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Cuticular Hydrocarbons as Potential Close Range Recognition Cues in Orchid Bees

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Abstract Male Neotropical orchid bees collect volatile chemicals from their environment and compose species-specific volatile signals, which are subsequently exposed during courtship display. These perfumes are hypothesized to serve as attractants and may play a role in female mate choice. Here, we investigated the potential of cuticular hydrocarbons as additional recognition cues. The cuticular hydrocarbons of males of 35 species belonging to four of the five extant euglossine bee genera consisted of aliphatic hydrocarbons ranging in chain lengths between 21 and 37 C-atoms in distinct compositions, especially between sympatric species of similar coloring and size, for all but one case. Cleptoparasitic *Exaerete* spp. had divergent profiles, with major compounds predominantly constituted by longer hydrocarbon chains (>30 C-atoms), which may represent an adaptation to the parasitic life history ("chemical insignificance"). Phylogenetic comparative analyses imply that the chemical profiles exhibited by *Exaerete* spp. are evolutionarily divergent from the rest of the group. Female hydrocarbon profiles were not identical to male profiles in the investigated species, with either partial or complete separation between sexes in multivariate analyses. Sexually dimorphic hydrocarbon profiles are assumed to be the basis for sex recognition in a number of insects, and thus may supplement the acquired perfume phenotypes in chemical information transfer. Overall, cuticular hydrocarbons meet the requirements to function as intraspecific and intersexual close range recognition signals; behavioral experiments are needed to determine their potential involvement in mate recognition.

Keywords Cuticular lipids · Recognition · Bees · Euglossini · Cleptoparasite

Introduction

Neotropical orchid bees are known for their unique interaction with fragrant orchids and other perfume flowers from which male bees collect volatile chemicals (Dodson et al. 1969; Dressler 1982; Roubik and Hanson 2004; Vogel 1966; Williams and Whitten 1983). The males acquire and store chemical compounds in specialized hind tibial pouches, thus concocting species-specific perfume blends (Eltz et al. 1999, 2005a; Zimmermann et al. 2009a). During courtship, the perfume is eventually exposed during a stereotypical territorial display behavior (Bembé 2004a; Eltz et al. 2005b). It is believed that the perfumes serve as chemical signals (Eltz et al. 2003, 2005b; Zimmermann et al. 2006), as their high volatility may allow the transfer of airborne chemical information. Male and female bees that come to inspect a display site usually arrive from downwind (Dodson 1966; Eltz et al. 2003; Kimsey 1980; Zimmermann et al. 2006; T. Pokorny, pers. obs.), thus lending support to the hypothesis that perfumes are used to transfer chemical information at medium to long ranges. The displaying male interacts with approaching conspecific male bees, sometimes engaging in complex, more or less ritualized flight contests, which in some species can last for up to over an hour (Kimsey 1980; T. Pokorny pers. obs.).

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When a female approaches the display site, copulation takes place at or near the male perching site (Dodson 1966; Eltz et al. 2003; Kimsey 1980; Zimmermann et al. 2006; T. Pokorny pers. obs.). Whereas male-male interactions are common and can be observed easily, the arrival of a female is a rare incident (only seven mating observations have been reported in the field: Dodson 1966; Kimsey 1980; Zimmermann et al. 2006; and six in caged bees: Eltz et al. 2003). For both, intra- and inter-sexual interactions, it is unclear which close range recognition processes are involved.

The cuticle of most terrestrial arthropods is coated with a layer of epicuticular lipids that prevent desiccation, while often also serving in chemical signaling (Chung and Carroll 2015). The chemical composition of this lipid layer is usually species-specific in both compound composition and the relative concentrations of individual lipids (see Bagnères and Wicker-Thomas 2010; Howard 1993; Howard and Blomquist 2005). Euglossine cuticular lipids have received limited attention. In a previous study, we examined the chemical composition of cuticular lipids in two species of orchid bees of the genus *Euglossa* (Pokorny et al. 2014). The cuticular profiles of *Euglossa dilemma* and *Euglossa viridissima* contain mainly long chain acetates, alcohols, and hydrocarbons. Whereas the combination of acetates and alcohols exhibited little differentiation between the two species, the relative abundances of hydrocarbons were species-specific (Pokorny et al. 2014). Cuticular hydrocarbons (CHCs) commonly are involved in mediating recognition of conspecifics, signaling of reproductive status, and establishing colony membership in social species (Ayasse et al. 2001; Blomquist and Bagnères 2010; D'Ettorre and Heinze 2005; Howard and Blomquist 2005). To elucidate whether CHCs may serve as recognition signals in euglossine bees, the present study aimed at determining whether (1) CHCs are chemically species-specific across a large sample of 35 species, and (2) CHC profiles differ between the sexes.

Methods and Materials

We sampled orchid bee CHCs by collecting bees directly in the field in Costa Rica (March to May 2013 and March 2014), French Guyana (August 2011), and the Yucantán peninsula of Mexico (March and April 2012). We used baits containing known orchid bee attractants (Roubik and Hanson 2004) to lure male bees. We captured bees with hand nets, and killed them by freezing at −10 to −20 °C. To avoid contamination with perfume or glandular compounds, we exclusively sampled CHCs from wings. Wings were excised close to the tegulae and transferred to 2 ml glass vials. Scissors and forceps were cleaned with hexane before and after each preparation of an individual bee. The vials were sealed airtight and kept refrigerated prior to further processing in Bochum, Germany. There, each wing sample (all four wings of one individual) was extracted in 250 μl of hexane (Rotisolv ≥99 %, Carl Roth). In order to concentrate samples for chemical analysis, we transferred the extracts into new clean glass vials and subsequently evaporated the solvent under a constant clean airstream. Samples were re-suspended with 50 μl of hexane. We collected a total of 5–15 individual wing samples per species for male bees, while obtaining as many samples of females as possible for each species. We obtained female bees from trap nests, natural nests, or by capturing them at flowers or other resources in the field. The sample preparation process used for female bees was identical to that used for males. Species determination of males was based on keys in Roubik and Hanson (2004) and Bembé (2004b), on identification tables/keys for the *Euglossa* species of Suriname by Bart De Dijn (unpublished, 2010), and on images compiled on the Discover Life web site (http://www.discoverlife.org).

Chemical analyses were conducted on an HP 5890II Gas Chromatograph coupled to an HP 5972 Mass Spectrometer (GC/MS, Agilent). Injection was splitless, the GC oven fitted with a DB-5MS column (30 m×0.25 mm×0.25 μm) and heated from 60 to 300 °C at 5 °C per min followed by 15 min isothermal at 300 °C. Eluted compounds were characterized by their mass spectra and retention indices, and considered identical between samples if both parameters matched. CHC identification was carried out by comparison to synthetic standards (Retention index mixture (Sigma R-8769) and a mix of synthetic alkenes obtained from M. Ayasse, University of Ulm) and mass spectral libraries (Adams 2001 and Wiley 275). We prepared dimethyl disulfide (DMDS) derivatives for selected samples to determine double-bond positions according to Dunkelblum et al. (1985). Ion currents of CHCs were integrated, and these peak areas subsequently were standardized by dividing the value of each compound over the total peak area of all CHCs of the chromatogram. Our final data matrix included all the CHCs with a relative amount higher than 0.5 %.

Multivariate analyses were conducted using the software Primer v6 (Clarke 1993; Clarke and Gorley 2006). Interspecific similarity was evaluated by non-metric multidimensional scaling (nMDS) and one-way analysis of similarities (ANOSIM). Both, nMDS and ANOSIM, were based on Bray-Curtis metrics of dissimilarity for multivariate datasets. The Bray-Curtis dissimilarity metric is unaffected by jointly absent (zero) values and only those compounds that are shared between individuals are considered. In nMDS plots, the spatial proximity of depicted samples ideally reflects their similarity to each other, with "stress" values indicating the goodness of fit between the two- or three-dimensional representation and the underlying similarity matrix. Values below 0.15 are considered a good fit. ANOSIM tests for differentiation between predefined groups. The resulting R value ($1>R>-1$) indicates the degree of separation between the groups

(complete separation of groups: $R=1$, no separation of groups: $R=0$, maximum separation within rather than between groups: $R=-1$) and therefore is at least as important as the corresponding P-values.

We conducted a series of comparative analyses to investigate the phylogenetic patterns of chemical trait evolution. To this end, we obtained a dated phylogenetic tree from the study of Ramírez et al. (2011), which includes 34 of the 35 species covered in our analysis and was trimmed accordingly. We conducted a Disparity Through Time (DTT) analysis using the R (R Core Team 2013) packages "ape" (Paradis et al. 2004), "ecodist" (Goslee and Urban 2007) and "geiger" (Harmon et al. 2008). We used a modified geiger source code based on the Bray-Curtis distance metric (kindly provided by Luke Harmon) in order to calculate pairwise distances. DTT plotting enables the exploration of multivariate trait diversification (chemical phenotypic characters) along the phylogenetic history of 34 species of euglossine bees included in our study. This method estimates the time course of phenotypic diversification by calculating DTT among all possible taxon pairs of each subclade. The relative disparity for each subclade is estimated by moving up the phylogeny from the root node to the tips of the tree, and at each node, the mean relative disparity is calculated as the average of the relative disparities of all subclades whose ancestral lineages were present at the time of that node. Observed disparity then can be compared to a Brownian null model of character evolution (Harmon et al. 2003).

To determine whether the CHC profiles exhibit a phylogenetic signal across lineages, we used nMDS and compared the stress values of different dimensionalities using the metaMDS function in the R package "vegan" (Oksanen et al. 2014). For downstream analyses, we used nMDS scores with minimum stress values from one- and two-dimensional analyses of 1000 iterations. We evaluated the strength of phylogenetic signal on the scores from one-dimensional analysis using Blomberg's K (Blomberg et al. 2003), and on the scores from the two-dimensional analysis using K_{mult}, a generalized K statistic designed for multi-dimensional data (Adams 2014). Statistical significance of phylogenetic structure was determined using 1000 phylogenetic permutations for both statistics.

In response to first results on CHC profiles of the species, we additionally tested whether patterns of CHC macroevolution supported a scenario in which the cleptoparasitic lineage (*Exaerete*) differed significantly from non-parasitic lineages (*Euglossa*, *Eulaema*, and *Eufriesea*) in its evolutionary trajectory. We compared several evolutionary models to find the best fit to explain the evolution of CHC phenotype across the Euglossini. First, we fit a single-rate multivariate Brownian Motion (BM) model, BM1. This model reflects a random walk process by which the probability of CHC divergence increases uniformly through time regardless of parasitic status. Second, we fit a single-optimum Orenstein-Uhlenbeck (OU) model, OU1, which has a global evolutionary rate parameter ($\sigma 2$), a global phenotypic optimum parameter (θ), and a global strength of selection (α) parameter. This model reflects a scenario where the evolutionary variance through time is narrowed compared to a pure BM1 model, with the strength of the pull towards an evolutionary mean proportional to α. We note that, although α is often interpreted in terms of stabilizing selection, we discuss it here in terms of an evolutionary pattern rather than a particular process, as support for a high α could result from several evolutionary mechanisms (not just stabilizing selection). We also evaluated a model in which the cleptoparasitic lineage's CHC phenotype trajectory is distinct from that of the non-parasitic lineages by comparing single-rate models to several different multi-rate scenarios. First, we assessed a two-rate BM model (BMM) in which the lineages exhibited distinct rates of evolution (different $\sigma 2$ parameters). Second, we fit a two-optima OU model (OUM) with separate chemical optima (θ) for cleptoparasitic and non-parasitic lineages, but global $\sigma 2$ and global α parameters. Third, we assessed a two-optima, two-rate OU model (OUMV) where cleptoparasitic and non-parasitic lineages had distinct θ and $\sigma 2$ parameters and a global α parameter. To account for uncertainty in the timing of evolution of cleptoparasitism, we fit all two-rate models to 100 stochastic character reconstructions of this trait generated using SIMMAP (Bollback 2006). To create stochastic character maps, we fixed the probability of the root state of Euglossini as non-parasitic at 0.95, and the transition rate from non-parasitic to cleptoparasitic as higher (0.95) than cleptoparasitic to non-parasitic (0.05) based on Cardinal et al. (2010). We assessed model fitting by comparing the mean Akaike information criterion (AICc) of each of the models. We fit each model in a univariate framework using the package OUwie (Beaulieu and O'Meara 2015) on scores of the one-dimensional nMDS analyses, and fit multivariate implementations of the BM1, BMM, OU1, and OUM models using mvMORPH (Clavel et al. 2015) on the two-dimensional nMDS results. We did not fit an OUMV multivariate model, as this method has yet to be implemented in mvMORPH.

While the above approach allowed us to test for a signature consistent with the *a priori* hypothesis that the cleptoparasitic lineage evolved in a distinct fashion from non-parasitic lineages in Euglossini, we also used a hypothesis-free data exploration approach to identify other potential shifts on the phylogeny regardless of *a priori* expectations. We used (1) the stepwise model comparison method traitMEDUSA implemented in the R package "motmot" (Thomas and Freckleton 2012) to identify the location and magnitude of rate shifts in a BMM model, and (2) the Bayesian Analysis of Macroevolutionary Mixtures (BAMM) framework (Rabosky 2014) to identify time-dependent and clade-specific phenotypic diversification rates. For the traitMEDUSA approach, we used a minimum clade size of two, placed no maximum on the total

number of rate shifts, and assigned a tree specific AICc cutoff value via trait simulation according to Thomas and Freckleton (2012). As it can accommodate multi-dimensional data, we applied the approach to the two-dimensional nMDS scores. For the BAMM analyses, we estimated the marginal densities of phenotypic rates for the one-dimensional nMDS scores using reversible jump Markov chain Monte Carlo (MCMC). We performed three BAMM runs in order to avoid getting stuck in local optima. Each BAMM analysis was run for 100,000,000 MCMC generations, sampling parameters every 50,000 generations. We computed tree scaled rate priors using the setBAMMpriors function in BAMMtools (Rabosky et al. 2015). We assessed convergence of the three BAMM runs for each rate by assuring the effective sample sizes of log-likelihoods, number of processes, and rate parameters were greater than 500 using the CODA library (Plummer et al. 2006). The first 10 million generations were discarded as burn-in for all analyses. For both, traitMEDUSA and BAMM analyses, we expected the best model to identify a shift at the base of the *Exaerete* clade, with or without other potential shifts elsewhere in Euglossini.

Results

Interspecific Comparisons of Male CHC Interspecific comparisons of CHC profiles were conducted for all species for which we were able to sample a minimum of five male individuals, leading to a total species count of 35 (24 from Costa Rica, 8 from French Guiana, and 3 from Mexico, see Online Resource 1). In extracts of the investigated species, between 11 and 34 hydrocarbons were identified, with chain lengths ranging from 21 to 37 C-atoms (Table 1). With the exception of C34:1, DMDS derivatives allowed the determination of double-bond positions in alkenes, but not in alkadienes and alkatrienes. Cuticular hydrocarbons of different samples that were not characterized through DMDS derivatization were treated as being identical when their retention indices proved to be identical. Most positional isomers of alkenes are more or less well separated by GC, but in some rare cases our approach may have led to grouping of different isomers.

To a certain extent, relative amounts of CHCs varied within species (see standard deviations, Table 1). Nevertheless, pairwise ANOSIM analysis identified significant differences between CHC profiles of different species in all cases but one (comparison between *E. augaspis* and *E. bursigera*, R=0.004, P=0.43, see Table 1 for average CHC compositions). All the other R values were considerably higher (R>0.174), indicating differentiation between species.

In nMDS analyses including all species, *Exaerete frontalis* and *Exaerete smaragdina* were separated clearly from all other species (Fig. 1). An analysis of compound contribution to similarity between species (SIMPER) and a comparison of relative compound concentrations showed that the main factor contributing to this differentiation was a predominance of long-chain CHCs in the two *Exaerete* species (Table 1), with the highest abundances provided by compounds exceeding chain lengths of 30 C-atoms. The CHC chain lengths of all other species ranged predominantly between 21 and 31 C-atoms, and the two-dimensional nMDS plots showed species-specific clustering but also substantial interspecific overlap (Fig. 1). Although the two *Exaerete* species showed the highest distinction regarding CHCs, with more than a third of the compounds either exclusive or shared with only one or two other species, 17 of the remaining species also exhibited at least one compound shared with two or less species. Six of these species exhibited an exclusive compound (Table 1).

To gain more insight into the species-level variation in these taxa, we separately analyzed groups of sympatric species (species sampled in the same country) that were similar in coloration and size, and thus might lack clear visual cues for close range recognition (Fig. 2 shows an example of small red bees from Costa Rica). Species clusters were separated clearly when considering these subgroups of sympatric taxa with similar coloration and external morphology (Figs. 2 and 3). All but one of the species pairs differed in their CHC profiles on a Bonferroni-corrected significance level (Online Resource 2). The exception was the comparison between *E. amazonica* and *E. cordata*, however, *E. cordata* had a low sample size of only five individuals.

The DTT plot showed that the CHC disparity was higher than expected under a null model of Brownian motion evolution at the base and at more recent branching nodes in the phylogeny (Fig. 4). This indicates that CHC profile diversification was high later in the evolutionary history of the group, with recently diverged subclades diversifying considerably, while overlapping one another in chemical space. However, irrespective of the CHC disparity in later subclades, comparative analyses revealed an overall phylogenetic signal across nMDS scores (K=1.03, P=0.002, K_{mult}=1.46, P<0.001), reflecting a pattern where closely related species tend to be more similar to one another in chemical space than more distantly related ones.

In both, univariate and multivariate analyses, single-rate models (BM1, OU1) poorly accommodated the data compared to multi-rate models, of which BMM and OUMV provided the best fits (Online Resource 3). In hypothesis-agnostic analyses, both traitMEDUSA and BAMM approaches independently identified the *Exaerete* lineage as having a distinct evolutionary rate from the rest of the phylogeny (Fig. 5b). For traitMEDUSA, the best fitting model identified the *Exaerete* clade as having an increased rate of evolution (relative rate increase=45.24) as compared to the core *Euglossa*/*Eulaema*/*Eufriesea* clade. BAMM results confirmed this pattern, also finding strong support for a model in which the *Exaerete*

Table 1 Average amounts in % (SD), rounded to the nearest integer, of cuticular hydrocarbons (CHCs) constituting the cuticular profile of the analyzed species

Compound	RI	*E. allosticta*	*E. amazonica*	*E. augaspis*	*E. bursigera*	*E. chalybeata*	*E. championi*	*E. cognata*	*E. cordata*	*E. crassipunctata*	*E. cybelia*	*E. dilemma*	*E. dodsoni*	*E. erythrochlora*	*E. flammea*	*E. hemichlora*	*E. gorgonensis*	*E. hansoni*	*E. heterosticta*
C21	2100												tr						
C22	2200									tr									
C23:2	2268																2 (2)		
9-C23:1	2274									tr							6 (3)		tr
7-C23:1	2281				tr												2 (1)		
C23	2300	3 (3)	2 (1)	11 (3)	8 (3)	tr	tr	tr	1 (1)	19 (9)	tr	8 (2)	5 (3)	5 (2)	tr	5 (2)	9 (4)	tr	1 (1)
9-C24:1	2374											tr							
7-C24:1	2381			tr	tr														
C24	2400	tr								tr		tr	tr						
C25:2	2470	7 (3)	tr	tr								tr					tr		
11-C25:1	2471													tr					
9-C25:1	2474	4 (3)	tr	4 (4)	2 (2)					2 (1)	tr	47 (11)	tr				5 (2)	tr	tr
7-C25:1	2482	tr	3 (2)	67 (7)	63 (9)	tr			tr	1 (1)	2 (1)	tr	2 (1)	17 (4)		33 (6)	5 (2)	1 (0)	tr
5-C25:1	2487			tr	2 (4)														
C25	2500	22 (4)	13 (4)	8 (2)	11 (4)	6 (3)	10 (6)	9 (2)	20 (11)	16 (3)	10 (2)	14 (2)	11 (2)	17 (5)	9 (2)	19 (6)	8 (2)	14 (4)	13 (2)
9-C26:1	2575	tr	tr								tr	tr					1 (0)		tr
7-C26:1	2582		tr		tr						tr		tr						
C26	2600									tr									
C27:2a	2655																		
C27:2b	2672	tr	1 (1)			2 (1)		tr	tr		3 (3)		2 (1)	4 (3)	tr	1 (2)		2 (2)	tr
9-C27:1	2675	6 (2)	15 (8)	1 (1)	1 (2)	10 (2)	tr	5 (3)	7 (5)	5 (2)	29 (13)	18 (7)	16 (13)	5 (3)	9 (2)	2 (2)	49 (8)	3 (1)	15 (2)
7-C27:1	2684	tr	19 (13)	2 (1)	2 (1)	11 (5)	20 (12)	3 (1)	4 (8)	1 (1)	30 (16)		52 (12)	5 (1)	1 (4)	6 (1)	2 (1)	26 (7)	1 (1)
5-C27:1	2691																		
C27	2700	3 (1)	4 (1)	3 (0)	3 (3)	3 (1)	12 (9)	6 (3)	3 (2)	8 (2)	5 (2)	3 (1)	4 (2)	2 (0)	4 (1)	2 (1)	3 (1)	7 (3)	5 (1)
9-C28:1	2776	tr	tr			1 (1)		1 (0)	tr	tr	tr				1 (0)				1 (1)
7-C28:1	2782					tr					tr							tr	
C29:3	2847																		
C29:2a	2848	tr																tr	
C29:2b	2850																	tr	
C29:2c	2855																	1 (0)	
C29:2d	2861																	2 (0)	
11-C29:1	2870										tr			14 (4)		3 (1)			
C29:2e	2873	tr			tr	tr	tr	tr	tr	tr	tr					tr		tr	tr
9-C29:1	2876	38 (6)	31 (11)		tr	59 (5)	16 (6)	65 (7)	53 (10)	36 (9)	9 (4)	tr	1 (1)	3 (2)	51 (13)	2 (3)	3 (1)	7 (3)	52 (5)
7-C29:1	2883	2 (1)	3 (3)		tr	2 (1)	6 (3)	3 (1)	2 (3)	tr	3 (2)		2 (0)	8 (2)	10 (4)	8 (3)		20 (4)	
C29	2900	4 (2)	2 (1)	2 (0)	2 (2)	2 (1)	2 (2)	3 (2)	5 (4)	5 (2)	1 (1)	1 (0)	2 (0)	3 (1)	4 (2)	3 (1)	tr	4 (2)	2 (1)
C30:2	2975																		
9-C30:1	2978	tr					tr												tr
C31:2a	3048	tr					tr											tr	
C31:2b	3052						3 (2)											1 (1)	
C31:2c	3060						4 (2)											1 (1)	
15-C31:1	3065																		
12-C31:1	3068						2 (1)												
11-C31:1	3069													5 (3)		7 (2)			
10-C31:1	3075																	2 (2)	
C31:2d	3075																		
9-C31:1	3077	6 (2)	4 (2)		tr	2 (1)	14 (6)	3 (2)	2 (1)	3 (2)	3 (3)	1 (1)		7 (3)	tr	3 (1)	2 (1)	3 (2)	4 (2)
7-C31:1	3085	tr					3 (2)				tr			2 (1)		2 (1)		tr	
C31	3100	1 (1)	tr	tr	1 (1)	tr	tr	tr	tr	tr	tr	1 (1)	tr	1 (0)	tr	2 (1)	tr	tr	tr
C32:2	3158																		
9-C32:1	3178																		
C33:2a																			

Table 1 (continued)

Compound	RI	*E. allosticta*	*E. amazonica*	*E. augaspis*	*E. bursigera*	*E. chalybeata*	*E. championi*	*E. cognata*	*E. cordata*	*E. crassipunctata*	*E. cybelia*	*E. dilemma*	*E. dodsoni*	*E. erythrochlora*	*E. flammea*	*E. hemichlora*	*E. gorgonensis*	*E. hansoni*	*E. heterosticta*
C33:2b		tr					tr												
C33:2c							2 (2)												
C33:2d							1 (1)												
14-C33:1																			
12-C33:1																			
11-C33:1														tr				tr	
10-C33:1																			
9-C33:1							1 (1)				tr	1 (1)		tr		tr	tr		
7-C33:1							tr												
C33					tr							tr							
C34:2a																			
C34:2b																			
C34:1																			
C35:3																			
C35:2a																			
C35:2b																			
C35:2c																			
C35:2d																			
10-C35:1																			
9-C35:1																			
C36:2																			
C37:2a																			
C37:2b																			
C37:2c																			

Compound	*E. ignita*	*E. imperialis*	*E. intersecta*	*E. milenae*	*E. mixta*	*E. prasina*	*E. sapphirina*	*E. tridendata*	*E. variabilis*	*E. villosiventris*	*E. viridissima*	*Ef. chrysopyga*	*Ef. pulchra*	*El. bombiformis*	*El. meriana*	*Ex. frontalis*	*Ex. smaragdina*
C21					tr	tr	tr							tr			
C22							tr										
C23:2							tr										
9-C23:1					tr	tr	tr							71 (12)	7 (8)	tr	tr
7-C23:1			tr				tr							tr	tr		
C23	tr			tr	tr	tr	39 (11)	1 (1)	tr	tr	4 (2)			5 (2)	2 (1)		tr
9-C24:1																	
7-C24:1			tr														
C24							tr										
C25:2			tr				tr				tr						
11-C25:1																	
9-C25:1		tr	3 (2)	tr		tr	tr	tr			6 (4)	1 (1)		tr	tr		tr
7-C25:1			50 (8)	1 (2)		tr	4 (2)	tr	2 (1)		tr						
5-C25:1			tr														
C25	12 (3)	6 (1)	10 (3)	12 (2)	12 (2)	14 (4)	17 (2)	14 (2)	16 (3)	7 (2)	13 (2)	12 (2)	15 (7)	7 (4)	7 (3)		1 (1)
9-C26:1	tr										1 (0)	tr	tr				
7-C26:1			tr	tr		tr			tr								
C26																	
C27:2a															2 (4)		
C27:2b	2 (1)		tr	2 (1)	tr	1 (1)			tr	tr	tr		tr		tr		
9-C27:1	7 (2)	4 (1)	3 (3)	3 (1)	5 (2)	26 (12)	tr	2 (1)	15 (4)	6 (2)	59 (8)	33 (4)	52 (11)	2 (1)	10 (9)		tr

Table 1 (continued)

Compound	*E. ignita*	*E. imperialis*	*E. intersecta*	*E. milenae*	*E. mixta*	*E. prasina*	*E. sapphirina*	*E. tridendata*	*E. variabilis*	*E. villosiventris*	*E. viridissima*	*Ef. chrysopyga*	*Ef. pulchra*	*El. bombiformis*	*El. meriana*	*Ex. frontalis*	*Ex. smaragdina*
7-C27:1	2 (1)	1 (1)	17 (3)	53 (6)	tr	36 (15)	8 (6)	17 (3)	36 (6)	7 (2)	3 (4)	tr		tr	tr		tr
5-C27:1						tr		tr									
C27	7 (2)	3 (1)	7 (3)	4 (2)	4 (1)	3 (1)	6 (2)	4 (1)	4 (1)	4 (1)	5 (3)	3 (1)	7 (3)	2 (1)	3 (1)		2 (1)
9-C28:1	1 (1)	tr			tr	tr				tr		tr	tr		tr		
7-C28:1																	
C29:3								tr									
C29:2a								2 (1)							6 (4)		
C29:2b								tr									
C29:2c								4 (2)							1 (1)		
C29:2d								3 (2)									
11-C29:1				4 (2)													
C29:2e	tr	tr			tr	tr	1 (1)	2 (1)						tr	tr		
9-C29:1	58 (7)	71 (7)		6 (3)	68 (8)	3 (2)	9 (2)	8 (3)	15 (3)	60 (6)	2 (1)	36 (4)	14 (7)	5 (3)	33 (12)	tr	4 (3)
7-C29:1	1 (1)	6 (4)	tr	8 (2)	2 (2)	4 (1)	3 (2)	21 (5)	5 (1)	10 (3)					tr		tr
C29	5 (3)	3 (2)	4 (2)	4 (1)	3 (1)	2 (0)	3 (1)	4 (1)	4 (1)	2 (2)	1 (1)	3 (1)	3 (2)	tr	3 (1)		2 (1)
C30:2								tr									
9-C30:1															tr		tr
C31:2a								1 (1)							4 (3)		
C31:2b								3 (2)							2 (2)		tr
C31:2c								1 (1)							tr		tr
15-C31:1																	tr
12-C31:1																	
11-C31:1				tr													
10-C31:1								1 (1)							tr		
C31:2d								tr									
9-C31:1	3 (1)	3 (1)		tr	3 (2)	tr	3 (2)	4 (3)		tr	2 (1)	6 (2)	3 (1)	2 (1)	7 (6)	tr	12 (10)
7-C31:1				tr			tr	2 (0)									1 (1)
C31	2 (2)	tr	1 (1)	tr		tr	tr	tr	1 (0)		1 (1)	2 (0)	3 (1)	1 (1)	2 (2)	tr	2 (1)
C32:2																	tr
9-C32:1																	tr
C33:2a																tr	2 (1)
C33:2b								tr							1 (1)	tr	12 (4)
C33:2c								tr							tr	1 (0)	7 (1)
C33:2d																tr	
14-C33:1															tr		
12-C33:1															tr		
11-C33:1								tr									
10-C33:1															tr		14 (11)
9-C33:1											1 (1)		tr	tr	tr	16 (2)	5 (6)
7-C33:1																3 (1)	2 (1)
C33													tr		tr	2 (1)	1 (1)
C34:2a																tr	
C34:2b																	tr

Table 1 (continued)

Compound	*E. ignita*	*E. imperialis*	*E. intersecta*	*E. milenae*	*E. mixta*	*E. prasina*	*E. sapphirina*	*E. tridendata*	*E. variabilis*	*E. villosiventris*	*E. viridissima*	*Ef. chrysopyga*	*Ef. pulchra*	*El. bombiformis*	*El. meriana*	*Ex. frontalis*	*Ex. smaragdina*
C34:1																tr	
C35:3																	tr
C35:2a																2 (1)	2 (2)
C35:2b																45 (3)	2 (1)
C35:2c																8 (2)	16 (8)
C35:2d																	3 (2)
10-C35:1																4 (4)	
9-C35:1															tr	10 (5)	2 (2)
C36:2																tr	
C37:2a																tr	
C37:2b																2 (1)	tr
C37:2c																1 (1)	

Compounds with average amounts below 1 % are indicated as trace compounds (tr). Compounds listed with Kovats' Retention Index (RI). For compounds of chain lengths larger than 32 C-atoms RI could not be calculated due to limits of the set of alkanes used for reference. Cn = Alkane with n C-atoms, x-Cn:1 = Alkene with n C-atoms, double-bond at position x, Cn:2 = Alkadiene with n C-atoms, Cn:3 = Alkatriene with n C-atoms. Double-bond position for compounds with multiple double-bonds unknown, compounds treated as identical if RIs coincide. Abbreviations of genera: *Euglossa* = *E.*, *Eufriesea* = *Ef.*, *Eulaema* = *El.* and *Exaerete* = *Ex*

lineage is assigned a different evolutionary process. The results suggest a substantial difference in the rate of CHC evolution between *Exaerete* and the rest of the group, with the posterior probability of a rate shift occurring either on the branch leading to *Exaerete* or at the base of the clade sister to *Exaerete* being cumulatively over 98 % (Fig. 5b). Overall, the best fitting models were consistently two-rate models in which cleptoparasitic lineages evolved at a higher (~50 to over >250 fold) rate than non-parasitic lineages, reflecting a rapid divergence of *Exaerete* away from the other orchid bee genera in chemical space. Support for a two-rate pattern also was present in the nMDS plot and a phenogram (Figs. 1 and 5a), where the *Exaerete* species occupied an area of chemical space separate from the rest of the Euglossini clade. Taken together, the results strongly suggest that CHC chemistry in the parasitic *Exaerete* lineages is evolving according to a distinct evolutionary process.

Female CHC Female bees were rarely encountered, and only between 1 and 8 females could be obtained for a total of ten species (Online Resource 1). Most taxonomic informative characters that are useful for species identifications are restricted to males, and even though females tend to look similar to conspecific males, females lack external diagnostic characters that define most euglossine bee species. Females could, therefore, only be unambiguously determined due to characterization of male siblings from the same nest in three species (*Euglossa dilemma*, *Euglossa townsendi*, *Euglossa viridissima*). For three additional species, species affiliation was inferred by their eclosion from the type of nest (characteristics like color of resin and size and shape of the brood cells) in combination with bee coloring (*Euglossa erythrochlora*) or through a combination of bee coloring and morphological characters (*Euglossa championi* and *Exaerete smaragdina*). The remaining four species could only be narrowed down to a number of possible species by the female's coloration compared to that of male bees that co-occur in the same area (female *Eulaema*: *El. bombiformis* or *El. meriana*; female *Euglossa* with red coloring: *E. bursigera*, *E. dodsoni*, *E. hansoni*, or *E. gorgonensis*; female *Euglossa* with blue coloring: *E. cybelia* or *E. villosiventris*). CHCs of males of the (potentially) corresponding species were used for comparisons by using nMDS even if the sample size was below five. ANOSIM analyses were conducted for *E. dilemma*, *E. viridissima*, *and E. erythrochlora* only, as these were the only species with at least five samples per sex.

The ANOSIM analyses revealed that a significant differentiation exists between female and male CHC of *E. dilemma* and *E. erythrochlora*, with the same trend in *E. viridissima* (P=0.053). CHC profiles of females were not positioned within the males' clusters in the corresponding two-dimensional nMDS representations. Clusters of female *E. dilemma* and

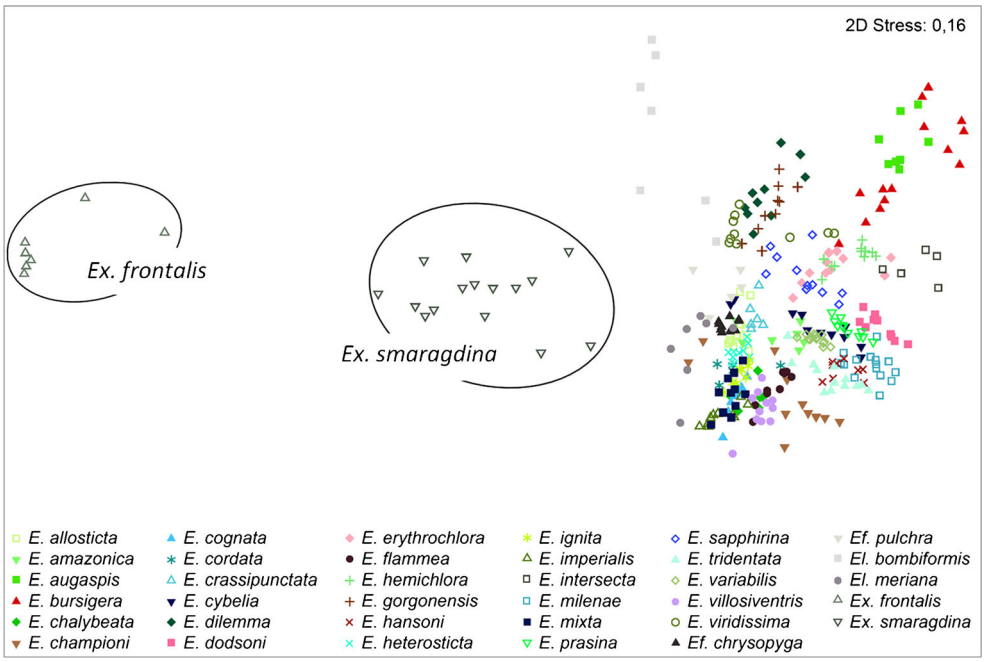

Fig. 1 nMDS plot of male cuticular hydrocarbon (CHC) profiles of 35 orchid bee species. The similarity of samples is conveyed by their proximity to each other. The two species of *Exaerete* that were clearly separated from all other samples are highlighted. Abbreviations of genera: *Euglossa*=*E.*, *Eufriesea*=*Ef.*, *Eulaema*=*El.* and *Exaerete*=*Ex*

E. viridissima partially overlapped with the male samples, yielding *R*-values below 0.5 (0.382 and 0.212, respectively), while female *E. erythrochlora* were separated clearly from the corresponding males (Fig. 6a). The same held true for female *E. championi*, *E. townsendi*, and *Ex. smaragdina*, which also were distinct from the male samples (Fig. 6b). Differences in the chemical profiles of males and females were due to compounds exclusive to one of the sexes and/or to differing relative amounts of some shared compounds (Online Resource 4). Thus no clear pattern in the compound types and relative amounts emerged for the differentiation between males and females, as in each species the differences between male and female CHC were due to different aspects of the chemical profile (see Online Resource 4).

The females of the genus *Eulaema* were positioned very close to the *El. meriana* cluster in the nMDS representation, and thus those samples likely belong to this species (Fig. 6c). The assignment of the other female samples based on CHC profiles is either tentative (*Euglossa* females blue and red2: *E. cybelia* and *E. gorgonensis*, Fig. 6d and e) or not possible given our dataset (*Euglossa* female red1, Fig. 6e).

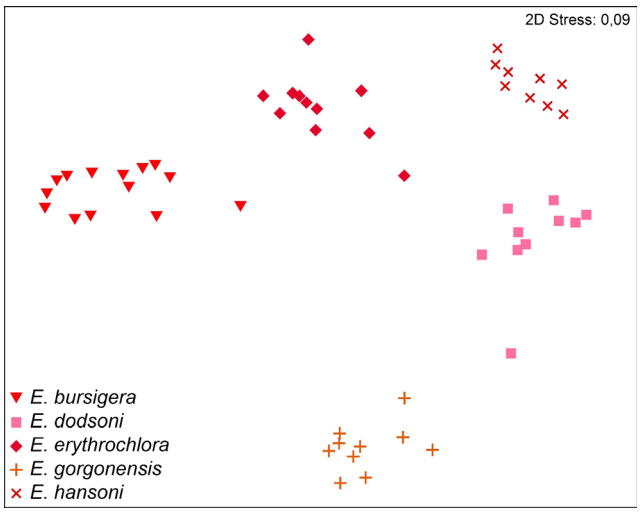

Fig. 2 nMDS plot of male cuticular hydrocarbon (CHC) profiles of sympatric species with similar visual appearance, here exemplary for small red orchid bees from Costa Rica. For the other nMDS plots, see Fig. 3

Discussion

Previous studies of euglossine chemical communication have focused on determining the variation and evolution of male acquired perfume traits (Eltz et al. 2008; Ramírez et al. 2010a; Zimmermann et al. 2009a). Perfume composition varies between populations (Ramírez et al. 2010a) and species, with pronounced differences among perfumes of closely related species (Zimmermann et al. 2009a). As male orchid bees actively expose their perfumes during territorial display (Bembé 2004a; Eltz et al. 2005b), it is hypothesized that the perfumes function as volatile signals mediating mate attraction and/or recognition. Up until now, no studies have examined the composition and variation of additional, close range recognition cues. The presence of multiple cues in the context of male-male contests and mate recognition, however, seems plausible. In Hymenoptera, close range sex pheromones can be present on a female's cuticle (see Ayasse et al. 2001), and in some bee taxa, pheromones have been shown to be

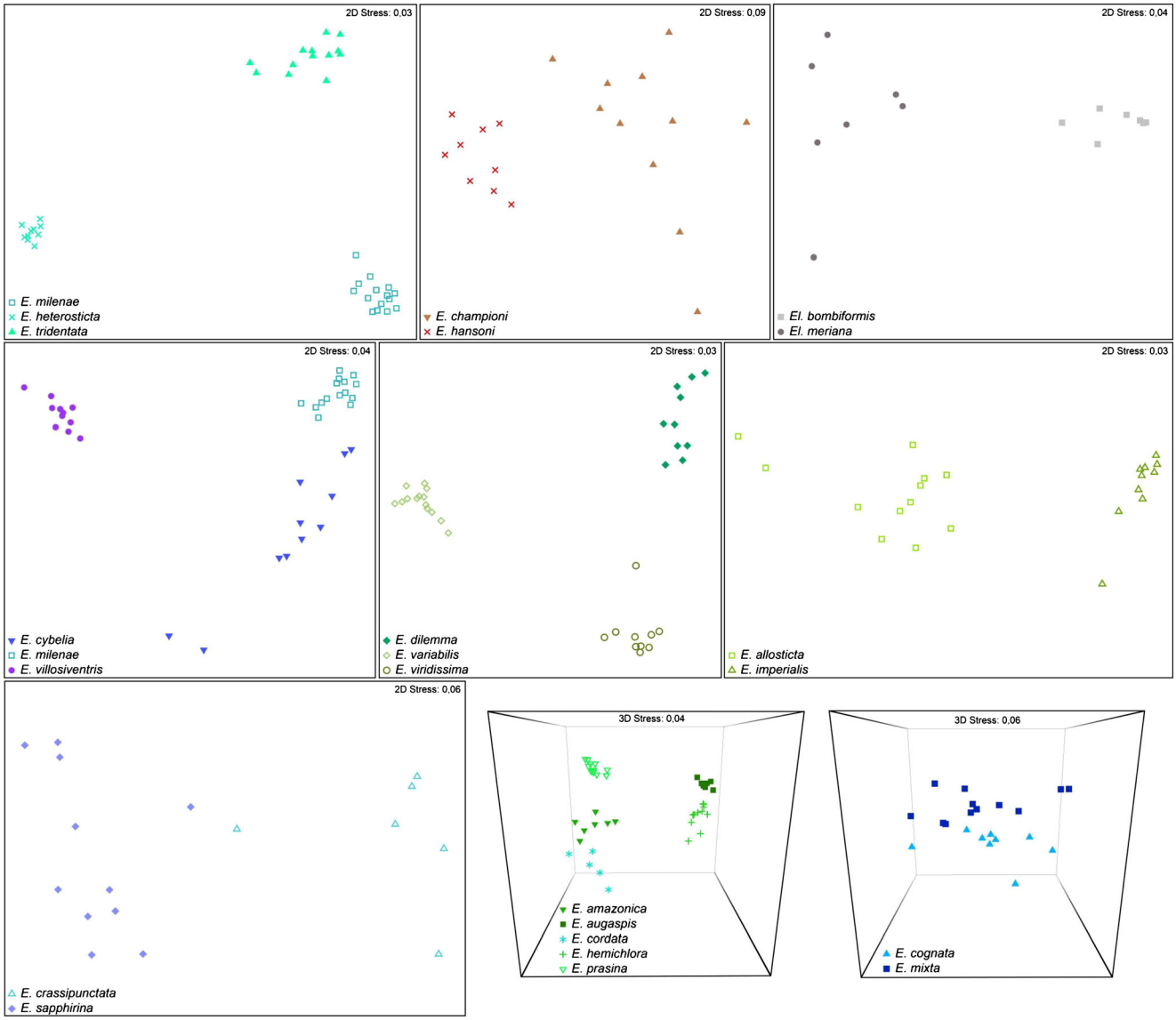

Fig. 3 nMDS plot of male cuticular hydrocarbon (CHC) profiles of sympatric species with similar visual appearance

components of the epicuticular lipid layer (e.g., Mant et al. 2005; Paulmier et al. 1999; Schiestl et al. 1999). CHCs are well-known pheromone signals in *Drosophila*, for example leading to initiation/enhancement or inhibition of courtship behavior (Ferveur and Sureau 1996). Although CHCs are usually characterized by their low volatility, it has been noted that wing fanning (as would be the case during flight encounters between orchid bees near a territorial male's perch) could help extend the range at which such chemicals could be detected (see discussion by Rybak et al. 2002 on signals involved in *Drosophila melanogaster* courtship).

Our analysis shows that CHC profiles of male orchid bees are, for the most part, species-specific in composition. One exception to this pattern was the observed similarity between male *E. augaspis* and *E. bursigera* CHCs, for which our analysis showed no differentiation. The two species are closely related, and perfume analyses have shown that the perfume phenotypes of these two species are highly differentiated (Weber et al., unpublished data). The lack of CHC profile differentiation in this pair of sibling species contrasts sharply with the clear differentiation of acquired perfumes as well as CHC profiles found for the sympatric sibling species *E. dilemma* and *E. viridissima* (Eltz et al. 2011; Pokorny et al. 2014). This might be explained by the distribution patterns of the respective species. Unlike *E. dilemma* and *E. viridissima*, *E. augaspis* and *E. bursigera* are not sympatric, with *E. augaspis* restricted to the Amazon Basin, while *E. bursigera* is reported for Central America and the Choco region (Ramírez et al. 2010b). It is possible that because speciation and reproductive isolation occurred without secondary contact, there were no selective pressures to drive character displacement of CHC profiles in this species pair.

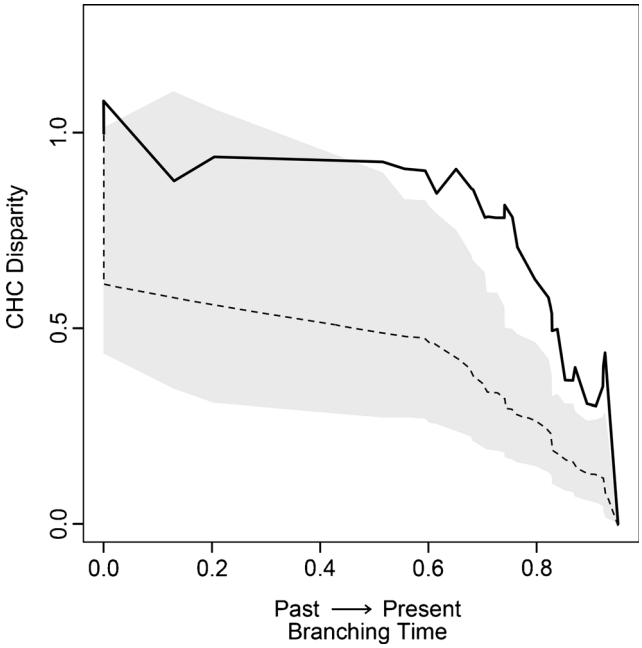

Fig. 4 Disparity-through-time (DTT) plot of cuticular hydrocarbon (CHC) profiles in relation to branching time across the evolutionary history of the lineages included in the analysis. The *dashed line* corresponds to the mean values obtained via simulated trait disparities (Brownian null model), the *grey* area corresponds to the 95 % confidence intervals, and the *solid line* to the empirical DTT

The remaining species included in our analysis could be distinguished clearly by their CHC profiles, and all sympatric species that were visually similar in size and coloration exhibited distinct CHC profiles. One of the pairwise ANOSIM comparisons did not reach the significance level after Bonferroni-correction, possibly due to the low sample size (five) of one of the species.

The intraspecific variation in relative amounts of the species-specific CHC might be caused by the age composition of the studied specimens. A number of studies have shown that insect CHC profiles can change depending on an individual's age/maturity (see e.g., Cuvillier-Hot et al. 2001; Vanickova et al. 2012; Wakonigg et al. 2000). In orchid bees, CHC have been shown to differ between females according to reproductive dominance status (Andrade-Silva and Nascimento 2015), but as CHC of Euglossini have only recently started to be investigated, there is as yet no information on potential CHC changes in males. Additionally, there is no reliable age indicator for orchid bees to date (although wing wear was tested by Eltz et al. 1999, individuals varied considerably in wing wear accumulation rates), thus we could not take the individual age of the analyzed specimens into account while obtaining sufficient sample sizes. Analyses using captive-reared orchid bees should allow elucidation of the question on age-related CHC profile differences.

Unlike a DTT analysis on perfume data, where chemical disparity clearly peaked at recent times, and thus between the most recently diverged subclades (Zimmermann et al. 2009a), CHC disparity declined during the second half of the evolutionary history, while nevertheless remaining higher than expected under a Brownian motion model. Together with the observed high disparity at the base of the phylogeny, the DTT dynamic was likely due to the combination of pronounced chemical differences between the genus *Exaerete* and all other euglossines (a basal split) and to the fact that many of the 35 studied species exhibited some rare or exclusive compounds.

Overall, the result suggests a certain level of divergent selection on CHC phenotypes. Disproportionally strong disparity of mate attraction/recognition signals between more closely related species is commonly considered important for reinforcement of premating isolation. However, if other mechanisms or signals that ensure the isolation of species exist, such as the perfume blends exposed by the male orchid bees, strong divergence in additional signals would be expected to be less likely (see Introduction in Symonds et al. 2009). The finding that CHC profiles of orchid bees are, and most likely have been, species-specific over evolutionary timespans thus supports the view that they have the potential to act as additional close range recognition cues in these bees. Such cues might be of particular importance in cases when several sympatric species are similar in size and coloration. Appropriate behavioral experiments need to be performed to test this hypothesis. Additionally, our findings suggest that analyses of CHC profiles could be a valuable tool for difficult species delimitations in orchid bee taxonomy.

The two *Exaerete* species included in this study exhibited unique CHC profiles that were clearly distinct from the profiles observed for species in the genera *Euglossa*, *Eufriesea*, and *Eulaema*. This separation was based on the predominant expression of compounds with chain lengths exceeding 30 C-atoms in *Exaerete* with very low relative amounts of shorter chain length CHCs, while *Euglossa*, *Eufriesea* and *Eulaema* rarely produced CHCs of chain lengths beyond 29 to 31 C-atoms. This chemical shift is likely related to the cleptoparasitic life style of *Exaerete* spp. Phylogenetic trait evolution model comparisons support this notion, as the best fit was constantly accommodated by scenarios in which the *Exaerete* lineage was evolutionarily distinct from the rest of the clade given its level of relatedness. Cleptoparasitic bees invade the nests of their hosts and lay their eggs into provisioned brood cells (Rozen 2003); in the case of *Exaerete*, the hosts are other species of euglossine bees in the genera *Eulaema* and *Eufriesea* (Roubik and Hanson 2004). Natural selection may have favored chemical profiles in *Exaerete* females that confer some degree of chemical inconspicuousness during encounters with their hosts. One way to avoid chemical recognition as an intruder is chemical mimicry, in which the parasite exhibits a cuticular profile similar to that of its host (Akino et al. 1999; Strohm et al. 2008), which, however, was

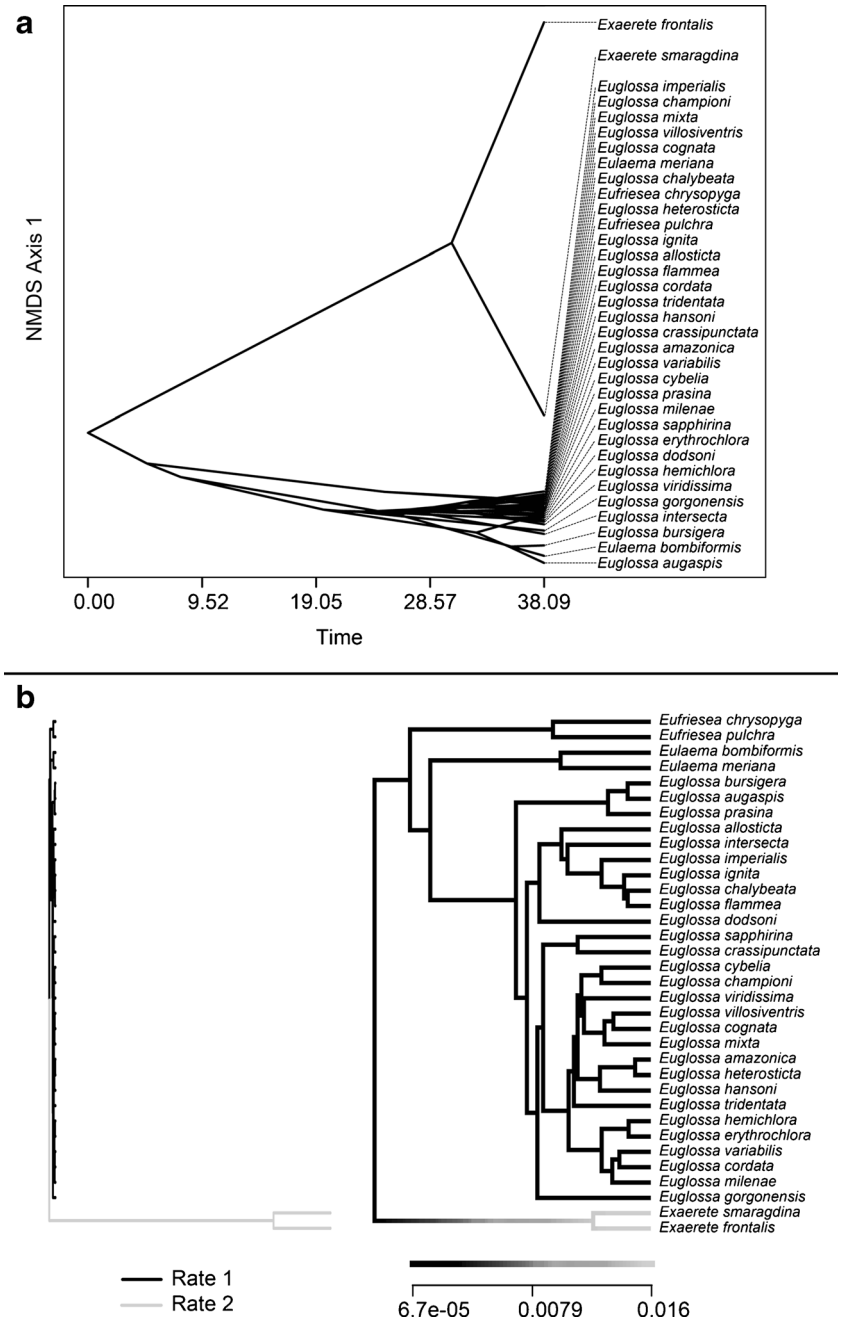

Fig. 5 **a** Phenogram of cuticular hydrocarbon (CHC) chemistry evolution within the Euglossini, species arranged on the y-axis according to one-dimensional nMDS analysis of CHC. **b** Results from traitMEDUSA (*left*) and BAMM (*right*). The *Exaerete* clade is consistently identified as having a high rate of CHC evolution compared to the rest of the group. TraitMEDUSA results scale the branch length according to the estimated rate of those branches. *Grey* represented clade shows an increased rate. BAMM results shade branches according to rates, with light greys representing a rapid rate of evolution and darker shades representing a slower rate of evolution

not the case in our study. Another strategy to avoid detection by the host is based on the lack of chemical recognition cues, chemical insignificance. In the case of CHCs, this can be achieved through either limited expression of CHCs or a shift to less volatile and thus less readily perceptible compounds. The latter has been proposed for some ants (Akino 2006; Lambardi et al. 2007), and might be the case for the *Exaerete* species of this study based on the observed predominance of CHCs of longer chain lengths. Although cleptoparasitic *Ex. smaragdina* seem to avoid direct confrontation with their hosts, and only enter the nest in the host's absence (Garófalo and Rozen 2001), chemical insignificance could reduce the risk of leaving easily perceivable CHC traces on the manipulated brood cells. Future work should include the CHC profiles of other *Exaerete* species and *Aglae caerulea*, the single species in the second genus of cleptoparasitic euglossine bees, in order to evaluate whether the shift to long chain CHCs is a general phenomenon among the parasitic orchid bees. Additional electroantennography and behavioral studies will be needed to evaluate whether such longer CHCs are indeed less readily perceived by the hosts. Although a benefit by chemical insignificance would apply only for female bees, the long chained CHC were common to both male and female *Exaerete* in our study. The observed shift to long chain CHCs may have

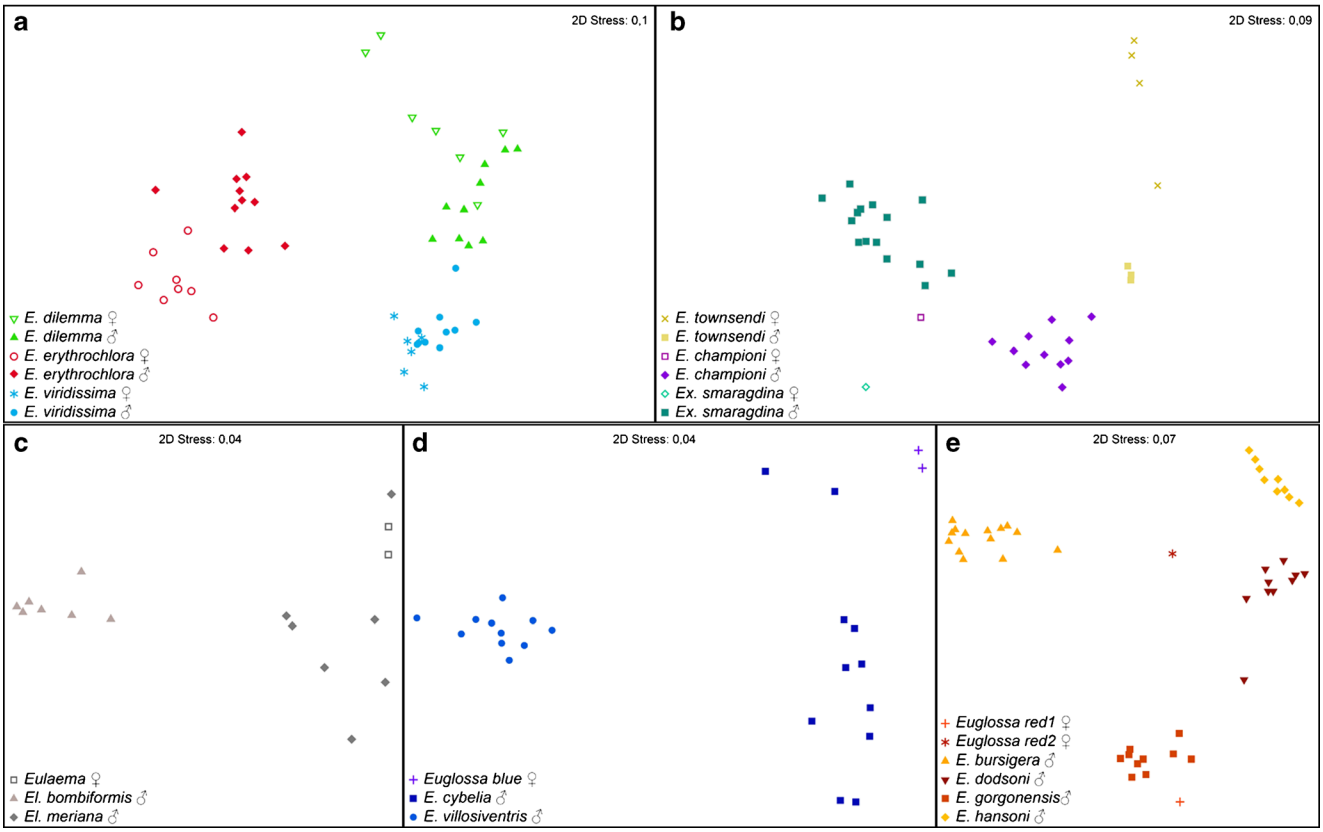

Fig. 6 nMDS plot of male and female cuticular hydrocarbon (CHC) profiles. **a** Comparisons of the species which could be analyzed using ANOSIM. **b** Comparisons of species with reliable species affiliation but smaller sample sizes. **c**, **d**, **e** Comparisons of female profiles with male profiles of potential species as inferred by female and male coloring

required a number of mutations that affect elongation processes during CHC biosynthesis, and such multiple changes likely would be expressed in both sexes due to ontogenetic constraints. If CHCs indeed serve as close range recognition signals, the impact of the shift towards long chain lengths in the *Exaerete* species on intraspecific recognition would be highly interesting to investigate.

Aside from species specificity, potential close range recognition cues involved in mate recognition need to be sex-specific. The chemical profiles of female bees tended to cluster near those of verified or potential male conspecifics, but there was no complete overlap in any case. Therefore, assignment of female bees to a particular species was not possible based on male CHC profiles alone, and our observations suggest that in most cases, orchid bees exhibit sex-specific profiles. This interesting finding deserves some explanation, however, at this point we can only speculate about the function of sexual dimorphic CHC profiles. One explanation could be that entire CHC profiles or components thereof function as intersexual recognition cues, as has been suggested for a number of insect taxa (Ayasse et al. 2001; Blomquist and Bagnères 2010; Howard and Blomquist 2005). For intersexual recognition to function effectively, chemical mate recognition could be based on the relative amounts or presence/absence of specific hydrocarbons (Mant et al. 2005; Paulmier et al. 1999; Tregenza and Wedell 1997; and discussed for sexual deception by Schiestl et al. 1999). In our analysis, no compositional pattern emerged (e.g., females with higher relative proportions of some alkenes, as found for *Megachile rotundata*, Paulmier et al. 1999), and no single compounds or compound classes stood out as being responsible for the differences between sexes across the tested species. This finding, however, is similar to studies on *Drosophila*, where many species exhibit sex dimorphic CHC profiles and have differing principal sex pheromones (different chain lengths/compound classes), while others hardly show chemical sex dimorphism (see Wicker-Thomas 2007 and references therein).

If CHC were to serve as close range sex pheromones in orchid bees, this might include a recognition function for the displaying territorial male. As the arrival of a female at a male's territory can be expected to be a rare event (orchid bees are singly mated, Zimmermann et al. 2009b), it might be crucial to recognize quickly whether the arriving bee is a male or a female individual. The number of females obtained for this study was low, and we only had satisfactory sample sizes for three species. Further studies including more females with confirmed species affiliations and corresponding behavioral and physiological experiments would be desirable.

Our study provides a first glimpse into the potential role of CHCs as close range recognition cues in orchid bees, and indicates that CHC could provide both species- and sex-specific recognition signals. Determining the potential behavioral effects of CHCs in orchid bee chemical communication is an interesting avenue of future research.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

Adams RP (2001) Identification of essential oil components by gas chromatography/quadrupole mass spectroscopy. Allured Publishing, Carol Stream

Adams DC (2014) A generalized K statistic for estimating phylogenetic signal from shape and other high-dimensional multivariate data. Syst Biol 63:685–697

Akino T (2006) Cuticular hydrocarbons of *Formica truncorum* (Hymenoptera: Formicidae): description of new very long chained hydrocarbon components. Appl Entomol Zool 41:667–677

Akino T, Knapp JJ, Thomas JA, Elmes GW (1999) Chemical mimicry and host specificity in the butterfly *Maculinea rebeli*, a social parasite of Myrmica ant colonies. Proc R Soc Lond B 266:1419–1426

Andrade-Silva ACR, Nascimento FS (2015) Reproductive regulation in an orchid bee: social context, fertility and chemical signaling. Anim Behav 106:43–49

Ayasse M, Paxton RJ, Tengö J (2001) Mating behavior and chemical communication in the order Hymenoptera. Annu Rev Entomol 46: 31–78

Bagnères AG, Wicker-Thomas C (2010) Chemical taxonomy with hydrocarbons. In: Blomquist GJ, Bagnères AG (eds) Insect hydrocarbons. Biology, biochemistry and chemical ecology. Cambridge University Press, Cambridge, pp 121–162

Beaulieu JM, O'Meara B (2015) OUwie: analysis of evolutionary rates in an OU Framework. R package version 1.45. http://CRAN.R-project.org/package=OUwie

Bembé B (2004a) Functional morphology in male euglossine bees and their ability to spray fragrances (Hymenoptera, Apidae, Euglossini). Apidologie 35:283–291

Bembé B (2004b) Revision der *Euglossa cordata*-Gruppe und Untersuchungen zur Funktionsmorphologie und Faunistik der Euglossini. Dissertation, University of Munich

Blomberg SP, Garland T Jr, Ives AR (2003) Testing for phylogenetic signal in comparative data: behavioral traits are more labile. Evolution 57:717–745

Blomquist GJ, Bagnères AG (2010) Insect hydrocarbons. Biology, biochemistry, and chemical ecology. Cambridge University Press, Cambridge

Bollback JP (2006) SIMMAP: stochastic character mapping of discrete traits on phylogenies. BMC Bioinforma 7:88

Cardinal S, Straka J, Danforth BN (2010) Comprehensive phylogeny of apid bees reveals the evolutionary origins and antiquity of cleptoparasitism. Proc Natl Acad Sci U S A 107:16207–16211

Chung H, Carroll SB (2015) Wax, sex and the origin of species: dual roles of insect cuticular hydrocarbons in adaptation and mating. Bioessays 37:822–830

Clarke KR (1993) Non-parametric multivariate analyses of changes in community structure. Aust J Ecol 18:117–143

Clarke KR, Gorley RN (2006) PRIMER v6: user manual/tutorial. PRIMER-E, Plymouth

Clavel J, with contributions from King A, Paradis E (2015) mvMORPH: multivariate comparative tools for fitting evolutionary models to morphometric data. R package version 1.0.5. http://CRAN.R-project.org/package=mvMORPH

Cuvillier-Hot V, Cobb M, Malosse C, Peeters C (2001) Sex, age and ovarian activity affect cuticular hydrocarbons in *Diacamma ceylonense*, a queenless ant. J Insect Physiol 47:485–495

D'Ettorre P, Heinze J (2005) Individual recognition in ant queens. Curr Biol 15:2170–2174

Dodson CH (1966) Ethology of some bees of the tribe Euglossini (Hymenoptera: Apidae). J Kansas Entomol Soc 39:607–629

Dodson CH, Dressler RL, Hills HG, Adams RM, Williams NH (1969) Biologically active compounds in orchid fragrances. Science 164: 1243–1249

Dressler RL (1982) Biology of the orchid bees (Euglossini). Annu Rev Ecol Syst 13:373–394

Dunkelblum E, Tan SH, Silk PJ (1985) Double-bond location in monounsaturated fatty acids by dimethyl disulfide derivatization and mass spectrometry: application to analysis of fatty acids in pheromone glands of four Lepidoptera. J Chem Ecol 11:265–277

Eltz T, Whitten MW, Roubik DW, Linsenmair KE (1999) Fragrance collection, storage, and accumulation by individual male orchid bees. J Chem Ecol 25:157–176

Eltz T, Roubik DW, Whitten MW (2003) Fragrances, male display and mating behaviour of *Euglossa hemichlora*: a flight cage experiment. Physiol Entomol 28:251–260

Eltz T, Roubik DW, Lunau K (2005a) Experience-dependent choices ensure species-specific fragrance accumulation in male orchid bees. Behav Ecol Sociobiol 59:149–156

Eltz T, Sager A, Lunau K (2005b) Juggling with volatiles: exposure of perfumes by displaying male orchid bees. J Comp Physiol A 191: 575–581

Eltz T, Zimmermann Y, Pfeiffer C, Ramirez Pech J, Twele R, Francke W, Quezada-Euan JJG, Lunau K (2008) An olfactory shift is associated with male perfume differentiation and species divergence in orchid bees. Curr Biol 18:1844–1848

Eltz T, Fritzsch F, Ramirez Pech J, Zimmermann Y, Ramírez SR, Quezada-Euan JJG, Bembé B (2011) Characterization of the orchid bee *Euglossa viridissima* (Apidae: Euglossini) and a novel cryptic sibling species, by morphological, chemical, and genetic characters. Zool J Linnean Soc 163:1064–1076

Ferveur J-F, Sureau G (1996) Simultaneous influence on male courtship of stimulatory and inhibitory pheromones produced by live sex-mosaic *Drosophila melanogaster*. Proc R Soc Lond B 263:967–973

Garófalo CA, Rozen JG Jr (2001) Parasitic behavior of *Exaerete smaragdina* with descriptions of its mature oocyte and larval instars (Hymenoptera: Apidae: Euglossini). Am Mus Novit 3349

Goslee SC, Urban DL (2007) The ecodist package for dissimilarity-based analysis of ecological data. J Stat Softw 22:1–19

Harmon LJ, Schulte JA, Losos JB, Larson A (2003) Tempo and mode of evolutionary radiation in iguanian lizards. Science 301:961–964

Harmon LJ, Weir JT, Brock CD, Glor RE, Challenger W (2008) GEIGER: investigating evolutionary radiations. Bioinformatics 24: 129–131

Howard RW (1993) Cuticular hydrocarbons and chemical communication. In: Stanley-Samuelson DW, Nelson DR (eds) Insect lipids: chemistry, biochemistry and biology. University of Nebraska Press, Lincoln, pp 179–226

Howard RW, Blomquist GJ (2005) Ecological, behavioural, and biochemical aspects of insect hydrocarbons. Annu Rev Entomol 50: 371–393

Kimsey LS (1980) The behaviour of male orchid bees (Apidae, Hymenoptera, Insecta) and the question of leks. Anim Behav 28: 996–1004

Lambardi D, Dani FR, Turillazzi S, Boomsma JJ (2007) Chemical mimicry in an incipient leafcutting ant social parasite. Behav Ecol Sociobiol 61:843–851

Mant J, Brändli C, Vereecken NJ, Schulz CM, Francke W, Schiestl FP (2005) Cuticular hydrocarbons as sex pheromone of the bee *Colletes cunicularius* and the key to its mimicry by the sexually deceptive orchid, *Ophrys exaltata*. J Chem Ecol 31:1765–1787

Oksanen J et al (2014) Vegan: community ecology package. R package version 2.2-0. http://CRAN.R-project.org/package=vegan

Paradis E, Claude J, Strimmer K (2004) APE: analyses of phylogenetics and evolution in R language. Bioinformatics 20:289–290

Paulmier I, Bagnères A-G, Afonso CMM, Dusticier G, Rivière G, Clément J-L (1999) Alkenes as a sexual pheromone in the alfalfa leaf-cutter bee *Megachile rotundata*. J Chem Ecol 25: 471–490

Plummer M, Best N, Cowles K, Vines K (2006) CODA: convergence diagnosis and output analysis for MCMC. R News 6:7–11

Pokorny T, Lunau K, Quezada-Euan JJG, Eltz T (2014) Cuticular hydrocarbons distinguish cryptic sibling species in *Euglossa* orchid bees. Apidologie 45:276–283

R Core Team (2013) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.r-project.org

Rabosky DL (2014) Automatic detection of key innovations, rate shifts, and diversity-dependence on phylogenetic trees. PLoS ONE 9: e89543

Rabosky DL, Grundler M, Title P, Anderson C, Shi J, Brown J, Huang H (2015) BAMMtools: analysis and visualization of macroevolutionary dynamics on phylogenetic trees. R package version 2.0.5. http://CRAN.R-project.org/package=BAMMtools

Ramírez SR, Eltz T, Fritzsch F, Pemberton R, Pringle EG, Tsutsui ND (2010a) Intraspecific geographic variation of fragrances acquired by orchid bees in native and introduced populations. J Chem Ecol 36: 873–884

Ramírez SR, Roubik DW, Skov C, Pierce NE (2010b) Phylogeny, diversification patterns and historical biogeography of euglossine orchid bees (Hymenoptera: Apidae). Biol J Linn Soc 100:552–572

Ramírez SR, Eltz T, Fujiwara MK, Gerlach G, Goldmann-Huertas B, Tsutsui ND, Pierce NE (2011) Asynchronous diversification in a specialized plant-pollinator mutualism. Science 333: 1742–1746

Roubik DW, Hanson PE (2004) Orchid bees of tropical America. Instituto Nacional de Biodiversidad (INBio), Heredia, Costa Rica

Rozen JG (2003) Eggs, ovariole numbers, and modes of parasitism of cleptoparasitic bees, with emphasis on Neotropical species (Hymenoptera: Apoidea). Am Mus Novit 3413:1–36

Rybak F, Sureau G, Aubin T (2002) Functional coupling of acoustic and chemical signals in the courtship behavior of the male *Drosophila melanogaster*. Proc R Soc Lond B 269:695–701

Schiestl FP, Ayasse M, Paulus HF, Löfstedt C, Hansson S, Ibarra F, Francke W (1999) Orchid pollination by sexual swindle. Nature 399:421–422

Strohm E, Kroiss J, Herzner G, Laurien-Kehnen C, Boland W, Schreier P, Schmitt T (2008) A cuckoo in wolves' clothing? Chemical mimicry in a specialized cuckoo wasp of the European beewolf (Hymenoptera, Chrysididae and Crabronidae). Front Zool 5:2

Symonds MRE, Moussalli A, Elgar MA (2009) The evolution of sex pheromones in an ecologically diverse genus of flies. Biol J Linn Soc 97:594–603

Thomas GH, Freckleton RP (2012) MOTMOT: models of trait macroevolution on trees. Methods Ecol Evol 3:145–151

Tregenza T, Wedell N (1997) Definitive evidence for cuticular pheromones in a cricket. Anim Behav 54:979–984

Vanickova L, Svatos A, Kroiss J, Kaltenpoth M, Do Nascimento RR, Hoskovec M, Brizova R, Kalinova B (2012) Cuticular hydrocarbons of the South American fruit fly *Anastrepha fraterculus*: variability with sex and age. J Chem Ecol 38:1133–1142

Vogel S (1966) Parfümsammelnde Bienen als Bestäuber von Orchidaceen und *Gloxinia*. Österr Bot Z 113:302–361

Wakonigg G, Eveleigh L, Arnold G, Crailsheim K (2000) Cuticular hydrocarbon profiles reveal age-related changes in honey bee drones (*Apis mellifera carnica*). J Apic Res 39:137–141

Wicker-Thomas C (2007) Pheromonal communication involved in courtship behavior in Diptera. J Insect Physiol 53:1089–1100

Williams NH, Whitten MW (1983) Orchid floral fragrances and male euglossine bees: methods and advances in the last sesquidecade. Biol Bull 164:355–395

Zimmermann Y, Roubik DW, Eltz T (2006) Species-specific attraction to pheromonal analogues in orchid bees. Behav Ecol Sociobiol 60: 833–843

Zimmermann Y, Ramírez SR, Eltz T (2009a) Chemical niche differentiation among sympatric species of orchid bees. Ecology 90:2994–3008

Zimmermann Y, Roubik DW, Quezada-Euan JJG, Paxton RJ, Eltz T (2009b) Single mating in orchid bees (*Euglossa*, Apinae): implications for mate choice and social evolution. Insect Soc 56:241–249