of Speciation. Evolution 64:295–315.

Symonds, M. R. E., and M. A. Elgar. 2008. The evolution of pheromone diversity. Trends Ecol. Evol. 23:220–228.

The Marie Curie Speciation Network. 2012. What do we need to know about speciation? Trends Ecol. Evol. 27:27–39.

Thomas, Y., M. T. Bethenod, L. Pelozuelo, B. Frerot, and D. Bourguet. 2003. Genetic isolation between two sympatric host-plant races of the European corn borer, *Ostrinia nubilalis* Hubner. I. sex pheromone, moth emergence timing, and parasitism. Evolution 57:261–273.

Vane-Wright, R. I., and M. Boppre. 1993. Visual and chemical signaling in butterflies—functional and phylogenetic perspectives. Philos. Trans. R. Soc. B Biol. Sci. 340:197–205.

Wicker-Thomas, C. 2011. Evolution of insect pheromones and their role in reproductive isolation and speciation. Ann. Soc. Entomol. Fr. 47:55–62.

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