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The Scent Chemistry of *Heliconius* Wing Androconia

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Abstract Neotropical *Heliconius* butterflies are members of various mimicry rings characterized by diverse colour patterns. In the present study we investigated whether a similar diversity is observed in the chemistry of volatile compounds present in male wing androconia. Recent research has shown that these androconia are used during courting of females. Three to five wild-caught male *Heliconius* individuals of 17 species and subspecies were analyzed by GC/MS. Most of the identified compounds originate from common fatty acids precursors, including aldehydes, alcohols, acetates or esters preferentially with a C_{18} and C_{20} chain, together with some alkanes. The compounds occurred in species-specific mixtures or signatures. For example, octadecanal is characteristic for *H. melpomene*, but variation in composition between the individuals was observed. Cluster analysis of compound occurrence in individual bouquets and analyses based on biosynthetic motifs such as functional group, chain length, or basic carbon-backbone modification were used to reveal structural patterns. Mimetic pairs contain different scent bouquets, but also some compounds in common, whereas sympatric species, both mimetic and non-mimetic, have more distinct compound compositions. The compounds identified here may play a role in mate choice thus helping maintain species integrity in a butterfly genus characterized by pervasive interspecific gene flow.

Keywords Pheromones · Mimicry · Male butterflies · Biosynthesis · Aldehydes · Alcohols

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Introduction

The Neotropical butterfly genus *Heliconius*, with its many (sub-)species and mimicry rings serves as a model to investigate fundamental questions of evolution and ecology, dating back to the times of Bates (1862). The diverse colour patterns of these butterflies and their role in mate choice and speciation has been investigated, showing that distinct genes code for different aspects of the butterflies' colour pattern (Merrill et al. 2013; Merrill et al. 2015) and are subject to strong natural selection for mimicry (Chouteau et al. 2016; Mallet and Barton 1989).

Wing colour patterns of *Heliconius* are used for signalling their toxicity to predators in mimicry rings and also as mating stimuli (Bates 1862). Mimetic convergence between co-occurring species may cause problems for male *Heliconius* butterflies, as they use visual cues to initiate courtship of females (Merrill et al. 2011). This is especially true for closely related species involved in the same Müllerian mimicry ring, since their courtship behaviour and habitat preferences are likely to be similar. One solution to overcome this ambiguous

mating cue is the use of sex pheromones emitted by the males (Mérot et al. 2015). While night-flying female moths usually use sex pheromones for long-range attraction, this is commonly not the case in day-flying butterflies. Generally, male pheromones seem to be less common in the Lepidoptera than female pheromones (Ando et al. 2004). Male pheromones of butterflies are typically used in short-range interactions during mating to serve either as aphrodisiacs, *e. g.* in *Danaus plexippus* (Pliske and Eisner 1969), *Bicyclus anynana* (Nieberding et al. 2008), and *Pieris* sp. (Yildizhan et al. 2009), or as antiaphrodisiacs, *e. g.* in *Pieris napi* (Andersson et al. 2000) or *Heliconius melpomene* (Schulz et al. 2008), although attraction over longer distances is also known from *Idea leuconoe* (Nishida et al. 1996).

The role of pheromones in mating of *Heliconius* is not well understood (Estrada and Jiggins 2008). Gilbert (1976) showed that male *H. erato* butterflies use an antiaphrodisiac to prevent other males from remating a female. These antiaphrodisiacs are transferred from abdominal clasper scent glands (CSG) of the male during mating to the abdominal pouch of the female. The composition of such CSGs varies greatly, comprising species-specific compound mixtures (Estrada et al. 2011; Mérot et al. 2015; Miyakado et al. 1989; Schulz et al. 2007; Schulz et al. 2008). The active antiaphrodisiac in *H. melpomene* is (*E*)-β-ocimene that is transferred to the female together with a range of less volatile lipidic compounds, likely modulating the evaporation rate of the pheromone (Schulz et al. 2008).

In addition to the antiaphrodisiacs, sex pheromones have been speculated to help in species recognition between *H. melpomene* and *H. cydno* (Jiggins 2008), *H. erato venus* and *H. erato chestertonii* (Muñoz et al. 2010) and between *H. erato* and *H. himera* (McMillan et al. 1997), which are all species pairs that differ in colour pattern. However, the role of pheromone discrimination is likely to be even stronger in cases of mimicry between sister species, such as *H. melpomene malleti*, *H. timareta florencia*, and *H. timareta thelxinoe*, *H. melpomene amaryllis* that are closely related sympatric species that share wing patterns. The presence of putative male sex pheromones has been identified in both *H. melpomene* and *H. timareta* (Mérot et al. 2015). These pheromones seem to enhance the mating success of the males, thus likely being used in species recognition or attraction of females (Mérot et al. 2015). The compound bouquet is specific to male hindwing androconia, not being found in females or other male wing regions (Darragh et al. unpublished data). In *H. timareta*, these compounds influence mating behaviour and are likely involved in species recognition or attraction of females (Mérot et al. 2015). The authors reported octadecanal, a range of hydrocarbons and several unidentified compounds from wing androconia of *H. melpomene* and *H. timareta.*

In order to better understand the chemical basis of species recognition in *Heliconius*, we characterize the androconial compounds in these scent glands and describe variation in their composition across the genus. We analyse a broad range of *Heliconius* species to begin to investigate the diversity of these compounds. Broadly, there are two hypotheses. First, the distribution of compounds might show a conserved phylogenetic pattern, consistent with gradual evolution over the last 20 million years of *Heliconius* evolution. Alternatively, if such compounds are commonly involved in species recognition and are driven by reinforcement, they might evolve rapidly and commonly differ between even closely related species. Such a pattern has been observed in other taxa, such as the genus *Bicyclus* (Bacquet et al. 2015). The main focus of the present paper is to identify and describe the diversity of compounds found across our samples. In addition, we wish to evaluate the biosynthetic relatedness of the compounds and evaluate whether certain structural features such as chain length, functional group, or basic structural features are used preferentially by certain species. Finally, a comparison with known compounds from CSGs will reveal chemical differences or similarities. A detailed evolutionary analysis is reserved for future work.

Methods and Materials

Butterfly Sampling Five individuals of the following taxa were collected in the wild: *H. melpomene rosina, H. cydno chioneus*, *H. hecale melicerta*, *H. doris, H. sara, H. ismenius* and *H. erato demophoon* from Gamboa, Panama; *H. cydno weymeri f. gustavi* from Topacio, Colombia; *H. timareta florencia*, and *H. melpomene malleti* from Florencia, Colombia; *H. elevatus* and *H. pardalinus* from near Tarapoto in Peru; and *H. himera* from Ecuador. We included three wild male specimens for the Colombian *H. cydno zelinde* from Queremal, *H. cydno weymeri f. weymeri* from Topacio and inter-racial wild hybrids of *H. cydno* (from a hybrid zone between *H. c. zelinde* and both forms of *H. c. weymeri* that occur in the pacific slopes from Calima). Finally, five individuals of *H. melpomene plesseni* and *H. melpomene malleti* derived from Ecuador were obtained from stock cultures in Cambridge and Panama, respectively (Darragh et al. unpublished data). The wing patterns, mimicry pairs/rings and distribution of species are shown in Fig. 1.

Chemical Analysis Androconia of the hindwings were dissected and placed into 300 μL of ultrapure dichloromethane (Merck UniSolv®) immediately after capture in the field. The samples were kept at −20 °C for storage and transported to Braunschweig for analysis. There the samples were concentrated to approximately 10 μL before analysis by evaporation at ambient temperature. At least three samples of each population were analyzed by gas chromatography/mass spectrometry (GC/MS) with a Hewlett-Packard model 5975 mass-

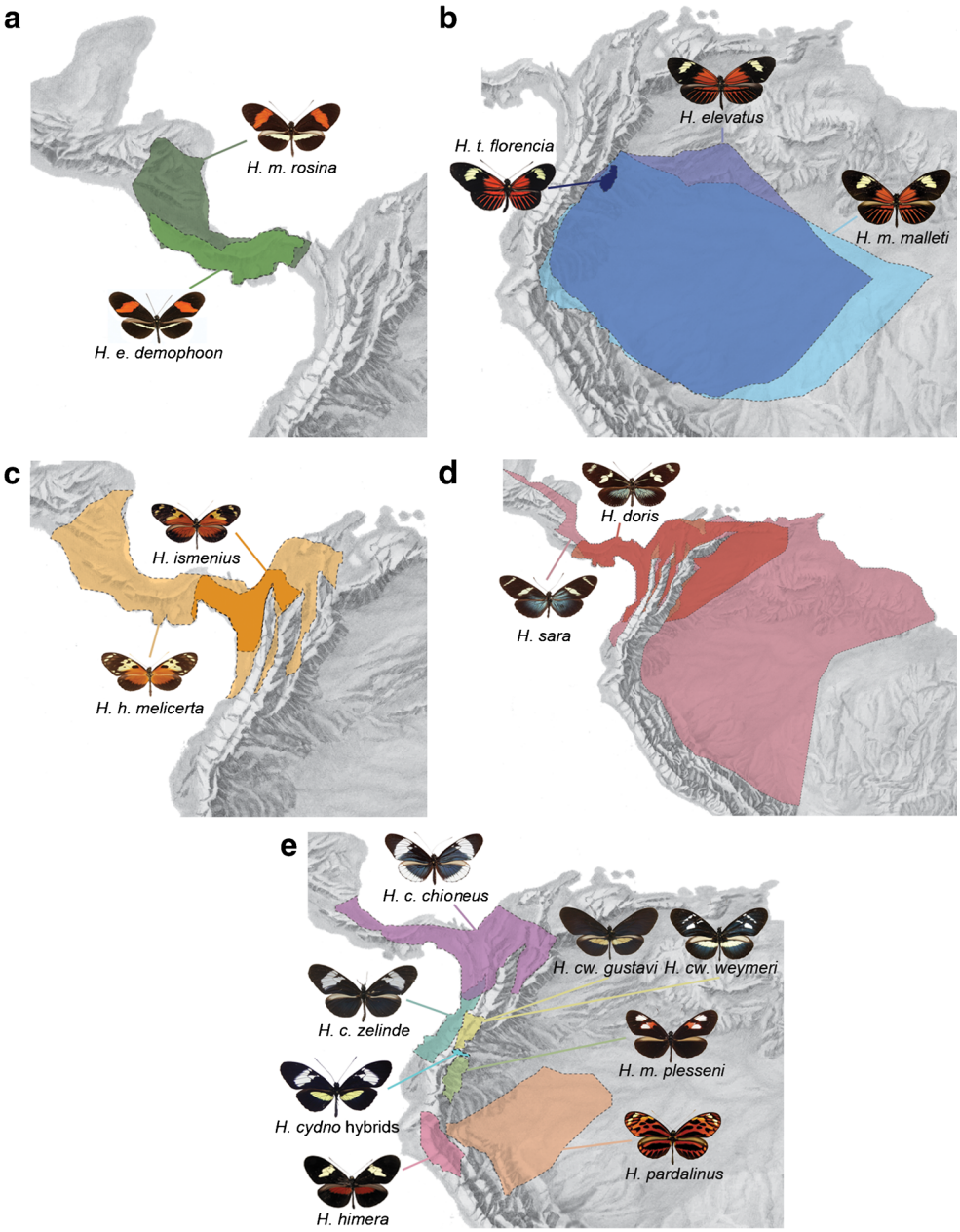

Fig. 1 Phenotype and geographic distribution of the *Heliconius* taxa investigated. **a** Mimicry ring between *H. melpomene rosina* and *H. erato demophoon* in Panama; **b** Mimicry ring between *H. elevatus elevatus*, *H. melpomene malleti* and *H. timareta florencia* in the Amazon basin; **c** Mimicry ring between *H. ismenius* and *H. hecale melicerta* in Panama; **d** Mimicry ring between *H. doris* and *H. sara*; **e** Additional species/races included: *H. cydno chioneus*, *H. cydno zelinde*, *H. cydno weymeri f. weymeri*, *H. cydno weymeri f. gustavi*, hybrids of *H. cydno*, *H. m. plesseni*, *H. himera* and *H. pardalinus butleri*. Distributions for the relevant subspecies are taken from Rosser et al. (2012)

selective detector connected to a Hewlett-Packard GC model 7890A equipped with a Hewlett-Packard ALS 7683B autosampler. A HP-5MS fused silica capillary column (Agilent, 30 m × 0.25 mm, 0.25 μm) was 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 program started at 50 °C. After 5 min the temperature was raised to 320 °C with a heating rate of 5 °C/min. Compounds were identified by comparison of mass spectra and gas chromatographic retention indices with those of authentic reference samples from our compound collection or obtained by synthesis, representing the different compound classes, and analysis of the mass spectra. Only compounds eluting earlier than pentacosane were included in the analysis because of the low volatility of later eluting compounds. Furthermore, the cuticular compounds eluting later would potentially skew the analysis towards differences in cuticular compounds instead of potential pheromone components. The double bond positions of unsaturated compounds were determined by derivatisation with dimethyl disulfide (DMDS) (Buser et al. 1983). 16-Methyloctadecan-1-ol was synthesised from the corresponding methyl ester by reduction with lithium aluminium hydride (Becker and Beckert 2004). 16-Methyloctadecanal was then obtained by oxidation of the alcohol with iodoxybenzoic acid (More and Finney 2002). Full experimental details and mass spectra are given in the

supplementary material (SM, Figs. S1 and S2). Relative concentrations of naturally occurring compounds were determined by integration, excluding obvious contaminants such as phthalates. In Tables 1, 2, 3 and 4 only compounds which occurred in at least two individuals of a (sub)species are listed. A full compound list can be found in the SM. Relative concentrations were determined by peak area analysis by GC/MS.

Statistics As compound concentrations were not normally distributed, median values were used to describe the relative concentrations found in the different species. We did not use absolute concentrations, because of the large variation between samples, perhaps reflecting unknown biological variables such as age, usage, previous matings, and other factors, or technical variability due to the wide variety of samples collected in different field expeditions.

To test whether the composition of the androconia is linked to any kind of relation of the butterfly species, e.g. shared ancestry or sympatry, relatedness between the butterfly scent bouquet was estimated. The compound composition of each individual was subjected to factor reduction (principal component analysis) using the software Past v3.0 (Hammer et al. 2001). As PC1 and PC2 account for most of the variation (44% and 14%, respectively), we used them to compute a hierarchical clustering (UPGMA method) with the Mahalanobis distance (Hammer et al. 2001) as a first approach to evaluate the similarity in chemical composition among the species. The same two components were also used to perform a generalized canonical discriminant analysis (DAPC) to further test whether species differ in their chemical composition, and statistical significance was also tested with an ANOVA. This analysis was done with the candisc package in R (Friendly et al. 2016). Then, to evaluate whether the differences found were due to the phylogenetic history of the species studied, we performed a phylogenetic ANOVA with 1000 simulations using the function 'phylANOVA' implemented in the R package phytools (Revell 2012). We also conducted a phylogenetic generalized least-squares analysis (PGLS) with the R package nlme (Pinheiro et al. 2017) and used the 'gls' function with the maximum-likelihood transformation to estimate Pagel's Lambda (Pagel 1999). Finally, we estimated Blomberg's K (Blomberg et al. 2003) with the 'phylosig' function in phytools (Revell 2012). We used a recently published

Table 1 Compounds identified in extracts of androconial organs of different *Heliconius melpomene* subspecies

compound	retention index	*H. m. plesseni*			*H. m. rosina*			*H. m. malleti*		
		median	percentile		median	percentile		median	percentile	
			25	75		25	75		25	75
octadecenol	2064	-			**0.32**	0.07	1.13	**0.00**	0.00	0.37
octadecenol	2067	-			**0.26**	0.09	0.96	**0.00**	0.00	0.51
octadecanol	2090	-			**22.73**	8.58	38.25	-		
methyloctadecanol	2144	-			**1.46**	0.18	2.35	-		
16-methyloctadecanol	2156	-			**0.26**	0.00	1.08	-		
(*Z*)-11-icosenol	2262	-			**6.27**	2.64	20.95	-		
(*Z*)-9-octadecenal	1989	**4.45**	0.63	10.44	-			-		
octadecanal	2019	**50.81**	18.32	65.77	**49.20**	22.51	51.26	**60.16**	20.90	72.20
methyloctadecanal	2074	-			**0.13**	0.00	0.43	**0.00**	0.00	1.34
methyloctadecanal	2080	-			**1.04**	0.31	1.85	-		
16-methyloctadecanal	2095	-			**1.05**	0.00	1.71	**0.00**	0.00	0.58
nonadecanal	2122	-			**0.26**	0.00	0.46	-		
(*Z*)-11-icosenal	2197	**9.74**	6.03	17.22	**0.85**	0.32	4.99	**7.62**	3.66	14.03
icosanal	2223	**1.79**	0.81	7.68	**0.97**	0.34	1.40	**1.40**	0.00	3.51
(*Z*)-13-docosenal	2401	**5.29**	1.49	7.20	**0.85**	0.27	1.61	-		
ethyl palmitate	1987	-			**0.00**	0.00	0.62	**0.91**	0.00	6.67
ethyl oleate	2160	-			**0.00**	0.00	0.65	**4.09**	0.00	7.36
ethyl stearate	2187	-			**0.00**	0.00	16.25	**0.92**	0.00	8.20
octadecane	1800	-			-			**0.23**	0.00	4.23
henicosane	2100	**32.64**	6.82	61.45	-			**7.33**	0.82	8.90

Number of individuals analyzed: *H. m. plesseni* 5, *H. m. rosina* 5, *H. m. malleti* 5

Medians (bold) and percentile values represent percentage contribution of each compound to the blend

phylogeny of *Heliconius* (Kozak et al. 2015) to implement the above tests.

We also tested whether the functional group, the chain length, or a 'basic modification' of a compound led to species-specific clusters and whether they had an influence on the outcome of the cluster analysis with the multivariate squared Euclidian distance using the medians of the relative concentrations (Đorđević et al. 2014; Schwander et al. 2013). To achieve this, each compound was sorted into various classes. The functional group classes were acetate, alcohol, aldehyde, alkane, alkene, ester, lactone, and terpene. The chain length of the aliphatic compounds was divided into the classes C9/10, C13/14, C15/16, C17/18, C19/20, C21/22, C23/24 and C25/26, in accordance with addition of acetate units during fatty acid biosynthesis. Basic modifications were defined as *n*-alkyl chain, methyl-branched alkyl chain, monounsaturated alkyl chain, diunsaturated alkyl chain, aromatic compound, monoterpenoid, sesquiterpenoid, and diterpenoid. The groups were derived from basic biosynthetic pathways (for a detailed discussion see Morgan 2010) likely operating in the biosynthesis of the target compounds in *Heliconius*. As an example, (*Z*)-11-icosenol is a member of the following classes: alcohol, C19/20, and monounsaturated chain. The medians of relative concentrations of all compounds of one class within one taxon were added and used for statistics calculated with the program IBM SPSS Statistics 23.

Results

A total of 77 wing androconia extracts of males from 17 *Heliconius* species and subspecies were analyzed. This number includes several different subspecies of *H. melpomene* and *H. cydno*, whose phylogenetic relationship is shown in Fig. S3 of the SM. The identification of the androconial compounds revealed the presence of long chain hydrocarbons, fatty acid esters, aldehydes, and alcohols as dominant compound classes. These compounds are biosynthetically derived from fatty acid biosynthesis (Morgan 2010). In addition, terpenoids as well as aromatic compounds were identified. The occurrence of the compounds in the various species are outlined below.

Aldehydes and alkanes represent the major compound classes present in the androconia of all *H. melpomene* subspecies (Table 1, Fig. S4), with octadecanal as the most prominent compound. *Heliconius m. plesseni* was the only subspecies with an alkane as the major constituent of the wing extract. *Heliconius m. rosina* also contained large amounts of alcohols, while in *H. m. malleti* fatty acid ethyl esters were present instead. All unsaturated compounds investigated by DMDS derivatization carried the double bond at the ω-9-position, as in (*Z*)-11-icosenal and (*Z*)-13-docosenal. In addition to these compounds, minor amounts of octadecenols and methyl-branched C_{18}-aldehydes and alcohols were identified in *H. m. rosina*. The mass spectra of the aldehydes were similar to those of nonadecanal (see SM), but they eluted earlier with retention indices between 2074 and 2095 (Table 1). Through use of the empirical gas chromatographic retention index calculation method developed by us (Schulz 2001), it became clear that these compounds are methyloctadecanals. The last eluting compound was identified as 16-methyloctadecanal, verified by its synthesis, as was the case for the respective alcohol 16-methyloctadecan-1-ol (Figs. S1 and S2). The location of the methyl group in the other methyloctadecanal isomers remained unclear, because the retention index pointed to an internal position, not deducible from the mass spectra. Terminal positions of the methyl group at C-2 or C-17 can be excluded, because synthetic reference material did not match with the natural compounds. Occasionally compounds such as the monoterpene limonene, ethyl *p*-ethoxybenzoate, and the fungal polyketide mellein[1] were found (see Table S1 in the SM).

The androconial bouquets of *H. cydno* are chemically more diverse compared to those of *H. melpomene*, with henicosane being the major component of all *H. cydno* subspecies (Table 2, Fig. S5). Besides alkanes, all subspecies contained ethyl fatty acid esters and occasionally isopropyl esters or trace amounts of aldehydes, as well as other components in varying composition. *Heliconius c. weymeri f. weymeri* extracts were generally of low concentration. *Heliconius c. zelinde* extracts also contained fatty acid derived macrocyclic lactones, isopropyl esters, and trace amounts of (*Z*)-9-tricosene. The lactones are also the major components of the male abdominal scent glands of *H. cydno* (Schulz et al. 2007). *Heliconius c. chioneus* contained a diverse blend of different compounds. *Heliconius cydno* is the only species that produced more than four compound classes. *Heliconius timareta florencia* is closely related to *H. cydno* (Fig. S1), reflected in its androconial composition (Table 2, Fig. S5).

The major component of *H. pardalinus butleri* was (*Z*)-11-icosenyl acetate, accompanied by smaller amounts of (*Z*)-9-henicosene and the phytol degradation product hexahydrofarnesylacetone (Schulz et al. 2011). In addition, icosyl propionate occurred exclusively in this species. The results are shown in Table 3 and Fig. S6, together with those from the related species *H. ismenius*, *H. elevatus*, and *H. hecale*, known as the 'silvaniforms' (SM, Fig. S3). The scent wing scales of *H. elevatus* contained only hydrocarbons, dominated by henicosane, similar to *H. cydno* (Table 3, Fig. S6). Despite the fact that *H. elevatus* is closely related to the sympatric *H. pardalinus butleri*, the only compounds these two species share are (*Z*)-9-henicosene and (*Z*)-9-tricosene.

[1] Compounds occurring in only one sample analyzed are not listed in the tables of the main text because they were not included in the cluster analysis. However, SM Table S1 shows all identified compounds.

Table 2 Compounds identified in extracts of androconial organs of different *Heliconius cydno* subspecies and *H. timareta*

compound	retention index	*H. c. zelinde*			*H. c. weymeri f. weymeri*			*H. c. weymeri f. gustavi*			*H. c. chioneus*			*H. cydno* hybrids			*H. timareta florencia*		
		median	percentile		median	percentile		median	percentile		median	percentile		median	percentile		median	percentile	
			25	100		25	100		25	75		25	75		25	100		25	75
(*Z*)-11-icosenol	2249	-			-			-			**2.55**	0.41	3.68	-			-		
unknown alcohol	2388	-			-			-			**0.29**	0.14	0.78	-			-		
tetradecyl acetate	1807	-			-			-			**0.27**	0.00	0.31	-			-		
octadecyl acetate	2195	-			-			-			**1.87**	1.11	4.60	-			-		
icosyl acetate	2394	-			-			-			**0.29**	0.13	1.99	-			-		
octadecanal	2012	**0.69**	0.00	0.93	**0.00**	0.00	1.82	-			**-**			**0.61**	0.00	1.23	-		
(*Z*)-11-icosenal	2185	**0.00**	0.00	5.72	**0.00**	0.00	2.20	-			**1.87**	1.69	2.84	**-**			-		
ethyl palmitate	1981	**1.53**	0.00	1.79	**0.00**	0.00	1.09	**1.19**	0.30	4.35	**0.00**	0.00	0.37	**0.00**	0.00	2.03	**7.50**	3.91	8.63
ethyl linoleate	2155	**-**			**-**			**-**			**0.00**	0.00	0.58	**0.00**	0.00	2.77	**7.59**	3.18	15.05
ethyl oleate	2160	**2.45**	0.00	3.33	**1.99**	1.57	7.09	**5.07**	2.64	12.95	**0.00**	0.00	0.75	**3.18**	0.00	12.02	**43.59**	30.32	56.50
ethyl stearate	2180	**0.66**	0.50	0.73	-			**0.00**	0.00	7.24	**0.00**	0.00	1.03	**0.73**	0.00	4.39	**5.41**	3.21	7.12
isopropyl oleate	2189	**5.68**	0.00	7.65	-			-			-			-			-		
isopropyl octadecadienoate	2231	**0.87**	0.00	2.55	-			-			-			-			-		
(*Z*)-octadec-9-en-11-olide	2023	**0.99**	0.00	7.22	-			-			-			-			-		
(*Z*)-octadec-9-en-13-olide	2027	**0.84**	0.00	2.51	-			-			-			-			-		
(9*Z*,11*E*,15*Z*)-octadeca-9,11,15-trien-13-olide	2061	**3.22**[1]	0.00[1]	22.29[1]	-			-			-			-			-		
(9*Z*,11*E*)-octadeca-9,11-dien-13-olide	2066				-			-			-			-			-		
limonene	1027	**-**			-			-			-			-			**2.12**	0.00	3.94
dihydroactindiolide	1528	**-**			-			-			-			-			**1.38**	0.00	2.85
ethyl *p*-ethoxybenzoate	1522	**-**			-			-			-			**0.00**	0.00	0.32	**1.15**	0.00	2.28
(*Z*)-9-tricosene	2259	**0.00**	0.00	1.12	-			-			**0.53**	0.36	1.49	**-**			**-**		
hexadecane	1600	**-**			-			-			**-**			**-**			**1.77**	1.12	3.52
nonadecane	1900	**0.00**	0.00	0.56	-			**0.49**	0.00	0.91	**0.00**	0.00	0.17	**1.01**	0.00	1.72	**-**		
icosane	2000	**0.92**	0.91	0.93	2.13	1.64	2.87	**1.59**	0.73	1.90	**1.15**	0.56	1.80	**1.22**	0.00	2.63	**-**		
henicosane	2100	**60.09**	47.51	72.37	86.07	83.58	88.20	**77.85**	67.26	86.33	**85.68**	65.08	86.90	**70.99**	0.47	79.02	**12.17**	9.48	28.90
docosane	2200	**0.00**	0.00	0.00	2.17	0.00	2.75	**1.51**	0.71	2.25	**-**			**2.11**	0.00	4.39	**1.79**	0.00	3.21
tricosane	2300	**2.28**	1.40	3.30	2.66	2.08	3.18	**3.27**	1.25	3.70	**4.93**	2.72	6.68	**-**			**0.00**	0.00	2.11
pentacosane	2500	**1.49**	0.76	1.63	-			**0.58**	0.00	2.42	**0.39**	0.30	2.15	**0.99**	0.00	1.50	**0.00**	0.00	0.64
octadecenoic acid	2119	**5.68**	0.38	9.11	-			-			**-**			-			-		
unknown terpenoid	2013	**0.69**	0.60	0.87	-			-			**-**			-			-		
unknown ketone	2103	-			-			-			**0.64**	0.00	1.44	-			-		

Number of individuals analyzed: *H. c. zelinde* 3, *H. c. weymeri f. weymeri* 3, *H. c. weymeri f. gustavi* 5, *H. c. chioneus* 5, *H. cydno* hybrids 3, *H. timareta florencia* 5

Medians (bold) and percentile values represent percentage contribution of each compound to the blend

[1] Individual integration impossible because of non-separated peaks

Table 3 Compounds identified in extracts of androconial organs of different silvaniform species of *Heliconius*

Compound	Retention index	*H. elevatus*			*H. hecale*			*H. ismenius*			*H. pardalinus*		
		median	percentile		median	percentile		median	percentile		median	percentile	
			25	75		25	75		25	100		25	75
octadecanol	2078	-			**0.18**	0.00	0.30	-			-		
(*Z*)-11-icosenol	2262	-			**10.79**	6.24	16.48	-			**0.00**	0.00	1.38
octadecyl acetate	2194	-			**0.57**	0.41	1.44	**94.70**	0.00	100	**-**		
(*Z*)-11-icosenyl acetate	2383	-			**4.61**	2.54	5.70	**-**			**68.34**	35.95	75.62
icosyl acetate	2409	-			**0.12**	0.00	0.54	**-**			**0.00**	0.00	0.23
methylicosyl acetate	2448	-			**3.49**	1.29	3.59	**-**			**-**		
(*Z*)-11-icosenyl propionate	2479	-			**-**			**-**			**1.66**	0.50	2.30
octadecanal	2006	-			**0.30**	0.13	0.62	**-**			**-**		
(*Z*)-11-icosenal	2184	-			**3.32**	2.14	4.16	**-**			**-**		
icosanal	2223	-			**0.15**	0.00	0.33	-			-		
(*Z*)-13-docosenal	2395	-			**2.13**	1.17	3.76	**-**			**-**		
hexahydrofarnesylacetone	1844	-			**16.13**	10.98	21.65	**0.00**	0.00	47.22	**6.47**	1.91	9.95
phytol	2210	-			**0.28**	0.05	0.42	**-**			**-**		
(*Z*)-9-henicosene	2071	**0.36**	0.01	0.54	**0.80**	0.67	1.14	**-**			**26.80**	12.20	39.20
(*Z*)-9-tricosene	2270	**0.89**	0.70	1.02	**1.86**	0.99	2.03	**-**			**2.08**	0.90	2.83
11-methyltricosane	2334	**-**			**0.41**	0.12	0.85	**-**			-		
11-methyltetracosane	2433	**-**			**0.22**	0.00	0.61	**-**			-		
icosane	2000	**0.36**	0.01	0.54	**0.34**	0.00	0.60	**-**			-		
henicosane	2100	**92.15**	87.42	94.69	**47.54**	41.68	58.51	**-**			-		
docosane	2200	**0.86**	0.39	1.08	**-**			**-**			-		
tricosane	2300	**5.20**	2.01	7.25	**2.81**	2.11	5.44	**0.00**	0.00	9.18	-		
pentacosane	2500	**0.73**	0.00	1.73	**0.29**	0.22	1.07	**5.30**	0.00	19.09	-		
2-henicosanone	2308	-			*0.22*	0.10	0.43	-			-		

Number of individuals analyzed: *H. elevatus* 5, *H. hecale* 5, *H. ismenius* 3, *H. pardalinus* 5

Medians and percentile values represent percentage contribution of each compound to the blend

The extracts of *H. ismenius* contained only a small number of volatiles, dominated by octadecyl acetate (Table 3, Fig. S6). *Heliconius hecale melicerta* is a mimic of *H. ismenius* in Panama. Similar to *H. ismenius,* the extracts contained alkanes, ester, and terpenoids, but the bouquet was more complex. Henicosane, hexahydrofarnesylacetone, and (*Z*)-11-icosenol were all major components. The relative proportion of hexahydrofarnesylacetone in both species was similar. In addition, different hydrocarbons and carbonyl compounds were present (Table 3, Fig. S6), leading to a broad variety of compound classes.

The androconial extracts of *H. doris* contained mainly alkanes, dominated by pentacosane. Fatty acid ethyl esters were minor constituents, but the fungal polyketide mellein was consistently present in moderate amounts, as was octadecyl acetate (Table 4, Fig. S7). Major constituents of *H. erato demophoon* were the alcohols hexadecanol and octadecanol, the diterpene geranylgeranylacetone, and an unknown macrolide. Minor components were octadecyl acetate, 4,8,12,16-tetramethylheptadecan-4-olide (Yildizhan et al. 2009), the terpenoids τ-cadinol and dihydroactinolide, as well as mellein (Table 4, Fig. S7). *Heliconius himera* is a member of the *H. erato* clade (Fig. S3). Its bouquet was dominated by an unknown diterpenoid, octadecyl acetate, hexadecanol, and pentacosane, but lactones were missing. Mellein was present as a minor component (Table 4, Fig. S7). Finally, *H. sara* containsed alkanes and octadecyl acetate in addition to other compounds (Table 4, Fig. S7).

Statistical Analysis In general, the dendogram of the analysis of the androconia composition of individuals revealed distinct groups largely consistent with species (Fig. 2). In the first cluster, most individuals of *H. melpomene* grouped together and were well differentiated from any other species. In the second cluster, *H. erato demophoon*, *H. paradlinus butleri* and *H. timareta florencia* appeared as closely related to each other, while maintaining their species identity. A third cluster was composed by *H. himera*, *H. ismenius*, *H. sara* and

Table 4 Compounds identified in extracts of androconial organs of different *Heliconius* species

Compound	Retention index	*H. erato demophoon*			*H. himera*			*H. sara*			*H. doris*		
		median	percentile		median	percentile		median	percentile		median	percentile	
			25	75		25	75		25	75		25	75
hexadecanol	1868	**21.10**	15.57	26.98	**25.94**	12.87	51.00	-			-		
octadecanol	2071	**24.33**	19.68	28.74	**-**			-			-		
octadecyl acetate	2195	**4.55**	2.21	19.54	**14.89**	7.06	17.92	**12.09**	1.13	35.97	**10.06**	4.07	23.07
icosyl acetate	2402	**-**			**0.00**	0.00	7.77	**0.00**	0.00	0.69	**1.23**	0.00	5.10
geranylgeranylacetone	2379	**10.74**	2.71	35.27	**0.00**	0.00	8.16	**-**			**-**		
unknown diterpenoid	2418	**-**			**26.23**	21.45	35.21	**-**			**-**		
ethyl palmitate	1987	**-**			-			**0.00**	0.00	1.60	**0.84**	0.00	1.94
ethyl linolate	2156	**-**			-			**0.00**	0.00	1.87	**0.61**	0.00	2.46
ethyl oleate	2162	**-**			-			**0.00**	0.00	1.10	**0.36**	0.00	1.39
ethyl stearate	2177	**-**			-			**0.00**	0.00	1.66	**0.75**	0.00	2.22
unknown C_{16} macrolide	1816	**14.41**	2.43	19.54	-			**-**			**-**		
4,8,12,16-tetramethylheptadecan-4-olide	2353	**2.27**	0.28	21.90	-			**-**			**-**		
dihydroactinolide	1532	**3.03**	0.67	4.05	-			**-**			**-**		
τ-cadinol	1645	**4.29**	0.00	9.24	-			**-**			**-**		
neophytadiene isomer	1829	-			-			**7.18**	0.00	37.58	**-**		
methylpentadecane	1531										**0.63**	0.26	2.49
tetradecane	1400	-			-			-			**1.14**	0.00	1.27
pentadecane	1500	-			-			-			**1.16**	0.00	2.39
heptadecane	1700	-			-			-			**0.84**	0.00	1.45
henicosane	2100	-			-			**5.15**	0.49	6.56	**1.34**	0.55	2.03
tricosane	2300	-			-			**16.91**	6.62	25.27	**7.49**	6.79	8.58
tetracosane	2400	-			-			**0.00**	0.00	7.44	**1.99**	1.95	5.46
pentacosane	2500	-			**11.84**	6.99	21.16	**11.66**	7.92	14.83	**51.40**	33.36	62.98
mellein	1839	**1.47**	0.00	4.33	**0.00**	0.00	6.47	**0.00**	0.00	1.45	**11.76**	1.51	27.05

Number of individuals analyzed: *H. erato demophoon* 5, *H. himera* 5, *H. sara* 5, *H. doris* 5

Medians and percentile values represent percentage contribution of each compound to the blend

H. doris, with the latter two species clearly differentiated from each other. Finally, the fourth cluster was mostly formed by *H. cydno*, but also included two silvaniform species, namely *H. hecale* and *H. elevatus*. Interestingly, we observed that mimetic species living in sympatry/parapatry (Fig. 1) were clearly differentiated based on their chemical composition. This is also the case for sympatric, non-mimetic and closely related species such as *H. cydno* and *H. melpomene* (Beltrán et al. 2007; Kozak et al. 2015). The discriminant analysis is consistent with these results (Fig. 3, ANOVA, $P = 2.10^{-16}$). None of the members of the monophyletic silvaniform clade (Fig. S3 SM), represented here by *H. elevatus, H. ismenus, H. hecale* and *H. pardalinus,* grouped together, implying rapid divergence in their androconial chemical composition. *Heliconius ismenius* was the only species that contained just two compounds, and showed no close relationship to any other species, while *H. pardalinus* was largely different to all other species due to the absence of alkanes.

The different subspecies in *H. melpomene* and *H. cydno* show variable composition. Races of *H. melpomene* are distinguishable from one another, with *H. melpomenen rosina* well resolved from the other two subspecies, likely due to the presence of alcohols. The subspecies of *H. cydno* are less well resolved, probably due to their high variability in compound composition, different from the more homogenous *H. melpomene.*

When we evaluated association between chemical identity and phylogeny, we obtained low values for both Pagel's Lambda ($\lambda = 0.04$, MLE: -252.13, $P = 0.14$) and Blomberg's K (K = 0.02, $P = 0.001$), indicating little phylogenetic signal in the data. However, Blomberg's K was significant, potentially as a result of departures from the Brownian motion model of trait evolution. Yet, further comparison of the variation in chemical composition accounting for phylogenetic signal revealed little evidence that differences in chemical composition among species and subspecies

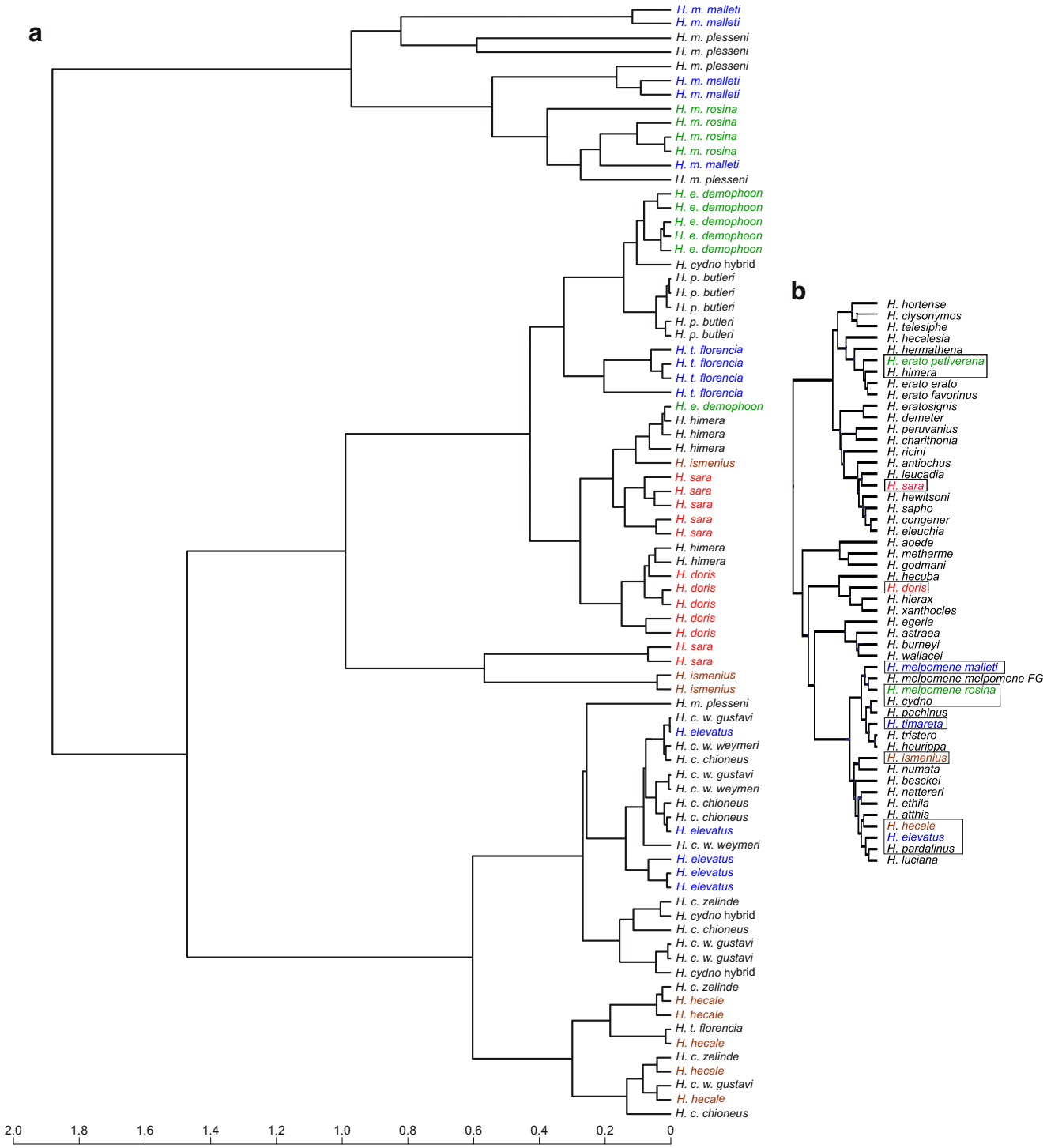

Fig. 2 **a** Full cluster analysis based on individual compositions of compounds occurring at least twice within analysed *Heliconius* individuals. Mimicry rings are colour coded; blue = rayed, green = postman, brown = silvaniform following colour code in Fig. 1. **b** Phylogenetic tree of heliconiines adapted from Kozak et al. (2015). Species investigated are boxed. *H. erato petiverana* = *H. erato demophoon*

are due to shared ancestry at 95% confidence (F = 34.57, $P = 0.01$). In summary, overall composition seemed to differ even between closely related species and showed little relationship to phylogeny.

As there was little evidence for phylogenetic signal, we carried out clustering analysis based on the samples without any further phylogenetic correction. We were interested in the similarity of blends with respect to their biosynthetic

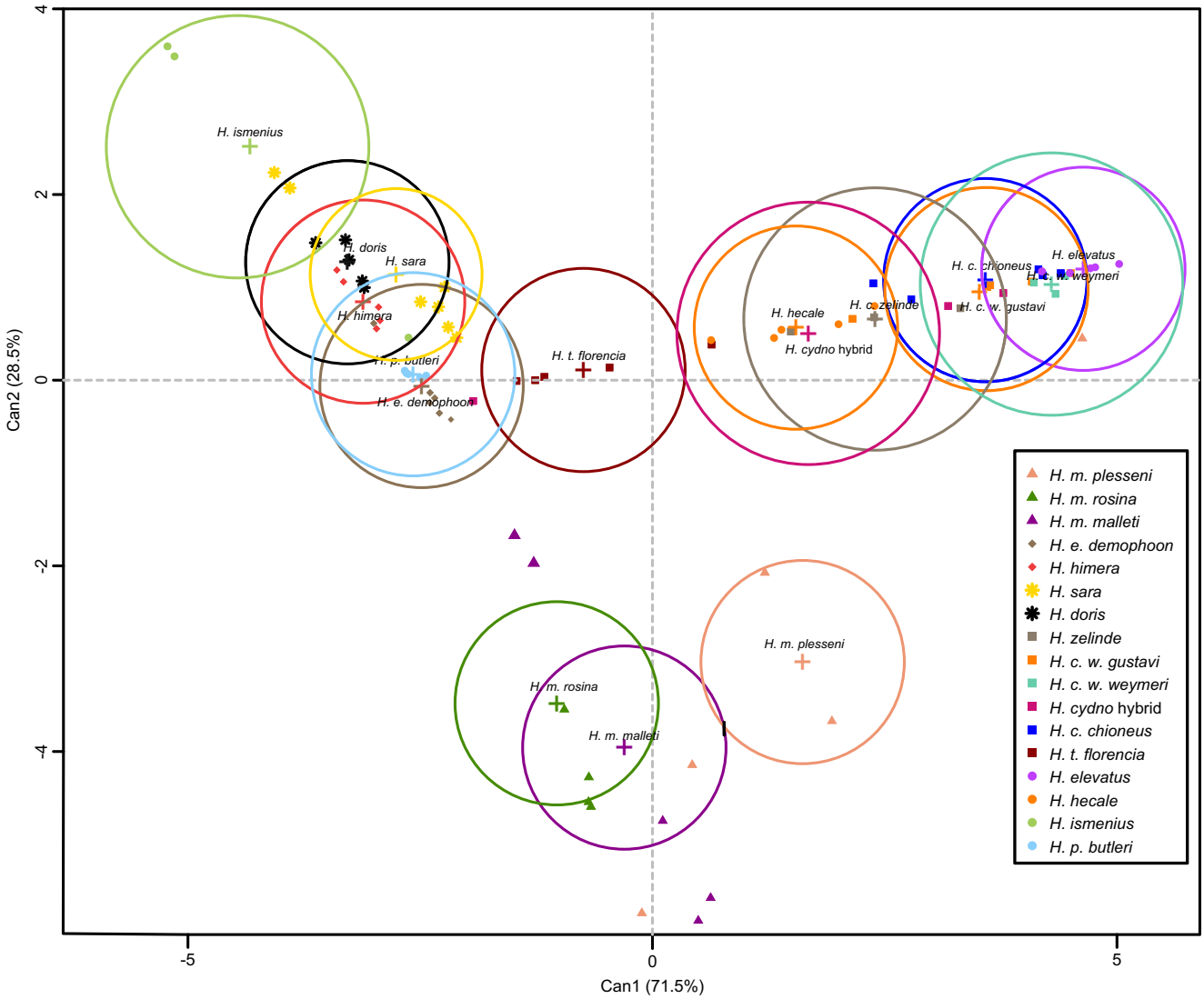

Fig. 3 Discriminant analysis based on individual compositions of compounds occurring at least twice within analysed *Heliconius* individuals. Circles correspond to the 95% of confidence around each group mean

pathways rather than solely considering individual components. Individual compounds share biosynthetic steps during their formation, assuming that most of the compounds identified are synthesized de novo by *Heliconius*. This approach revealed insights not only into the presence of a specific biosynthetic transformations, but also their importance for the specific composition of blends. The androconial bouquet usually consists of closely related compounds in *Heliconius*, differing in three aspects: 1) chain length of aliphatic compounds and terpenes, 2) terminal functional group, and 3) modifications in the carbon backbone in fatty acid derivatives, such as *n*-alkyl, alkenyl, alkadienyl, or methyl-branched chain, or terpenes, such as mono-, sesqui-, or diterpene (called here basic modification). These aspects characterize the biosynthesis of the gland constituents (Fig. S8, SM). Changes in gene activity, probably only by a small degree, or genes encoding the enzymes can have a big impact on the species-specific bouquet composition of a species or even an individual (e. g. Lassance et al. 2013). Therefore, we classified each compound according to its functionality, chain length, and backbone modification (see SM Table S2). Cluster analysis of each of the three categories was then performed.

The cluster analysis of the basic modifications revealed a large group using mostly saturated and ω-9 unsaturated chains including all *H. cydno* (Fig. S9, SM). There was a second large cluster determined by methyl-branches in the alkyl chain and double unsaturation. Further differentiation is due to terpenes. The three *H. melpomene* are divided into the two main clusters and *H. pardalinus* is very different to all other species because of the high proportion of diterpenes. The mimetic species do not cluster together.

The cluster analysis according to chain length (Fig. S10, SM) showed three clusters and again *H. pardalinus* as single species. The *H. cydno* cluster is again visible, characterized by

a high abundance of the C21/22 class. The second group contained a high proportion of the C17/C18 class, while the third cluster was more diverse. Mimetic species as well as phylogenetic neighbouring species do not cluster together.

The cluster analysis based on functionality (Fig. S11, SM) was the only one of the three reduced analyses that clustered *H. melpomene* together in a homogenous group, likely because of the dominance of aldehydes, indicating their important role in the specific odor composition of this species. The closely related *H. timareta* appears close to *H. melpomene*. *Heliconius cydno* forms a distinct group that clusters all its subspecies, whereas *H. ismenius* and *H. pardalinus* now occurred in a cluster together with *H. erato* and *H. himera*. The cluster analysis based on functionality was the only analysis that recovered phylogenetic similarity between *H. melpomene, H. cydno* and *H. timareta* and recovered a homogeneous *H. melpomene*-cluster.

Overall, the different cluster analyses did not group mimetic species together, underlining the diversity of their androconial chemistry. The chain length cluster showed similarities to the full cluster analysis of the individuals, exemplified by the *H. cydno*-cluster containing *H. elevatus* and *H. hecale* and the *H. e. demophoon*, *H. himera*, *H. sara* and *H. doris* group, but lacked a homogeneous *H. melpomene* cluster. These cluster analyses revealed that chain length is an important variable for the specificity of bouquets.

Discussion

In summary, 68 different compounds were identified in the wing androconia of the 17 species and subspecies investigated, adding 51 to the seven compounds, octadecanal and heptadecane to pentacosane hydrocarbons reported by Mérot et al. (2015). A few compounds with high volatility were found, e.g. limonene. Compounds of medium to low volatility dominated the bouquets. Henicosane was the most common of them, but preferentially C_{18}- and C_{20}-aldehydes, alcohols and esters contributed to species-specific mixtures. These compounds are produced via the fatty acid biosynthetic pathway (Morgan 2010). With the exception of minor methyl branched compounds such as 16-methyloctadecanal/ol and the macrolides of *H. cydno*, these compounds are not very specific components, because they can be readily formed in a few biosynthetic steps from commonly available fatty acids. Minor constituents, on the other hand, originate from terpene biosynthesis, fungal polyketide biosynthesis (mellein), or are aromatic compounds.

Androconial Composition and Biosynthesis in *Heliconius*

The dominant fatty acid derived long chain aldehydes, alcohols, acetates and ethyl esters resemble long-range sex pheromones of night-flying moths (Ando et al. 2004) and have been shown to act as sex pheromones in the butterfly *Bicyclus anynana* (Nieberding et al. 2008), but occur in other butterflies as well, e.g. the danaine *Lycorea ceres* (Meinwald et al. 1966; Schulz 1987).

Generally, male butterfly pheromone components are structurally more diverse compared to those of moths. Some are derived from plant products, such as pyrrolizidine alkaloids, as found in danaines and ithomiines (Eisner and Meinwald 1987; Nishida et al. 1996; Pliske and Eisner 1969; Schulz et al. 2004), or are terpenoids (Francke et al. 1989; Honda et al. 2006; Nieberding et al. 2012; Nishida et al. 1996; Schulz et al. 1988a; Schulz et al. 1988b; Yildizhan et al. 2009). We found that nearly 70% of the wing androconial compounds in *Heliconius* likely originate from fatty acid biosynthesis, which agrees with recent findings in *Bicyclus*, where about a half of all potential pheromone components originate from the same pathway (Bacquet et al. 2015). All compounds previously reported from *H. melpomene* and *H. timareta* fall into this category (Mérot et al. 2015).

Figure 4 shows a general hypothesis for the biosynthetic pathway leading to the metabolites used by *Heliconius* based on a wide variety of biosynthetic studies (see e.g. Morgan 2010 for detailed references; Liénard et al. 2014). Common fatty acid synthase (FAS) builds up saturated fatty acid up to a C_{18} chain that serve as a starter for further transformations. More detailed biosynthetic pathways leading to the aliphatic, fatty acid derived compounds identified in the *Heliconius* wing androconia are shown in Figs. S12 to S22 (SM), emphasizing the close chemical relationship between the compounds. A high chemical diversity of the bouquet useful for the generation of species-specific mixtures can be obtained by activating or deactivating enzymes/genes responsible for certain biosynthetic transformations (e.g. Lassance et al. 2013). For example, an alcohol oxidase acting on saturated alcohols seems to be active in *H. melpomene*, but inactive in *H. cydno*.

Interestingly, all double bonds of the fatty acid derivatives in *Heliconius* are located at the ω-9 position,[2] indicating activity of a common Δ9-desaturase active in production of these compounds. This is different from the Δ11-desaturase present in *Bicyclus* (Liénard et al. 2014). Subtle changes in a few enzyme activities might therefore result in different scent gland mixtures in *Heliconius*, leading to differences between species, subspecies or even individuals. Similar effects have been observed in other systems such as moths (Albre et al. 2012), flies (Takahashi et al. 2001) and *Bicyclus* (Bacquet et al. 2015). Interestingly, Liénard et al. (2014) present evidence for extensive duplication of FAR genes in *Heliconius melpomene* as compared to moths and even the monarch butterfly (Davey et al. 2016), and this may explain some of the diversity of compounds found in *Heliconius* (Lassance et al. 2013; Liénard et al. 2014).

[2] The character ω denotes the aliphatic end of an aliphatic chain, while Δ is counted from the carboxylate head group.

ile Δ is counted from the carboxylate head group.

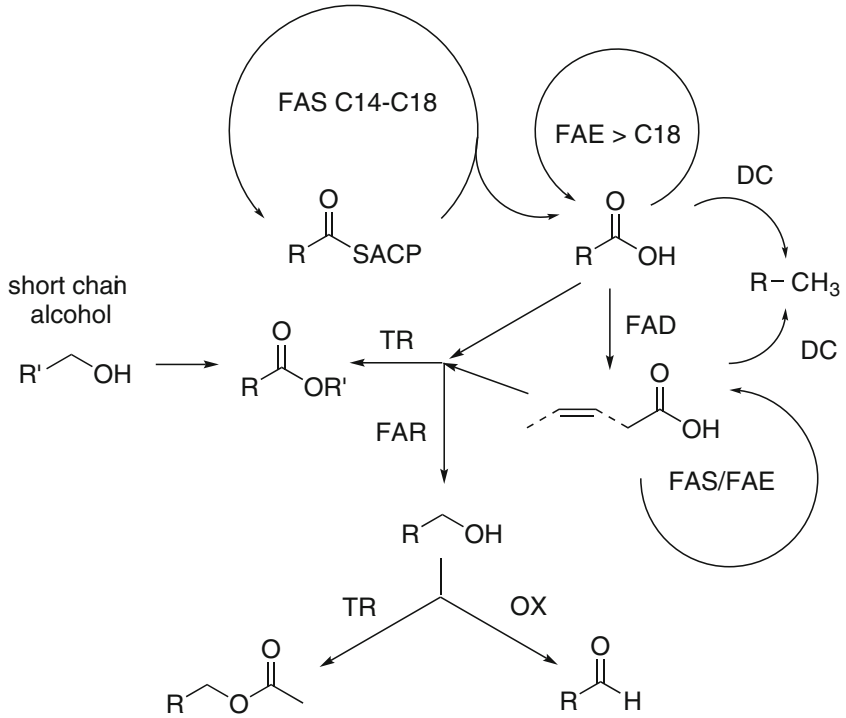

Fig. 4 Proposed biosynthetic pathways to different fatty acid metabolites used by *Heliconius*. FAS fatty acid synthase; DC decarboxylase; FAD fatty acid desaturase; TR transferase; FAR fatty acid reductase; FAE Fatty acid elongase; OX oxidase; ACP acyl carrier protein. Common FAS builds up saturated fatty acid precursors bound to ACP. The chain length can be extended beyond C_{18} by FAE. The resulting fatty acids are further modified by enzymatic activity. A DC leads to hydrocarbons under formal release of CO_2. Double bonds can be introduced by fatty acid dehydrogenases, and the chain length again modified by FAS/FAE (Liénard et al. 2014). The carboxylic acid group can be reduced to an alcohol (or probably directly to an aldehyde). The alcohol, on the other hand, can either be oxidised to an aldehyde by an OX, or converted by a TR using acetyl coenzyme A to form acetates. Additionally and in contrast to biosynthetic pathways operating in moths, the esterification of an acid with short chain alcohols can form different fatty acid esters (Wang et al. 2014)

The remaining 30% of the compounds identified constitute terpenoids or aromatic compounds. Of special interest is mellein, present in five species. Mellein is a typical fungal polyketide metabolite, but serves as a pheromone of males of the oriental fruit moth, *Grapholita molesta* (Nishida et al. 1982) and of the greater wax moth, *Aphomia sociella*. A mellein producing fungus was isolated from the wings of the latter (Kunesch et al. 1987). Mellein is also a phagostimulant for the danaine butterfly, *Idea iasonia* that actively stores it in the male pheromone glands (Nishida et al. 1996) and an ant trail pheromone (Bestmann et al. 1992). Polyketide synthases are not known from insects (Pankewitz and Hilker 2008), therefore it seems likely that mellein is produced by a fungus. It was found solely in the androconia potentially indicating a symbiotic interaction or even a fungal infection. The scattered occurrence in the individuals analyzed seems to rule out an obligate symbiotic interaction.

Wing Androconial Vs. Clasper Scent Glands Compounds

In addition to wing androconia, male heliconiines also possess clasper scent glands (CSG), which are used to transfer antiaphrodisiacs during mating (Gilbert 1976; Schulz et al. 2008). The concentration of the compounds in these glands are usually higher and the chemical diversity is larger than in the wing androconia (Estrada et al. 2011; Mérot et al. 2015; Miyakado et al. 1989; Schulz et al. 2007; Schulz et al. 2008). When comparing the results in this study with a previous comprehensive study of CSGs by us (Estrada et al. 2011), CSGs usually contain some compounds of higher volatility (RI < 1800) than those found in the wing androconia. They are embedded in a less volatile mixture of esters as well as cuticular alkanes and dialkyltetrahydrofurans (Schulz et al. 1998).

Hydrocarbons occur in most species in both wing androconia and CSGs because of the epicuticular wax layer. Nevertheless, the major aliphatic aldehydes and alcohols we found in the androconia of *H. melpomene* do not occur in the respective CSGs (Mérot et al. 2015; Schulz et al. 2008). This is also the case for *H. hecale*, *H. ismenius*, *H. erato*, and *H. sara*, for which data on CSG constituent composition also exist (Estrada et al. 2011). Similarly, ethyl esters are restricted to the wing androconia. Nevertheless, the C_{18}-macrolides identified only in *H. cydno zelinde* androconia also dominate the CSG composition of this species (Schulz et al. 2007) and two acetates of *H. hecale* were also reported from the CSG (Estrada et al. 2010). More volatile compounds are

characteristic for CSGs and are mostly absent in the wing androconia. In summary, it is evident that with few exceptions the wing androconia and the CSGs contain a distinct set of compounds, indicating different functions of the constituents.

Blend Differences between Closely Related Species In *Heliconius*, closely related species usually differ in wing colour phenotype and belong to different mimicry rings, while co-mimics generally belong to distant lineages (Turner 1976). For instance, the closely related species *H. cydno* and *H. melpomene* are usually sympatric and display divergent colour patterns, with the former characterised by yellow/white and the latter by red/orange wing patterns. These species diverged recently (~2.8 Mya) and different barriers contribute to their reproductive isolation (Jiggins 2008). However, recent evidence of mimetic convergence between closely related species of the *melpomene/cydno* group has been documented in east Andean Colombia and Peru (Giraldo et al. 2008; Mérot et al. 2013). One of the cases involves *H. melpomene malleti* and *H. timareta florencia*, which share a '*dennis-ray*' wing pattern in Florencia, Colombia. Where such closely related species share a mimetic wing phenotype, visual mate recognition is not possible so pheromone signals may be required to avoid hybridisation.

In the genus *Bicyclus*, there is evidence for character displacement in pheromone blend between sympatric species, perhaps due to reinforcement (Bacquet et al. 2015). Similarly here, we show marked differences in the bouquets between sympatric, closely related species, e. g. *H. melpomene malleti* and *H. timareta florencia*, sharing a wing colour pattern. A recent analysis of *H. melpomene amaryllis* and *H. timareta thelxinoe* wing androconia showed that octadecanal was only present in *H. melpomene*, while henicosane was present in both species (Mérot et al. 2015). We could confirm these results, but additionally significant amounts of unsaturated aldehydes were present in *H. melpomene malleti* and ethyl esters in *H. timareta florencia*, which have not been reported previously. In contrast, Mérot et al. (2015) reported unspecified benzoate esters in wing androconia. The occurrence as four homologs with retention indices consistent with a C_{12}- to C_{15}-chain suggests these compounds to be contaminants, because they are known skin cosmetic constituents (Becker et al. 2012). Unspecified methyl esters were reported as well, likely formed from common fatty acids. We could not confirm the presence of these compounds in our samples.

One could argue that closely related species differing in colour pattern do not need different attractive blends. This is not the case in the species we investigated. Large composition differences are also found between non-mimetic *H. cydno* and *H. melpomene/H. timareta*, differentiating all three species (Fig. 2).

The non-mimetic *H. elevatus pseudocupidineous* and *H. pardalinus butleri* represents another closely related pair that also show strong differences in their chemical blends. *Heliconius pardalinus butleri* contains acetates, which are absent in any other east Andean species and also lacks any alkanes, which contrasts with almost all other species analyzed. In these species pairs, it seems likely that their bouquets might be useful for species discrimination, and that character displacement might have led to rapid divergence between closely related and sympatric species.

The compounds that differ between the sympatric and mimetic species are strong candidates for being species-recognition signals, in the absence of wing colour patterning cues for mate recognition. These include octadecanal and henicosane, but minor components are most likely important as well to ensure species differentiation.

Blend Differences between Mimetic Species There is an intriguing pattern whereby sympatric mimetic species often share components of their bouquets. *H. erato demophoon* and *H. melpomene rosina* are part of the same mimicry ring in Panama. Both species produce large amounts of octadecanol, but specific mixtures of other alcohols and acetates. In contrast to all other *H. melpomene*, *H. m. rosina* is the only subspecies which produces both alcohols and acetates. This is quite surprising as its sympatric co-mimic also produces these compound classes. Nevertheless, there are considerable differences in the composition of the scent bouquets between the two species that likely contribute to discrimination in mating. Another mimetic species pair are *H. doris* and *H. sara*, although *H. doris* is polymorphic and not all forms are mimetic with *H. sara*. The compounds of both butterflies were surprisingly similar, despite the fact that these species are not particularly closely related. The bouquets were dominated by alkanes, but both also shared terpenoids and acetates. While all *H. doris* samples contained small amounts of fatty acid ethyl esters, they were also present in two of five samples of *H. sara*. *Heliconius doris* produces large amounts of the fungal metabolite mellein that occurred also in two of five *H. sara* individuals. *H. sara* also produces large amounts of neophytadiene. Both species contain octadecyl acetate, while icosyl acetate occurred in all *H. doris* and in two of five *H. sara*. There seems to be more variation between individuals, especially in *H. sara*, than between species.

In contrast, where mimicry pairs are more related to each other they seem less likely to share compounds, as exemplified by *H. ismenius* and *H. hecale melicerta*, which show major differences in their scent bouquets, with various acetates, alcohols and esters of *H. h. melicerta* and the unknown diterpene of *H. ismenius* (Fig. 2). The other closely related mimicry pair are *H. m. malleti* and *H. t. florencia* with very divergent pheromone blends that has been discussed in the previous section.

Overall, there is an intriguing pattern whereby sympatric and rather distantly related mimetic species share some components of their bouquets. This raises the possibility that mimicry might be associated with convergence in some aspects of chemical signals, perhaps as part of the aposematic signal to predators. A general odor of a mimicry ring could add to the visual effect of the common wing pattern, enhancing signal effectiveness and potentially memory of the predator, although this remains to be tested.

Conclusion

The majority of the 68 compounds we identified in male heliconiine wing androconia seem to be biosynthesized by the butterflies, originating from the fatty acid biosynthetic pathway. The dominant aliphatic compounds derived from fatty acids are a variable source of bouquet constituents because only a few enzymatic steps are needed to alter the composition in a specific signature. These compounds and the other compounds identified show species-specific compositions and might be potentially used as pheromones. A comparative analysis showed that individual species mostly possess unique blends which potentially can be used as chemical signals for species recognition, although some less well separated species exist.

The lack of phylogenetic signal and similarity of compounds between relatively distant species and differences between closely related species demonstrates rapid evolution of the bouquet among the latter, driven by reinforcement. The results imply that all *Heliconius* butterflies carry the same biosynthetic machinery capable of producing a diverse range of compounds. We hypothesise that the androconial bouquets in *Heliconius* can evolve rapidly due to minor regulatory changes that switch on or off different parts of the biosynthetic pathways, while keeping the underlying biosynthetic machinery unchanged. This would allow repeated loss and gain of compounds along different branches of the phylogeny.

Mimetic species often produce some shared compounds, but differ at the individual compound level, although more species sampling will be required to test whether sharing of compounds between mimetic species is greater than expected by chance.

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