Species specificity and intraspecific variation in the chemical profiles of *Heliconius* butterflies across a large geographic range

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Abstract

Traits important for mate choice and behavioural isolation are predicted to be under strong stabilising selection within species. These traits can also exhibit variation at the population level that can be driven by both neutral and adaptive evolutionary processes. Here, we investigate the patterns of androconial and genital chemical profile inter- and intra-specific divergence in mimetic *Heliconius* butterflies across the Neotropics. We find evidence for species-specific compounds which are consistent across a large geographic range, suggesting a role in behavioural species isolation. At the population level, we also find chemical bouquet differences, but, contrary to predictions, these do not appear to be stronger in sympatric populations of co-mimetic species. The strength of the correlation between chemical and the genetic divergence varies between species and is generally stronger for genital chemical profiles than for androconial chemical divergence. Together, these patterns support that multiple evolutionary processes shape the evolution of chemical bouquets in *Heliconius* butterflies.

Keywords

Mate choice, reproductive isolation, chemical ecology, Lepidoptera, pheromones, signalling

Introduction

Reproductive isolation between lineages is important for the maintenance of biodiversity (Coyne and Orr 2004). In many systems, mate choice provides a strong pre-mating barrier to hybridisation (Nagel and Schluter 1998; Gray and Cade 2000; Ready et al. 2006; Friberg et al. 2008; Seehausen et al. 2008; Selz et al. 2014; Martin and Mendelson 2016). Closely related species often differ in traits important for mate choice, with individuals displaying a preference for conspecific phenotypes (Jiggins et al. 2001; Mas and Jallon 2005; Yildizhan et al. 2009; Ryan and Guerra 2014; Saveer et al. 2014). These traits

can also exhibit variation both within and between populations of the same species, either due to genetic drift and/or varying selective regimes across their ranges (Ryan and Rand 1993; Ryan et al. 1996; Bolnick and Kirkpatrick 2012; Ryan and Guerra 2014). Thus, to understand the evolution of reproductive isolation between lineages it is important to study phenotypic and genetic variation both within and between species.

Traits involved in behavioural isolation are predicted to show strong species-specific differences (Gerhardt 1982). Traits and preferences impose selection on one another, resulting in stabilising selection which can act to decrease intraspecific phenotypic variation (Butlin et al. 1985; Pfennig 1998; Ptacek 2000). As a consequence, we would expect to find little trait variability across species' large geographic ranges (Ferreira and Ferguson 2002; McPeek et al. 2011; Weber et al. 2016). Even if the signal as a whole varies geographically, certain invariant features may be important for successfully distinguishing species identities (Benedict and Bowie 2009).

Signals important for behavioural isolation could arise from the divergence of traits used in intraspecific communication between populations (Ryan and Rand 1993; Johansson and Jones 2007; Smadja and Butlin 2008; Mendelson and Shaw 2012). Signal divergence can be driven by various factors, both neutral and adaptive, usually involving multiple evolutionary forces (Leonhardt et al. 2013; Sun et al. 2013). A positive correlation between genetic distance, geographic distance, and phenotypic variation is consistent with stochastic processes, such as genetic drift, playing a prominent role (Irwin et al. 2008). In contrast, a lack of correlation between phenotypic and genetic divergence suggests that selection is shaping the phenotypic variation, perhaps driving divergence in different directions in each population (Hankison and Ptacek 2008; Mullen et al. 2009; Campbell et al. 2010; Conrad et al. 2018).

Chemical compounds, such as sex pheromones, mediate intraspecific communication in many systems (Wyatt 2003, 2014). The role of chemical signalling in behavioural isolation is also well established, especially among moth species (Löfstedt 1993; Smadja and Butlin 2008). Due to the coordination between detection and production, these pheromone blends are traditionally regarded as being under stabilising selection towards a species stereotype (Löfstedt 1993). Even when species-specific characteristics are present, chemical compounds can exhibit intraspecific variation, with both qualitative and quantitative differences found across a species' range (Ryan et al. 1995; Huang et al. 1998; Gemeno et al. 2000; McElfresh and Millar 2001; Groot et al. 2009; Weber et al. 2016; Conrad et al. 2018).

Studies of *Heliconius* butterflies have greatly contributed to our understanding of adaptation and speciation (Jiggins 2008, 2017; Merrill et al. 2015). Despite the high reliance of this group on visual cues for mating (Jiggins et al. 2001; Bybee et al. 2012; Merrill et al. 2014; Sánchez et al. 2015; Finkbeiner et al. 2017), it has long been suggested that male pheromones play a role in pre-mating barriers (Jiggins 2008; Merrill et al. 2015), but so far tests of their role has been limited and restricted to just a few species. Chemical signalling in *Heliconius* is important for female mate choice (Mérot et al. 2015; Darragh et al. 2017), and compounds differ between species (Mérot et al. 2015; Mann et al. 2017), suggesting a potential role in reproductive isolation. This seems especially important in closely-related species which are also co-mimics, such as *H. timareta* and *H. melpomene,* where visual signals alone may not be sufficient to successfully identify conspecifics (Giraldo et al. 2008; Mérot et al. 2013; Sánchez et al. 2015).

Interspecific confusion due to wing pattern mimicry could lead to selection for chemical recognition between species (Estrada and Jiggins 2008). This could result in reproductive character displacement between chemical profiles, whereby chemical bouquets differ more between sympatric than allopatric populations (Butlin 1987; Liou and Price 1994;

Höbel and Gerhardt 2003; Dyer et al. 2014). Alternatively, the chemical compounds could be part of a multimodal aposematic warning signal (Rothschild 1961; Rojas et al. 2018). In this case, we might expect co-mimics to exhibit more similar chemical bouquets to aid recognition with predators. These two opposing hypotheses have not been formally tested in *Heliconius*, and their relative importance could have great impacts on our understanding of the role of chemical signalling in speciation.

To investigate the evolution of *Heliconius* chemical profiles, we collected samples from seven species across the Neotropics, representing all major clades of the genus. We analysed both wing androconial and genital compounds of male butterflies to identify differences between species and evaluated consistency in the compound blends across different localities of the same species. We then investigated intraspecific variation in chemical profiles of *H. melpomene* and *H. erato* from across a large geographic range. We correlated these data with both geographic and genetic distances between different localities to test the hypothesis of selective evolutionary forces driving the observed variation in sex pheromones.

We used samples of *H. timareta*, *H. cydno* and *H. melpomene* from either side of the Andes in western Ecuador and Colombia to test for reproductive character displacement. *Heliconius timareta* and *H. cydno* are equally related to *H. melpomene*, but whilst *H. cydno* and *H. melpomene* have different wing warning colouration, *H. timareta* and *H. melpomene* are visual co-mimics (Fig. 1). We predicted that species pairs living in sympatry would be more divergent in terms of their chemical bouquet, and that this divergence would be greater between *H. timareta* and *H. melpomene* than between *H. cydno* and *H. melpomene*, as maladaptive mating choices would be unlikely in the latter pair due to the differing colour patterns. We used samples from two different mimicry rings in western Ecuador and Panama to test if the chemical compounds are part of the co-mimicry signal.

Methods

Sampling

Between one and fifteen males of *Heliconius* were collected from each of the following wild populations for chemical analysis (Fig. 1, Table S1). We include *H. tristero* as a subspecies of *H. timareta*, referred to hereafter as *H. timareta tristero* (Mérot et al. 2013).

Extraction and chemical analysis of tissues

The androconial region of the wing, previously described as the grey-brown overlapping region of the hindwing (Darragh et al. 2017), as well as the genitalia, were dissected for analysis immediately after collection. To extract pheromones, the tissue was soaked in 200µl dichloromethane containing 200ng 2-tetradecyl acetate (internal standard) in 2ml glass vials with PTFE-coated caps (Agilent, Santa Clara, USA) for one hour. The solvent was then transferred to new vials and, maintained cool in the field and stored at -20°C upon return. Androconial samples were evaporated to a reduced volume at room temperature prior to analysis. Pheromone extracts were analysed by GC/MS using an Agilent model 5977 mass-selective detector connected to an Agilent GC model 7890B and equipped with an Agilent ALS 7693 autosampler. HP-5MS fused silica capillary columns (Agilent, Santa Clara, USA, 30 m × 0.25 mm, 0.25 µm) were used. Injection was performed in splitless mode (250°C injector temperature) with helium as the carrier gas (constant flow of 1.2 ml/min). The temperature programme started at 50°C, was held for 5 minutes, and then rose at a rate of 5°C/minute to 320°C, before being held at 320°C for 5 minutes. Components were identified by comparison of mass spectra and gas chromatographic retention index with those of authentic reference samples and also by analysis of mass spectra. Components were quantified using 2-tetradecyl acetate as an internal standard. Only compounds eluting earlier than hexacosane were analysed in androconial samples, and those earlier than nonacosane

used in genital samples (Darragh et al. 2017). We removed compounds that were not found in at least half of all individuals from a given population.

Calculation of genetic and geographic distance matrices

To explore genetic distance among the studied *H. erato* and *H. melpomene* subspecies, we computed genetic covariance matrices and performed MDS for each species separately. A whole-genome sequence from a representative individual from each subspecies was used (Table S2). Genotypes were inferred from reads mapped to the *H. melpomene* (v2.5) and *H. erato demophoon* genome scaffolds (Heliconius Genome Consortium 2012; Challis et al. 2016; Davey et al. 2017; Van Belleghem et al. 2017) with bwa v0.7.15 (Li and Durbin 2009). We computed a pairwise identical by state (IBS) matrix with a random sampled read from each position in the genome, implemented in ANGSD v0.912 (Korneliussen et al. 2014). An interspecific genetic distance matrix was constructed using the function "cophenetic.phylo" from the *ape* package (Paradis and Schliep 2018) with a previously published phylogeny (Kozak et al. 2015). Geographic distance matrices were created by inputting the co-ordinates of collection localities into the function "distm" in the *geosphere* package to calculate the Haversine great-circle-distance between points (Hijmans 2017).

Statistical analyses

Variation in chemical profiles

Divergence in chemical profiles across species and populations was estimated with non-metric multidimensional scaling (NMDS) ordination in three dimensions, based on a Bray-Curtis similarity matrix. We used the "metaMDS" function in the *vegan* package version 2.5-1 (Oksanen et al. 2017), and visualised the NMDS using the *ade4* package (Dray and Dufour 2007).

We assessed the relative importance of relevant factors in driving the variation in chemical profiles with multivariate statistical analyses. These factors included: species identity, geographic region and individual locality. To compare overall variation in chemical composition between groups, we carried out PERMANOVA (permutational multivariate analysis of variance) testing base on a Bray-Curtis distance matrix, using the "adonis2" function in the *vegan* package (Oksanen et al. 2017) with 1000 permutations. We investigated each term in the model sequentially, starting with species identity, the main clustering factor found from visualisation with NMDS, followed by geographic region (Panama *vs* Western Andes *vs* Eastern Andes *vs* Amazon), and finally individual collecting localities. We followed these PERMANOVA tests with *post hoc* pair-wise testing using the function "pairwise.perm.manova" in the *RVAidememoie* package, with Bonferroni correction, to identify which grouping factors were significantly different (Hervé 2018). We repeated this within species, in *H. erato* and *H. melpomene,* to investigate fine-scale intraspecific geographic patterns. In the within species analysis we included geographic region (Panama *vs* Western Andes *vs* Eastern Andes *vs* Amazon), and individual collecting localities as the two factors.

One issue with distance-based analyses such as PERMANOVA is that differences in dispersion between groups can be confounded with differences in location (Warton et al. 2012). To confirm these analyses and account for this issue, we implemented multivariate generalised linear models using the function "ManyGLM" from the *mvabund* package (Wang et al. 2012). We modelled the data using a negative binomial distribution, which we found to be appropriate through examination of residual plots. For interspecific analyses we included species, region, and locality nested within region in the model. For intraspecific analyses we included region and locality nested within region. The "ManyGLM" function fits models to each chemical compound, summing the test statistics to give a multivariate test statistic

known as Sum-of-LR. This statistic can be tested for significance using resampling methods. We can also determine which compounds are driving between-group differences by looking at the individual contribution of each compound to the Sum-of-LR, with p-values adjusted for multiple testing using the "adjust" option.

Inter- and intra-specific indicator compounds

We carried out indicator analysis using the *indicspecies* package (Cáceres and Legendre 2009). Groupings are decided *a priori* and components are determined which act as indicators of these groups. The best indicators are those which are only found in a single group and all group members possess the compound; such a compound would have an indicator value of 1. We used the function "indicators" to investigate both which single compounds and combinations of compounds best predict group membership. We used the function "pruneindicators" to find the single or combinations of compounds which had the highest indicator values.

Phylogenetic and geographic distance

Shared ancestry can explain part of the variation in a species' chemical profile. We tested for a correlation between phylogenetic distance and chemical profile divergence with Mantel tests using the *vegan* package (Oksanen et al. 2017). To investigate the role of geographic distance in chemical profile divergence, we compared geographic and chemical distances matrices with partial Mantel tests. To visualise the species phylogeny we used the "plot.phylo" function from the ape package (Kozak et al. 2015; Paradis and Schliep 2018).

Genomic and chemical distance within species

We calculated intraspecific genetic distances using genome sequences from 11 *H. erato* and 13 *H. melpomene* populations. We visualised genetic distances in two dimensions using MDS with the function "cmdscale". We tested for a correlation between intraspecific

genetic distance and chemical profile divergence with mantel tests using the *vegan* package (Oksanen et al. 2017). Hybrids between populations of the same species were excluded from this analysis. We used partial Mantel tests to investigate the role of geographic distance.

Character displacement of chemical profiles

We used samples of four populations from two localities: *H. timareta tristero* and *H. melpomene bellula* from the foothills of the eastern Colombian Andes, *H. cydno alithea* and *H. melpomene cythera* from the foothills of the western Ecuadorean Andes. We calculated pairwise Bray-Curtis distances between individuals of *H. timareta tristero* and *H. cydno alithea* with the two *H. melpomene* populations. We tested for differences between these distances using a one-way ANOVA. For *post hoc* analysis, we used the "HSD.test" function in the *agricolae* package to carry out a Tukey's honestly significant difference test to determine which groups were significantly different from each other (de Mendiburu 2019).

Co-mimics and similarity of chemical profiles

We used samples of two mimicry rings from two localities, Panama and western Ecuador. *H. melpomene* and *H. erato* form one mimicry ring, whilst *H. cydno* and *H. sapho* form another, with the addition of *H. eleuchia* in western Ecuador. To test which factors most influenced the variation in chemical profiles we used both PERMANOVA and multivariate GLM. We included species, region, mimicry group, and interactions between these terms in the models.

All statistical analyses were performed with *R* version 3.5.1 (R Core Team 2018). Figures were made using a palette of colours optimized for colour-blindness (Wong 2011).

Results

Chemical compounds in androconia and genitals:

We sampled 252 androconia and 275 genitals across 42 populations of 33 subspecies of seven species, and identified 349 compounds in the genitals and 157 in the androconia. Of the total number of androconial compounds, 38% are fatty acid derivatives, 20% aromatics, 10% terpenoids, 1% macrolides, <1% lactones and 31% unknown or unidentified compounds. Of the genital compounds, 17% are fatty acid derivatives, 7% aromatics, 10% terpenoids, 1% lactones, 12% macrolides and 44% unknown or unidentified compounds. The main difference is that there are more macrolides in the genitals than androconia.

Heliconius species varied considerably in the amount and abundance of compounds (Fig. 2). Between species there was variation in the number of compounds per individual, and the overall amount of compounds detected (Table S3, S4). For the androconia, there were the fewest compounds in *H. eleuchia* (13±5), and the most in *H. melpomene* (32±11). *H. sapho* had the lowest total abundance of compounds at 1,458±1,606 ng, and *H. melpomene* the most at 7,412±16,484 ng. The species with the fewest genital compounds was *H. sapho* with 32±7, and the most *H. cydno* with 102±21. *H. sapho* also had the lowest total amount of compounds at 6,991±7,950 ng, and *H. cydno* the highest at 91,517±134,225 ng. In general, a higher number of compounds and amount of each is found in the genitals than androconia of *Heliconius*.

What factors affect interspecific variation in chemical profiles?

Our sampling provided us with the context to investigate the degree to which variation in chemical composition is partitioned within or between species, in order to understand the extent to which chemistry is a species-diagnostic trait. Visualisation of the chemical profiles reveals that individuals mostly group by species for both androconial and

genital chemical bouquets (Fig. 3). Species significantly differ in their androconial bouquet, with species identity accounting for 58% of the overall variation in chemical profiles (PERMANOVA, Species, $F_{6,251}$=74.23, p<0.001). All pairwise comparisons of species are significant (Table S5). A further 4% of variation can be explained by region (Amazon/Eastern Andes/Western Andes/Panama), and 3% by locality nested within region (PERMANOVA, Region, $F_{3,251}$=10.24, p<0.001; (Region/Locality), $F_{8,251}$=2.72, p<0.001). Finally, 5% of variation is explained by an interaction between species and region, and 2% between species and locality nested within region (PERMANOVA, Species*Region $F_{6,251}$=4.96, p<0.001; Species*(Region/Locality) $F_{8,251}$=1.82, p<0.001).

The results were similar for the genital bouquets, with species identity accounting for 51% of the variation in chemical profiles (PERMANOVA, Species, $F_{6,274}$=61.29, p<0.001). All pairwise comparisons are significant apart from *H. elevatus* and *H. melpomene* (Table S6). A further 5% of variation can be explained by region (Amazon/Eastern Andes/Western Andes/Panama), and 3% by locality nested within region (PERMANOVA, Region, $F_{3,274}$=12.73, p<0.001; (Region/Locality), $F_{8,274}$=2.99, p<0.001). Finally, 6% of variation is explained by an interaction between species and region, and 2% between species and locality nested within region (PERMANOVA, Species*Region $F_{6,274}$=6.68, p<0.001; Species*(Region/Locality) $F_{8,274}$=1.77, p<0.001). For both androconial and genital chemical profiles, most variation is explained by species identity, rather than geographic location, as confirmed by ManyGLM (Tables S7, S8).

Are there species-specific chemical compounds?

In order to identify candidate recognition pheromones, we examined our data for species specific compounds using an indicator analysis. In most species that we examined, there were single androconial compounds that were strong indicators of species identity (Table 1). For example, Geranylgeranylacetone was found only in *H. erato*. Similarily,

Octadecanal was found almost exclusively in *H. melpomene* (specificity=0.999). *H. cydno* and *H. eleuchia* had the weakest indicator scores; in *H. cydno* because the compound was not found in all individuals examined members (coverage= 0.667), and in *H. eleuchia* because the compound was also found in other species (specificity= 0.747). There were similarly species-specific genital compounds in all species except *H. sapho* and *H. timareta*, where a combination of two compounds is the best predictor (Table 2). For *H. erato* we identified a terpene ester which is only found in *H. erato* individuals and no other species. Other terpene esters are also almost perfect indicators in *H. erato*. For *H. melpomene*, the known anti-aphrodisiac, (*E*)-β-ocimene, is the best predictor.

Does phylogenetic distance explain chemical profile divergence?

Using genomic sequence data, we explored the degree to which variation between species can be explained by geographic and genetic distance among the samples. We carried out partial Mantel tests to investigate the correlation between two variables whilst controlling for a third variable. When controlling for geographic distance, genetic and androconial chemical divergence are correlated (Mantel test: r=0.1088, p=0.0001), and there is an even stronger correlation between genetic and genital chemical divergence (Mantel test: r=0.6936, p=0.001). When controlling for genetic distance, geographic distance is significantly but weakly correlated with androconial chemical divergence (Mantel test: r=0.043, p=0.002), with similar results for genital chemical divergence (Mantel test: r=0.046, p=0.007).

What factors affect intraspecific variation in chemical profiles of H. erato *and* H. melpomene*?*

As well as investigating between-species differences, we wanted to determine sources of variation within species using our broad sampling of distinct color pattern forms of *H. erato* and *H. melpomene*. For *H. erato* there was a strong grouping of individuals by region

(Fig. 4), with 27% of variation in androconial profiles being explained by region and 11% by locality nested within region (PERMANOVA, Region $F_{3,87}$=11.16, p<0.001, Locality $F_{6,87}$=2.35, p<0.001). All four regions are significantly different from each other (Pairwise permutation MANOVAs, p<0.01). For *H. erato* genital compounds, 37% of variation is explained by region, and 11% by locality nested within region (PERMANOVA, Region $F_{3,91}$=19.01, p<0.001, Locality $F_{6,91}$=2.83, p<0.01). All four regions are significantly different from each other (Pairwise permutation MANOVAs, p<0.05).

These geographic differences in chemical profiles are not as strong in *H. melpomene* (Fig. 5). For *H. melpomene* androconial compounds, 18% of variation is explained by region and 13% by locality nested within region (PERMANOVA, Region $F_{3,86}$=6.73, p<0.01, Locality $F_{8,86}$=1.82, p<0.001). The West Andes subspecies (*H. m. cythera*) is not significantly different from either East Andes (multiple populations) or Panama (*H. m. rosina*) (Pairwise permutation MANOVAs, p=0.072), however, the other comparisons are significantly different (Pairwise permutation MANOVAs, p<0.05). For *H. melpomene* genital compounds, 20% of variation is explained by region, 12% by locality nested within region (PERMANOVA, Region $F_{3,103}$=8.91, p<0.001, Locality $F_{7,103}$=2.34, p<0.001). All regions are significantly different from each other (Pairwise permutation MANOVAs, p<0.05), apart from West Andes and Amazon (Pairwise permutation MANOVAs, p=0.120). Within region the amount of variance explained by locality is the same in both *H. erato* and *H. melpomene* but at the larger geographic scale of region, *H. erato* is more structured, with more variance explained. These results were confirmed by ManyGLM (Tables S11, S12, S13, S14).

Do we find subspecies-specific chemical compounds?

We used an indicator analysis to search for compounds unique to specific populations of *H. erato* and *H. melpomene*. Most intraspecific differences are due to quantitative rather than qualitative differences between populations, perhaps explaining why many population

indicators were weak as they are also found in other regions at different amounts (Table S9, S10). The only exception is *H. e. cyrbia* (Western Ecuador) that has many genital compounds unique to this region (Table S9).

Does intraspecific genetic distance explain chemical divergence in H. erato *and* H. melpomene*?*

In *H. erato* both androconial and genital chemical distance are positively correlated with genetic distance, even when we account for geographic distance (Mantel test, androconia, R=0.255, p=0.001; genitals, R=0.352, p=0.001; partial Mantel test, androconia, R=0.169, p=0.001; genitals, R=0.348, p=0.001). When we account for genetic distance, geographic distance is negatively correlated with androconial chemical distance and not correlated with genital chemical distance (partial Mantel test, androconia, R=-0.149, p=0.001; genitals, R=-0.078, p=0.962).

In *H. melpomene* both androconial and genital chemical distance are correlated with genetic distance (Mantel test, androconia, R=0.0467, p=0.019; genitals, R=0.146, p=0.001). When accounting for geography, this correlation is still present for the genital bouquet, but disappears for the androconia (partial Mantel test, androconia, R=0.0135, p=0.264, genitals, R=0.120, p=0.001). When we first consider genetic distance, geographic distance is not positively correlated with genital chemical distance, however, it is positively correlated with androconial chemical distance (partial Mantel test, androconia, R=0.231, p=0.001; genitals, R=-0.004, p=0.526). *Heliconius melpomene* genitals show similar patterns to *H. erato* genitals, although the correlation is weaker. *Heliconius melpomene* androconia, however, show a different pattern, with intraspecific variation explained by geographic but not genetic distance.

Is there evidence for reproductive character displacement of chemical profiles?

We found that, as expected, individuals group by species, with *H. melpomene* populations (*H. m. cythera* and *H. m. bellula*) grouping together, and *H. cydno* and *H. timareta* grouping together, as predicted by phylogeny (Fig. 6). To test for reproductive character displacement, we compared the pairwise chemical distance between (i) sympatric; and (ii) non-sympatric species pairs. For androconial bouquets, we found that *H. melpomene cythera* and *H. cydno alithea* (which are sympatric in western Ecuador) are more different from one another than the allopatric *H. melpomene cythera* and *H. timareta tristero* (Fig. S1), consistent with reproductive character displacement. However, this was not true in the other comparison. Specifically, *H. melpomene bellula* and *H. timareta tristero*, which are sympatric in Colombia, are not more different than the allopatric *H. melpomene bellula* and *H. cydno alithea* (Fig. S1).

Genital bouquets also do not follow a consistent trend of reproductive character displacement (Fig. 6). Again, we found that *H. melpomene cythera* and *H. cydno*, which are sympatric in western Ecuador, are more different than the allopatric *H. melpomene cythera* and *H. timareta* (Fig. S2). However, we also found *H. melpomene bellula* and *H. timareta*, which are sympatric in Colombia, to be more similar to one another than allopatric *H. melpomene bellula* and *H. cydno* (Fig. S2), in contrast to expectations. In both genital comparisons *H. melpomene* is more similar to *H. timareta* than *H. cydno*, regardless of geography.

Is there evidence for similarity between co-mimics of chemical profiles?

Individuals collected in Panama and western Ecuador from two mimicry rings group by species (Fig. 7). In a PERMANOVA model including species, region and co-mimicry group as terms, as well as interactions between these terms, we did not find co-mimicry

group to be a significant term. The final model including significant terms included species, region, and an interaction between species and region (PERMANOVA, Species $F_{4,85}$=42.94, p<0.001; Region $F_{1,85}$=3.39, p=0.004; Species*Region $F_{3,85}$=2.81, p=0.002). We confirmed these results using ManyGLM (Table S15).

We found similar trends for genital bouquets. Again, we did not find co-mimicry group to be a significant term, with the final model including species, region, and an interaction between these two (PERMANOVA, Species $F_{4,88}$=42.19, p<0.001; region $F_{1,88}$=8.29, p<0.001; Species*Region $F_{3,88}$=5.01, p<0.001). We confirmed these results using ManyGLM (Table S16). In both PERMANOVA and in multivariate GLMs, variation in both androconial and genital bouquets is explained mostly by the species of the butterfly.

Discussion

The species-rich genus *Heliconius* is an excellent example of a continental-scale adaptive radiation in the Neotropics (Kozak et al. 2015). Speciation in this group is often associated with divergence in wing colour pattern and there has been a lot of research into the importance of pattern variation in speciation and mate preference (Jiggins et al. 2001; Jiggins 2008; Merrill et al. 2011, 2015; Sánchez et al. 2015). However, one of the surprising findings to emerge from comparative genomic analysis is the wealth of genes involved in chemosensory reception (Heliconius Genome Consortium 2012), suggesting that chemical signalling may play an important role in the reproductive biology of the group. To begin to understand the role of chemical signalling in this radiation we have surveyed both inter- and intraspecific variation of *Heliconius* androconial and genital chemical profiles across the Neotropics. We find that most of the variation in chemical profile across our samples is explained by species, and identify key chemicals serving as indicators for each species. Nonetheless, there is also abundant intraspecific variation in chemical profiles. This variation

was mainly quantitative in nature, with the exception of *H. erato cyrbia* which produces some compounds not found in other *H. erato* populations, and strongly correlated with genetic distance. Within this backdrop, we find little evidence for reproductive character displacement among closely related species. Moreover, the hypothesis that co-mimics might also show converge in their chemical profiles was similarly not supported. Our work sets the stage for further investigation of the function of chemical profiles, and their role in within and between-species signalling.

Chemical profiles are predicted to be highly species-specific if they are involved in species recognition during mating. Orchid bee chemical blends, important for mating and species recognition, show high species-specificity, as well as within-species variability, which can be partly explained by geography (Zimmermann et al. 2006; Weber et al. 2016; Brand et al. 2019). We see similar patterns in *Heliconius*, with greater interspecific differences than intraspecific in chemical profiles. Species identity is the best predictor of chemical divergence, with geographic location able to explain some intraspecific differences. One exception to this is *H. elevatus* which does not group separately from its co-mimic *H. melpomene* for genital compounds. Further samples are needed to confirm that this result is not due to the small sample of *H. elevatus* in this study. As in orchid bees, these species differences are consistent across a large geographic range, suggesting that they could be important for reproductive isolation between species (Weber et al. 2016).

Consistent species-specific compounds are likely to be biologically important. In *H. melpomene* we already know that the compounds identified as an indicator for the genitals, (*E*)-β-ocimene, is biologically active (Schulz et al. 2008). Combining broad geographic sampling with indicator analysis provides a promising approach to determine potential biologically active compounds in other species, which could then be tested behaviourally.

Androconial and genital bouquets show different patterns of evolution. Whilst androconial chemical distance was only weakly correlated with genetic distance between species, genital chemical distance was very strongly correlated. This suggests that neutral evolutionary forces are more important in driving genital chemical evolution. The correlation between genital chemical distance and genetic distance is a much stronger correlation than previously reported (Estrada et al. 2011), possibly due to the quantitative nature of our data. One point to consider is that this could be due to the fact that there are more compounds in the genitals than androconia. Most of these compounds are probably neutrally evolving and therefore drive the correlation to be stronger. For example, in *H. melpomene*, one compound, (*E*)-β-ocimene, can alone act as an anti-aphrodisiac, with other components of the bouquet thought to moderate its evaporation rate (Schulz et al. 2008). Looking at the evolutionary patterns of only the compounds we know to be biologically active, rather than the entire bouquet, may allow us to disentangle the processes involved in the evolution of these profiles.

Heliconius erato and *H.melpomene* both exhibit extensive colour pattern variation across their geographic range (Sheppard et al. 1985). We show that these populations also differ in their androconial and genital bouquets. Whilst traditionally predicted to be under stabilising selection, intraspecific variation between populations in chemical profiles has been documented in other Lepidoptera (Huang et al. 1998; McElfresh and Millar 2001; Takanashi et al. 2005; Groot et al. 2009; Bacquet et al. 2016). Chemical divergence in putative male sex pheromones between populations of *Bicyclus anynana* is reported to be as large as differences between *Bicyclus* species, and is greater than predicted by genetic divergence (Bacquet et al. 2016). This is in contrast to what we find here, where interspecific differences are much greater than intraspecific. *Heliconius erato cyrbia*, found west of the Andes, produces many unique genital compounds. These populations are also the most genetically

divergent *H. erato* population in our study, suggesting that genetic drift could be important for the evolution of chemical profiles in *Heliconius*. Across all *H. erato* populations in our study we find a correlation between chemical distance, both androconial and genital, and genetic distance. These correlations suggest that the geographic variation between populations could be neutral, with stochastic processes important for bouquet evolution in *Heliconius*.

In *H. melpomene*, correlations between chemical and genetic distance are weaker, with androconial variation better explained by geographic distance. This does not match our expectations of neutrality and might imply that other evolutionary forces are important for chemical profile evolution in *H. melpomene*. One factor which could vary is larval host plant use, known to affect compound production in *H. Melpomene* (Darragh et al. 2019). *Heliconius* butterflies use different host plants across their geographic range, and vary in their degree of specialisation (Benson et al. 1975; Benson 1978); this may be responsible for some of the variation explained by geographic distance. Age differences across study individuals could also result in pheromone variation both within and between populations, as these bouquets are only found in sexually mature males, and may change throughout their lifetime (Darragh et al. 2017).

Strong species differences in bouquets and the presence of species-specific compounds, combined with mate choice studies, suggests that chemical signalling plays a role in reproductive isolation in *Heliconius* butterflies (Mérot et al. 2015; Darragh et al. 2017). One related prediction is that we might expect to detect a pattern of reproductive character displacement, as is found in *Bicyclus*, with sympatric species pairs exhibiting more differences in male volatile compounds than allopatric pairs (Bacquet et al. 2015). However, we find little evidence for reproductive character displacement between *H. melpomene*, *H. cydno* and *H. timareta*. We predicted more chemical divergence between sympatric species,

and also greater divergence between *H. timareta* and *H. melpomene* which are sympatric closely-related co-mimics in Colombia (Estrada and Jiggins 2008; Giraldo et al. 2008). Instead we find that *H. timareta* and *H. melpomene* are more similar in their chemical profile regardless of sympatry.

A lack of reproductive character displacement in chemical bouquets is surprising as patterns of character displacement in male mate preference have been previously described in *Heliconius* (Jiggins et al. 2001; Kronforst et al. 2007). The displacement in preference is therefore not accompanied by differences in chemical bouquets between populations. One hypothesis to explain the pattern of similarity between *H. melpomene* and *H. timareta* is that introgression is more prominent in the direction from *H. melpomene* into *H. timareta*. This combined with the fact that *H. timareta* has a smaller range and lower effective population size than *H. cydno* may mean it is to some extent swamped by gene flow, contributing to why it is more similar to *H. melpomene* than expected (Martin et al. 2019).

Heliconius butterflies are an excellent example of visual mimicry, with different species converging on the same warning colour patterns (Sheppard et al. 1985; Sherratt 2008; Merrill et al. 2015). It has been suggested that chemical compounds could also contribute to mimicry between species (Dettner and Liepert 1994; Mann et al. 2017). We find that individuals within co-mimicry group do not have more similar chemical profiles than expected. This suggests that selection for similarity between co-mimics is not the driving force of chemical bouquet evolution. Most known examples of chemical mimicry come from systems of deception, for example, mimicry of ant alarm pheromones by rove beetles to avoid predation, rather than mimicry of aposematic warning signals (Dettner and Liepert 1994; Stoeffler et al. 2007; Vereecken and McNeil 2010). The extreme selection pressure on wing colour and pattern mimicry in *Heliconius* might counteract the potential for chemical

mimicry, as chemical bouquets might be critical to species recognition in this highly mimetic group.

Even if the compounds are not part of the mimicry, this does not rule out a role in predator deterrence. Genital compounds were originally suggested to form part of the anti-predation signal (Eltringham 1925). More recently, it has been shown that these compounds act as anti-aphrodisiacs, transferred to females during mating (Gilbert 1976; Schulz et al. 2008); however, they could be important for both functions. We detected 2-sec-butyl-3-methoxypyrazine in the genitals of *H. melpomene*, *H. cydno* and *H. timareta*, and 2-isobutyl-3-methoxypyrazine in the genitals of *H. melpomene* and *H. cydno*, both compounds that are known to act to deter predators in the wood tiger moth (Rojas et al. 2017, 2018, 2019; Burdfield-Steel et al. 2018). More generally, methoxypyrazines are known to act as warning odours in many other insects (e.g. Lepidoptera, Rothschild, Moore & Brown, 1984; fireflies, Vencl et al., 2016), effective, for example, for avian predators (Guilford et al. 1987). Further investigation will be required to determine if odours of *Heliconius* butterflies act as anti-predation signals.

Overall, our study reveals that the chemical profiles of *Heliconius* butterflies are consistent with a role in both species recognition and intraspecific mate choice. A pattern of species-specificity alongside intraspecific variation geographically has also been found in bird song (Benedict and Bowie 2009). This has been proposed to be a result of a balance between stabilising selection towards a species stereotype, sexual selection promoting diversity, and geographic segregation alongside selection and drift. Chemical bouquet evolution is also likely to be driven by a combination of these evolutionary processes, both neutral and adaptive. Future steps will involve behavioural and electrophysiological tests to determine the biologically active chemical compounds for different species, which may

reveal different evolutionary processes shaping them to those affecting the bouquet as a whole.

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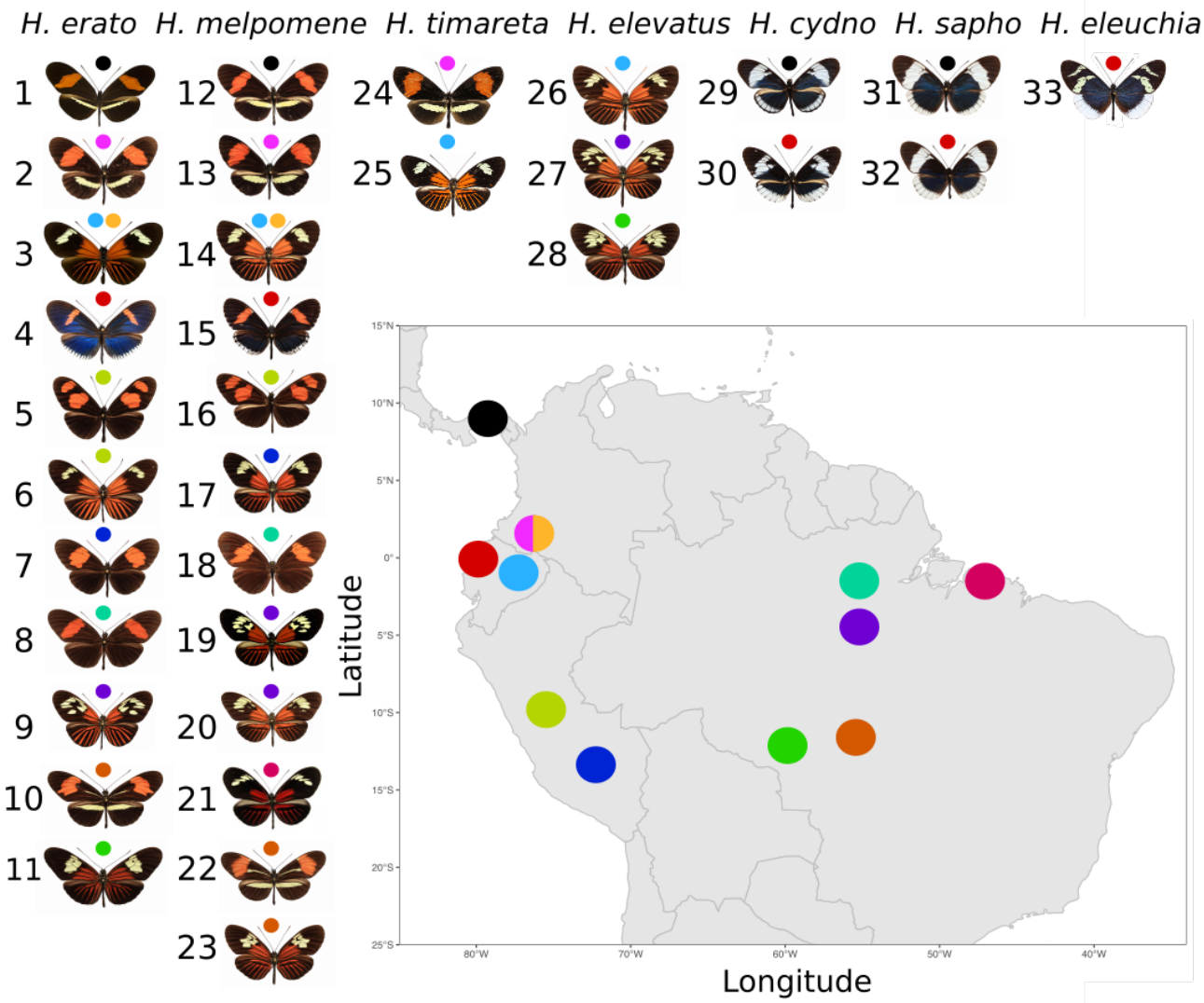

Figure 1. Map indicating which species and intraspecific populations were collected at twelve localities across the Neotropics. *H. erato* (1-11) 1) *H. e. demophoon* 2) *H. e. dignus* 3) *H. e. lativitta* 4) *H. e. cyrbia* 5) *H. e. microclea* 6) *H. e. emma* 7) *H. e. amphitrite* 8) *H. e. hydara* 9) *H. e. amazona* 10) *H. e. phyllis* 11) *H. e. venustus*. *H. melpomene* (12-23) 12) *H. m. rosina* 13) *H. m. bellula* 14) *H. m malleti* 15) *H. m cythera* 16) *H. m. xenoclea* 17) *H. m. schunkei* 18) *H. m. melpomene* 19) *H. m. madeira* 20) *H. m. thelxiope* 21) *H. m. intersectus* 22) *H. m. penelope* 23) *H. m. burchelli*. *H. timareta* (24-25) 24) *H. t. tristero* 25) *H. t. nov. spp. H. elevatus* (26-28) 26) *H. e. willmotti* 27) *H. e. schmassmanni* 28) *H. e. perchlora*. *H. cydno* (29-30) 29) *H. c. chioneus* 30) *H. c. alithea*. *H. sapho* (31-32) 31) *H. s. sapho* 32) *H. s. candidus*. *H. eleuchia* 33) *H. e. primularis*. See Table S1 for sample numbers and details of hybrids.

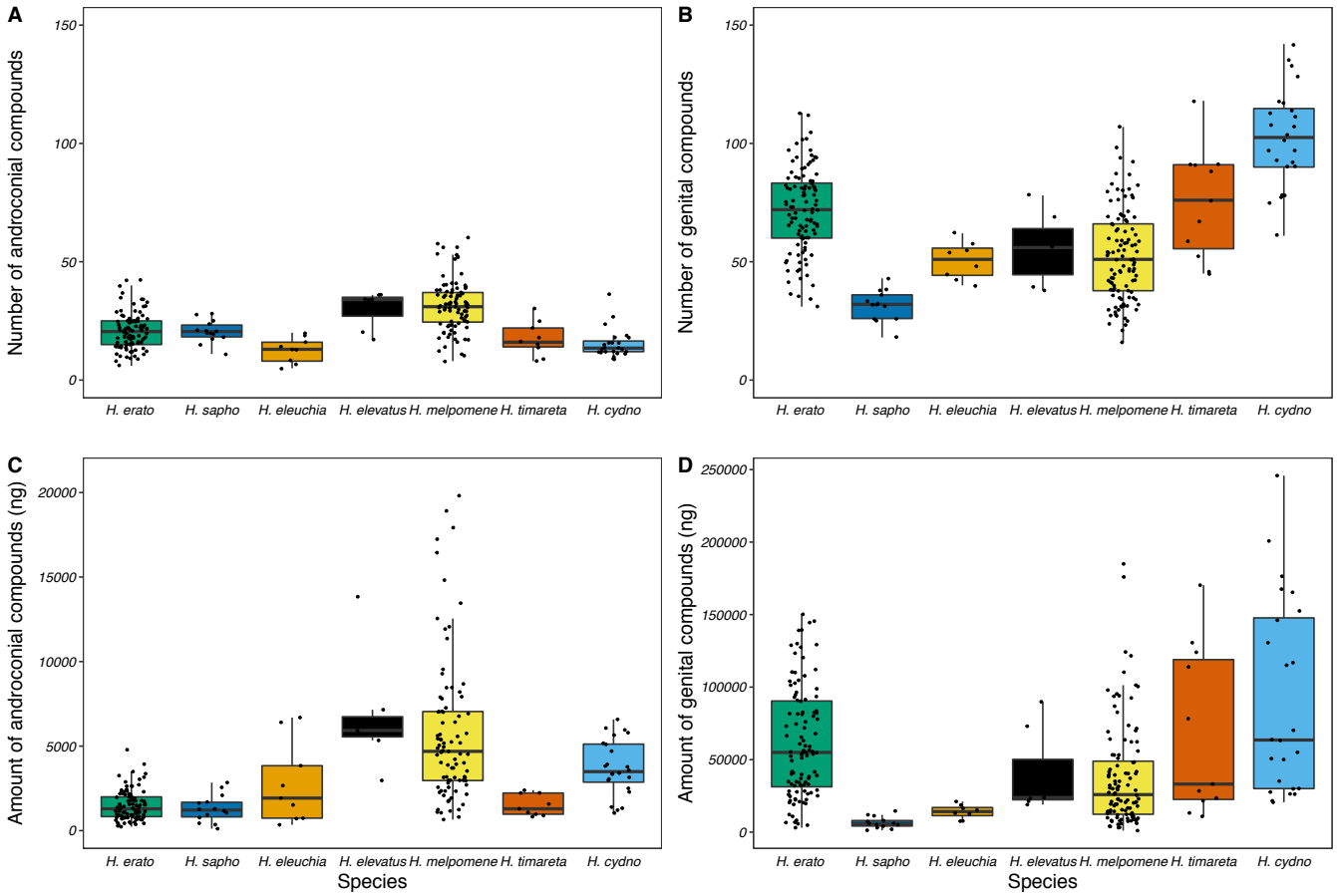

Figure 2. Composition of androconial and genital bouquets across seven *Heliconius* species. Species significantly differ in: (A) number of androconial compounds (anova, $F_{6,245}$=21.54, p<0.001), (B) number of genital compounds(anova, $F_{6,268}$=36.15, p<0.001), (C) amount of androconial compounds (anova, $F_{6,245}$=11.55, p<0.001), (D) amount of genital compounds (anova, $F_{6,268}$=11.62, p<0.001). Four outlier individuals were removed from C.

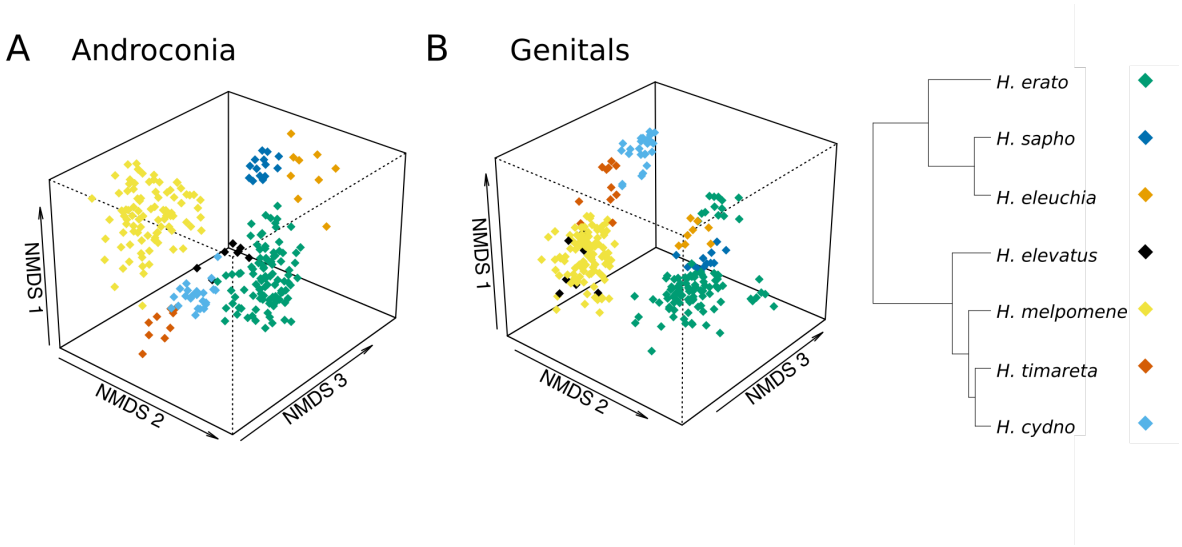

Figure 3. NMDS (non-metric multidimensional scaling) plot illustrating in three dimensions the variation in chemical compounds of male *Heliconius* of different species. A) Androconial compound bouquets differ significantly between species. Stress=0.155. B) Genital bouquets also differ significantly between species. Stress=0.121.

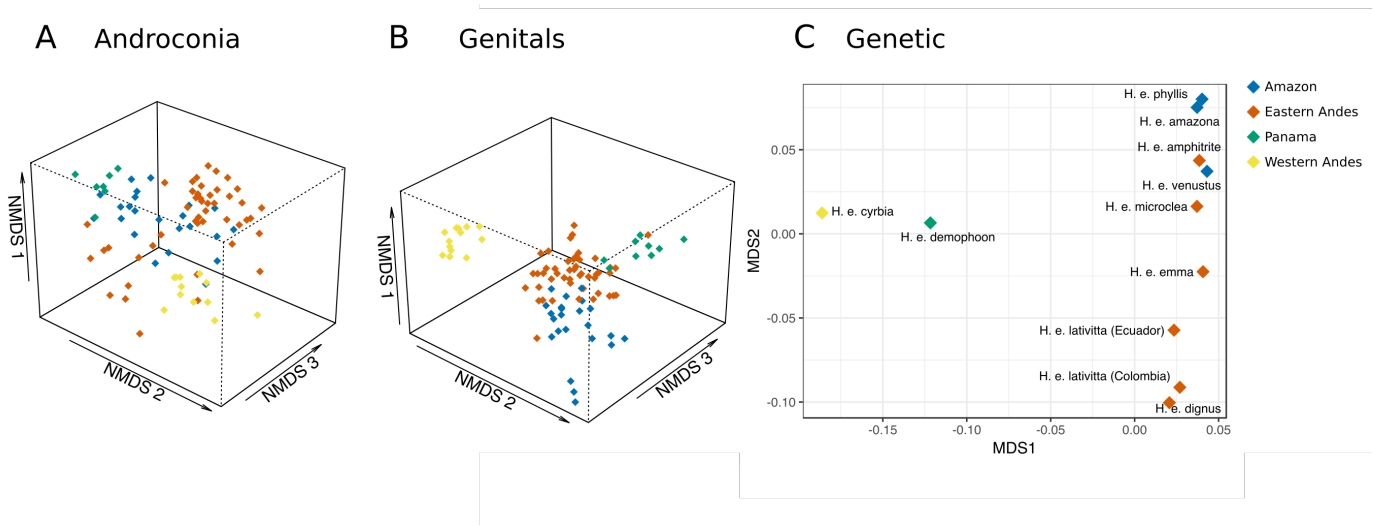

Figure 4. Plots of androconial, genital and genetic distance between *H. erato* populations. A) NMDS (non-metric multidimensional scaling) plot illustrating in three dimensions the variation in androconial chemical compounds. Stress=0.174. B) NMDS plot illustrating in three dimensions the variation in genital chemical compounds. Stress=0.118. C) MDS plot illustrating in two dimensions the genetic distance between populations of *H. erato*.

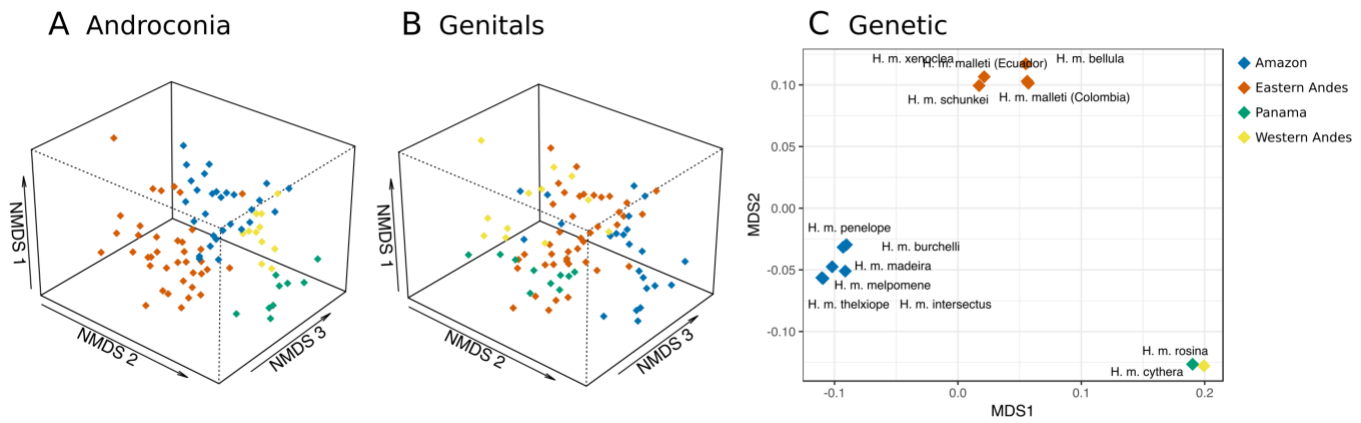

Figure 5. Plots of androconial, genital and genetic distance between *H. melpomene* populations. A) NMDS (non-metric multidimensional scaling) plot illustrating in three dimensions the variation in androconial chemical compounds. Stress=0.151. B) NMDS plot illustrating in three dimensions the variation in genital chemical compounds. Stress=0.161. C) MDS plot illustrating in two dimensions the genetic distance between populations of *H. melpomene*.

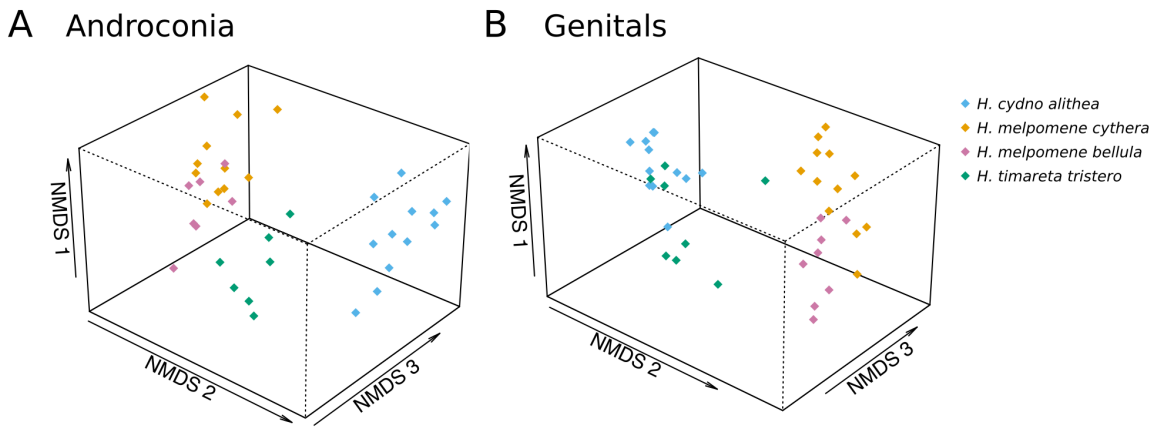

Figure 6. NMDS (non-metric multidimensional scaling) plot illustrating in three dimensions the variation in chemical compounds of male *H. cydno, H. melpomene* and *H. timareta* from Western Ecuador and Colombia. A) Androconial compound bouquets, stress=0.092. B) Genital bouquets, stress=0.076.

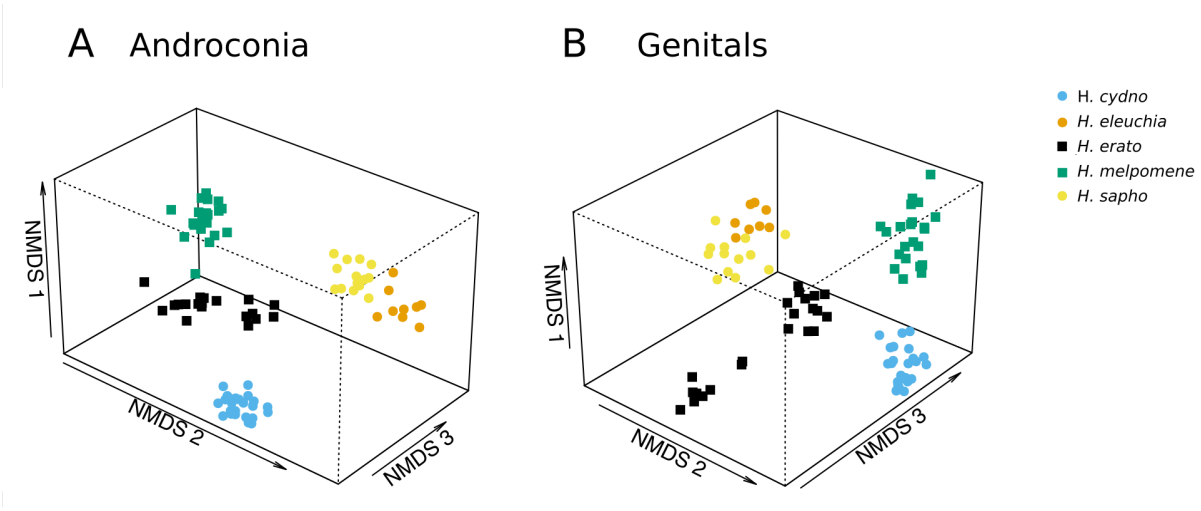

Figure 7. NMDS (non-metric multidimensional scaling) plot illustrating in three dimensions the variation in chemical compounds of male *Heliconius* from Panama and Western Ecuador. *H. erato* and *H. melpomene* are co-mimics (squares), whilst *H. cydno*, *H. eleuchia* and *H. sapho* form a second co-mimicry group (circles). A) Co-mimicry group contributes 1% of variation in androconial chemical bouquets. (Stress=0.098. B) Co-mimicry group contributes 1.8% of variation in genitals chemical bouquets. Stress=0.094.

Table 1: Androconial compounds which are the best indicators of species identity. A is a measure of group specificity of the compounds, B is a measure of group coverage, and sqrtIV is the indicator value which considers both A and B and ranges from 0 (compound not present in any individuals of that species) to 1 (compound only present in that species, and present in all individuals).

Species/compound	A: specificity	B: coverage	sqrtIV
H. cydno			
Unknown aromatic (RI=2130)	1	0.667	0.816
H. eleuchia			
Hexahydrofarnesylacetone	0.747	1	0.864
H. elevatus			
Homovanillylalcohol	0.912	1	0.955
H. erato			
Geranylgeranylacetone	1	1	1
H. melpomene			
Octadecanal	0.999	1	1
H. sapho			
Methyl 4-hydroxy-3-methoxybenzoate	0.866	1	0.931
H. timareta			
5-Decanolide	1	0.889	0.943

Table 2: Genital compounds which are the best indicators of species identity. A, B, and sqrtIV as in Table 1.

Species/compound	A: specificity	B: coverage	sqrtIV
H. cydno			
Unknown ester (RI=1390)	0.999	1	0.999
H. eleuchia			
Unknown macrolide RI=1878	0.969	1	0.984
H. elevatus			
Icosenol	0.908	1	0.953
H. erato			
Unknown terpene ester (RI=2494)	1	1	1
H. melpomene			
(*E*)-β-ocimene	0.865	1	0.930
H. sapho			
(*Z*)-3-hexenyl isobutyrate & Unknown (RI=1691)	0.957	0.923	0.940
H. timareta			
Butyl oleate & (*Z*)-9-Octadecen-13-olide	0.915	1	0.956