Title

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Abstract

Traits that mediate reproductive isolation between species, such as those involved in mate choice and/or recognition, are predicted to experience stabilizing selection towards the species mean. Male orchid bees collect chemical compounds from many sources, such as plants and fungi, which they use as a perfume signal (pheromone) during courtship display and are suggested to contribute to reproductive isolation between species. Environmentally acquired signals are more prone to variation as source availability can vary through space and time. If orchid bee perfumes are important for reproductive isolation between species, we expect them to exhibit stable species-specific differences in time and space. Here, we describe phenotypic patterns of inter- and intraspecific variation in the male perfumes of three sympatric species of *Euglossa* orchid bees across an entire year, investigating both their seasonality and species-specificity. Our analysis revealed considerable within-species variation in perfumes. However, species-specificity was maintained consistently throughout the year, supporting the idea that these perfumes could play an important role in reproductive isolation and are experiencing stabilizing selection towards a species mean. Our analysis also identified strong correlations in the abundance of some compounds, possibly due to shared collection sources between species. Our study suggests that orchid bee perfumes are robust in the face of environmental changes in resource availability and thus can maintain reproductive isolation between species.

Keywords: mate choice; signals; pheromone; courtship; reproductive isolation; seasonality

Introduction

The maintenance of distinct species relies on reproductive isolating barriers that reduce or prevent gene flow between diverging lineages (Coyne and Orr 2004). A key barrier to gene flow in animals is mate choice (Jiggins et al. 2001; West and Kodric-Brown 2015; Martin and Mendelson 2016; Shahandeh et al. 2018). For mate choice to effectively maintain reproductive isolation among closely related lineages, each species must differ in traits associated with mating and/or courtship behavior, and individuals must exhibit a preference for the conspecific phenotype (Mas and Jallon 2005; Ryan and Guerra 2014; Saveer et al. 2014; Shahandeh et al. 2018). Due to their importance in reproductive isolation, traits associated with courtship display and/or mate recognition are expected to experience stabilizing selection, resulting in reduced intraspecific variation and consistent species differences (Gerhardt 1982; Pfennig 1998; Benedict and Bowie 2009; McPeek et al. 2011).

Detection of chemical signals (Robertson 2019) is considered to be the most ancient and widespread sensory system, playing a key role in communication (Ache and Young 2005; Amo and Bonadonna 2018). Of particular relevance to mate choice are sex pheromones: chemical signals that mediate intraspecific communication in the context of mating (Wyatt 2003, 2014). Due to their important role in mating, divergence in signals and preferences between populations can lead to reproductive isolation (Schneider 1992; Johansson and Jones 2007; Smadja and Butlin 2008; Saveer et al. 2014). The role of chemical signaling in speciation has been well-studied in moths, where pheromones experience stabilizing selection towards the species mean (Löfstedt 1993; Smadja and Butlin 2008). However, even with high species-specificity, pheromones exhibit qualitative and quantitative differences within and between populations of the same species which may be due to genetic drift or varying selection pressures either in space or time (Carde and Allison 2016).

The term pheromone refers to the role of the chemical signal but does not address the source of the compound. The mechanisms by which pheromones are acquired, or produced, could impact the amount of intraspecific variation they exhibit depending on the availability and quality of the sources. Some of the most well-studied insect pheromones are biosynthesized *de novo*, for example, many lepidopteran pheromones (Roelofs and Rooney 2003; Liénard et al. 2008; Groot et al. 2016; Darragh et al. 2020, 2021). These genetically controlled pathways could reduce the amount of intraspecific variation due to a lack of reliance on source availability. However, some pheromone compounds are not biosynthesized by the insect itself and instead originate exogenously. For example, arctiid moths, such as *Uthetheisa ornatrix,* sequester alkaloids as larvae which they then process to produce pheromone compounds as adults (Conner et al. 1981).

A unique example of compound acquisition comes from the orchid bees, a group of insect pollinators found throughout the lowlands of tropical America, from Mexico to Brazil. Male orchid bees collect compounds from environmental sources, such as flowers and fungi, and store them in specialized hindleg pouches for use as a pheromone (perfume) during mating displays (Vogel 1966; Dressler 1982; Eltz et al. 1999, 2005b). While male orchid bees can mate multiple times (Henske et al. 2022), female orchid bees only mate once (Zimmermann et al. 2009b), with males competing for female attention. Perfumes are important for female choice, with males supplemented with perfumes mating more and siring more offspring in two-choice trials (Henske et al. 2022).

In addition to the perfume compounds that orchid bees collect, male bees accidentally incorporate many additional by-product compounds that co-occur with the compounds they actively search for (Eltz et al. 2005a). These additional compounds may vary between individuals as male bees collect perfume compounds from multiple sources (Ramírez et al. 2002; Pemberton and Wheeler 2006). Due to the reliance of orchid bees on environmental

74 sources these signals could be prone to exhibiting a substantial amount of variation across
75 both space and time.
76 The stability and species-specificity of orchid bee perfumes has mainly been
77 investigated with respect to geography. Orchid bees can be found in areas with differing plant
78 communities. For example, an introduced population of *Euglossa dilemma* in Florida, a
79 region lacking perfume orchids, has a high level of perfume similarity compared to bees from
80 the native range in Mexico and Central America (Pemberton and Wheeler 2006; Ramírez et
81 al. 2010a). *Euglossa dilemma* and *Euglossa viridissima*, a recently diverged pair of orchid
82 bees, exhibit consistent species-specific differences across their ranges (Brand et al. 2020).
83 Moreover, these perfume difference coincide with rapid evolution of odorant receptor genes
84 that mediate both perfume acquisition by males and perfume preference by females, resulting
85 in reproductive isolation (Brand et al. 2020). Comparisons across more distantly related
86 lineages have also found evidence for species-specificity of perfumes, with much greater
87 variation between species than within species (Zimmermann et al. 2006; Weber et al. 2016).
88 In addition to variation in space, orchid bee perfumes could vary in time. The
89 availability of chemical compounds may change throughout the year as source abundance
90 fluctuates due to phenological cycles. Although orchid flowers provide only a fraction of the
91 compounds collected by orchid bees in their perfumes (Whitten et al. 1993; Ramírez et al.
92 2011), many orchid species exhibit a pronounced flowering peak in the dry season in
93 Panama, with few species exhibiting year-round blooming patterns (Ackerman 1983). A peak
94 in orchid diversity within bee-orchid interaction networks also occurs during the dry season
95 in Costa Rica (Ramírez 2019). Despite these seasonal changes, orchid bees have been found
96 to build nests year round (de J. May-Itzá et al. 2014), and carry out courtship displays in both
97 the rainy season (Kimsey 1980) and the dry season (Pokorny et al. 2017), suggesting that
98 mating occurs year round.

Relatively little is known about orchid bee perfume dynamics during these seasonal changes. Perfume compounds are stored relatively efficiently by male orchid bees over a timescale of days to weeks (Eltz et al. 2019; Henske et al. 2022). However, this is not enough to buffer against changes in resource availability as orchid bees live for a few months in the wild which is less than the length of a wet or dry season (Ackerman and Montalvo 1985). Studies comparing one timepoint per season, find mixed evidence of seasonal effects. *Euglossa dilemma* has a more complex perfume in the rainy season, but only marginal effects on complexity are seen in *Euglossa viridissima* (Eltz et al. 2015). The same dataset did not find seasonality of individual compounds (Pokorny et al. 2013). However, these studies of two timepoints do not represent a true time series.

Here, we investigate the stability of orchid bee perfumes through time. We hypothesize that for perfumes to be important in reproductive isolation, species specificity need to be stable in time with consistent differences between species. Our extensive dataset allows us to use phenotypic patterns to test evolutionary hypotheses and provides candidates for future behavioral studies. We conducted a year-long analysis of perfume variation in three co-occurring species of orchid bees. We analyze perfume composition of 572 individual male bees from two closely related species, *Euglossa imperialis* and *Euglossa flammea*, and a more distantly related euglossine bee, *Euglossa tridentata* (Ramírez et al. 2010b). Samples were collected at monthly intervals over a year, resulting in a time series dataset which we use to study the seasonality of orchid bee perfumes. We describe how species differ in their perfumes, which compounds contribute to these differences, and how consistent these differences are through time. We also carry out intraspecific analyses to investigate the seasonality of the perfume of each species and whether compound collection exhibits seasonal trends.

Methods

Sample collection

Samples were collected in La Gamba field station, Puntarena, Costa Rica (8°42′03′′ N, 83°12′06′′ W) from 28th August 2015 (referred to as September 2015 samples), until 30th August 2016 (referred to as September 2016 samples) between 8am and 12pm. Samples were collected at approximately one-month intervals (exact dates found in sample information at https://osf.io/rwxv6/?view_only=1851fc1b29de4b8eaaf1f0657d9ee876). For most analyses these timepoints were considered separately, however, for seasonality analyses where 12 timepoints (one per month) are required, we combined samples from September 2015 and September 2016. Bees were collected by netting at chemical baits on filter paper using cineole, eugenol, and methyl salicylate. Precipitation data is available from La Gamba field station (https://www.lagamba.at/en/research/scientific-data-of-the-golfo-dulce-region/).

Chemical analysis

Hindlegs were placed in 500µl hexane and stored at -20°C. For analysis, 50µl was transferred to a vial containing 15µl of a 16.5ng/µl solution of 2-undecanone in hexane as an internal standard. Samples were analyzed using Agilent model 5977A mass-selective detector connected to Agilent GC model 7890B, with a HP-5 Ultra Inert column (Agilent, 30 m × 0.25 mm, 0.25 µm). 1µl of each sample was injected using Agilent ALS 7694 autosampler in split mode with a 5:1 ratio with helium as the carrier gas (250°C injector temperature, split flow of 3.5 ml/min). The temperature program started at 55°C for 3 minutes, and then rose at 10°C/min to 300°C. The temperature was held at 300°C for 1 minute and 315°C for 5 minutes.

Compounds were quantified using the internal standard 2-undecanone to calculate the amount in nanograms. Compounds were identified by comparing mass spectra and gas chromatographic retention index with previous analyses. Compounds not thought to be perfume compounds, such as hydrocarbons or compounds also found in head extracts, were

removed. Many are likely to be derived from labial gland compounds which the male bees release to dissolve volatiles before transferring this mixture to the hindlegs and recycling the labial compounds (Eltz et al. 2007). We included volatile/semi-volatile compounds eluting before a retention index of 2400. We removed compounds found in less than five percent of individuals from the overall dataset and repeated this when analyzing data from each species.

Statistical analyses

Do species differ in their perfumes?

To measure perfume divergence, we carried out nonmetric multidimensional scaling (NMDS) (Bray-Curtis similarity matrix, lowest k value with stress<0.2 was k=4) using the "metaMDS" function in *vegan* with absolute peak areas (Oksanen et al. 2020). For visualization we used the *ade4* package (Dray and Dufour 2007; Thioulouse et al. 2018).

We used multivariate analyses to investigate perfume variation. We carried out a PERMANOVA (permutational multivariate analysis of variance) using the "adonis2" function in *vegan* (Bray-Curtis distance matrix, 1000 permutations). We tested each term sequentially, starting with species, as this was the main clustering factor identified through visualization, followed by month, and an interaction term. To evaluate model fit, we used Akaike's information criterion (AIC)(Table S1). To identify which groups were significantly different from each other we carried out Bonferroni-corrected *post hoc* pairwise testing using the "pairwise.perm.MANOVA" function in the *RVAideMemoire* package (Hervé 2021).

Distance-based analyses can lead to false-positives by confounding differences in dispersion and location (Warton et al. 2012). We tested for differences in variance using the "betadisper" and "permutest" functions in *vegan*. To confirm the results of the PERMANOVA analysis, we used multivariate generalized linear models using the function "ManyGLM" from the *mvabund* package (Wang et al. 2012). We rounded our data to integers and modelled using a negative-binomial distribution. The "ManyGLM" function fits

models to each compound in the dataset and then sums the test statistics producing a multivariate test statistic known as Sum-of-LR, which can be tested for significance using resampling. We included species, month, and an interaction term. We used backward elimination and compared model fit with a likelihood ratio test (Table S2). The output includes the contribution of each compound to the Sum-of-LR, allowing us to determine which compounds drive group differences. P-values were adjusted for multiple testing.

Which compounds contribute to these species differences?

In addition to identifying the compounds driving group differences using ManyGLM, we also carried out an indicator analysis using the *indicspecies* package to determine which compounds contribute to species differences (Cáceres and Legendre 2009). The groups of interest are the species, and the goal is to identify compounds which indicate group membership. The best indicator would be a compound which is found in a single species (specificity) and in all members of that species (coverage), resulting in a perfect indicator value of one. Compound specificity is calculated using amounts, while coverage only includes presence/absence data. We used the function "multipatt" to investigate which single compounds are the best predictors of membership to each species (De Cáceres et al. 2012).

Do species share perfume motifs?

It has been suggested that closely-correlated compounds are likely derived from the same perfume sources (Zimmermann et al. 2009a). To determine if the species in our analysis shared groups of correlated compounds, we created correlation matrices using the "cor" function in the *corrplot* package (Wei and Simko 2021). We tested for significant correlations using the "cor.mtest" function. We plotted the significant strong correlations (with a cut-off of $p=0.01$ and $R<0.8$) using hierarchical clustering in the "corrplot" function and compared clusters between species.

Are species differences consistent through time?

To visualize differentiation between species throughout the year we calculated Bray-Curtis differences in a pairwise fashion each month and plotted the resulting differences to show how average species differences change over time.

We conducted statistical analyses to determine how species differences change over time. The dynamics of a particular species over time can be considered as a trajectory through space using community trajectory analysis (De Cáceres et al. 2019; Sturbois et al. 2021). We reduced each time point to the average compound amount for all compounds for each species so that each month only has one multivariate datapoint per species. We used the function "trajectoryPCoA" from the package *ecotraj* to display the trajectories for each species. To investigate the geometric properties of each trajectory, we used the functions "trajectoryLengths" and "trajectoryDirectionality" to determine trajectory length and directionality. To compare trajectories between species we used the functions "trajectoryDistances" to calculate the average distance between each species, and "trajectoryConvergence" to test for convergence between species over time.

In this analysis we assume that species would either converge or diverge over time, however, species differences could vary seasonally. To test this, we calculated the centroid of all individuals of each species per month in the NMDS ordination space. For each month, we then calculated the Euclidean distance between cluster centroids (using all 4 NMDS axis) resulting in one distance value for each species-comparison per month (McLean et al. 2019). For each species-pair comparison we then used the "cosinor" function in the *season* package (Barnett and Dobson 2010; Barnett et al. 2012, 2021). This function fits a cosinor model as part of a generalized linear regression, assuming a sinusoidal pattern of seasonality. We log-transformed our data and used the gaussian distribution, found to be appropriate based on residual plots. We assumed that one cycle occurs per year, with one peak and one trough,

explained by the phase of the model. The model is fitted using a cosine and sine term which define the sinusoid. *p*-values are provided for both the sine and cosine terms in the model and so the threshold for significance is reduced to 0.025. We also corrected for multiple testing due top number of compounds using the "p.adjust" function in R with the false discovery rate option.

Does compound collection exhibit seasonality?

In addition to testing whether overall species differences exhibit seasonality, we wanted to investigate whether compound collection within species exhibits seasonality. Including month in the PERMANOVA and ManyGLM models tests whether compounds change over time, however, this ignores the order of the months, instead of including the likely correlation between consecutive months. To account for this correlation we used the "cosinor" function in the *season* package (Barnett and Dobson 2010; Barnett et al. 2012, 2021), assuming one cycle per year. We did this both for the amount of each individual compound collected by a species throughout the year, and for NMDS dimensions for each species. The NMDS analyses were run for each species (k=2 *E. flammea* and E. *imperialis*, k=3 *E. tridentata*, lowest k value with stress<0.2 chosen). We log-transformed our data (+2 to allow us to log the negative values from NMDS dimension scores and +1 to allow us to log the zero values for the individual compounds) and used the gaussian distribution, found to be appropriate based on residual plots. As above, the significance threshold is reduced to 0.025 to account for multiple testing. We corrected for multiple testing across multiple compounds per species using the "p.adjust" function R with the false discovery rate option.

Plotting and data manipulation

Plots were made using *ggpubr* (Kassambara 2019), *cowplot* (Wilke 2020), and *ggplot2* (Wickham 2009). Additional packages used for data transformation were *MASS*

(Venables et al. 2002), *dplyr* (Wickham et al. 2021), *tibble* (Müller and Wickham 2022), and *usedist* (Bittinger 2020). Analyses were carried out in R version 4.1.2 (R Core Team 2021).

Results

Do species differ in their perfumes?

We sampled 572 male orchid bees of three species across one year (12-16 individuals per species per month) and identified 222 compounds. All species differed both in the total number of compounds, and the total amount of compound present in their perfume (Figure S1). Overall, *E. tridentata* had both the highest number of compounds and the largest quantities of the combined compounds. While there was some overlap in the compounds found in each species, the most abundant compounds differed considerably (Table 1).

Both *E. flammea* and *E. imperialis* have simpler perfumes, dominated by one or a few compounds, in contrast to the more diverse perfume of *E. tridentata* (Figure S2). The perfume of *E. flammea* is the simplest, with (*Z*)-Carvone oxide averaging 52% of the perfume (Table 1). The perfume of *E. tridentata* is more complex, and includes many low-abundance compounds; the most abundant compound is only 12.7% of the total perfume (Table 1). The most abundant compounds in *E. flammea* are also found in 99% of individuals, showing that these are the primary focus of male collection (Table 2). In contrast, the compound with highest frequency in *E. tridentata,* (*Z*)-linalool oxide, found in 91% of individuals, is not included among the five most abundant compounds (Table 2). In general, the frequency of compounds shows a different pattern to compound abundance since many compounds that are found at high frequency are not present in high abundance (Figs. S2, S3).

To determine the species-specificity of perfumes, we investigated how variation is partitioned between and within species. Individuals mostly cluster by species (Fig. 1). Species have significantly different perfumes, with species accounting for 37% of variation in perfume (PERMANOVA $F_{2,571} = 174.28$, $p < .001$). All pairwise comparisons of species are

significantly different (Bonferroni-corrected pairwise PERMANOVA, p=0.003). A further 3% of the variation is explained by collection month (PERMANOVA, $F_{12,571} = 2.29$, $p < 0.001$). Since species also differed in their dispersion (permutation test of homogeneity of dispersion, $F_{2,569}$=19.86, p=0.001; Table S3) we confirmed these results with multivariate generalized linear models using the *mvabund* package (Wang et al. 2012; Warton et al. 2012). We found the best model included species and month, with more variation explained by species, as detected by PERMANOVA (Table S4).

Which compounds contribute to species differences?

To determine which compounds best predict membership to a particular species we carried out an indicator analysis. The best predictors of species identity are those which are found in every individual of a species and in no individuals of any other species. Therefore, it is not always the case that the most abundant compound in a species in the best indicator as it may also be found in other species. For example, cineole is the most abundant compound in *E. imperialis* but is also found in *E. flammea*, making it a poor predictor of species identity. We found that 2/3 of indicator compounds in *E. flammea* and *E. tridentata*, and 1/3 in *E. imperialis* were also in the top five most abundant compounds for those species (Table 1, Table 3). Some of these compounds were also found to be major contributors to deviance due to the species term in the ManyGLM model (Table S4).

Do species share perfume motifs?

It has been suggested that closely correlated compounds (known as motifs) are likely to be derived from the same perfume source (Zimmermann et al. 2009a). This implies that motifs shared among individuals of the same species (or different species) correspond to compounds obtained from the same perfume sources. To test this, we calculated inter-compound correlation within each species. Overall, as expected due to the fact that orchid bees use a diverse range of sources for collection, we found that most compounds vary

297 independently, with a low level of correlation between compounds (*E. imperialis, R*=0.09; *E.*
298 *tridentata*, *R*=0.1; *E. flammea*, *R*=0.14). The biggest motif found in *E. imperialis* is formed of
299 eight sesquiterpenes and similar to a six-compound motif found in *E. flammea* (Figs. S4, S5).
300 Another motif, this time of acetates, is also shared between *E. imperialis* and *E. flammea*. In
301 addition, *E. flammea* has a species-specific motif consisting mostly of carvone and limonene
302 compounds (Fig. S2). The main motifs identified in *E. tridentata* are smaller and generally
303 not shared with the other species (Fig S4). Some motifs made up of only two compounds
304 were shared between all three species such as α-terpinene and γ-terpinene (Figs. S4, S5, S6).
305 Are species differences consistent through time?
306 Visualization of species differences through time revealed that interspecific
307 differences are maintained throughout the year for all three species pairs (Figure 2). We used
308 community trajectory analysis to track the trajectory of each species through time in our
309 study period. We found that while *E. flammea* and *E. tridentata* have similar trajectory
310 lengths, meaning change in perfume composition between months, *E. imperialis* has a
311 trajectory length of less than one third of the other two species (Figure S7). *Euglossa*
312 *flammea* changes most over PCoA1 which accounts for a higher percentage of variation
313 suggesting that this species exhibits the biggest changes. All three species exhibit low levels
314 of directionality, suggesting little overall change in perfume composition through time (Fig.
315 S7). Similar to our NMDS visualization, we found that *E. flammea* and *E. tridentata* were the
316 most dissimilar (average distance between trajectories: *E. flammea – E. tridentata*, 110,750;
317 *E. flammea – E. imperialis*, 94,116; *E. imperialis – E. tridentata*, 86,438). Finally, we found
318 no evidence for convergence or divergence in chemical similarity between species (Mann-
319 Kendall trend test, p=NS). We followed up this linear analysis with a seasonality analysis
320 where species differences through the year are modelled as a sinusoidal curve. We found no
321 evidence for seasonal changes in species differences throughout the year (Table S5).

322 `Does compound collection exhibit seasonality?

323 To test whether compound collection exhibits seasonality, we used cosinor model
324 analyses. Firstly, we took a multivariate approach by looking for seasonal patterns in the
325 NMDS ordinations of each species. We found seasonal effects for the first NMDS dimension
326 of both *E. flammea* and *E. tridentata*, as well as the second NMDS dimension of *E.*
327 *tridentata*, while no dimension in *E. imperialis* exhibited seasonal variation (Table S6).

328 We then tested individual compounds for evidence of seasonality. We found that 39%
329 of *E. flammea* compounds (41/105), 35% of *E. imperialis* compounds (48/139), and 22% of
330 *E. tridentata* compounds (40/184) exhibit a pattern of seasonality. The seasonal compounds
331 found in each species are not mutually exclusive, with eight shared between all three species
332 (RI=1203.5, ethyl,4-ethoxybenzoate, cineole, geranyl linalool, α-terpineol, α-phellandrene,
333 RI=1081.5 (acetate), and phenyl acetaldehyde). A similar peak phase across species was
334 found for most compounds, suggesting that seasonality could be due to environmental
335 abundance of the compounds. Despite compound seasonality, species differences are
336 maintained throughout the season, for example, cineole is always found in higher absolute
337 and relative abundance in *E. imperialis* even during seasonal fluctuations (Fig. 3). Of the ten
338 compounds which contributed most to the deviance explained by "month" in the Many GLM
339 model, seven were also identified as seasonal compounds, with four identified as seasonal in
340 all three species. We found that fewer compounds exhibit a pattern of seasonality when
341 analyzing relative abundance: 21% of *E. flammea* compounds (23/105), 12% of *E. imperialis*
342 compounds (16/139), and 13% of *E. tridentata* compounds (23/184) exhibit seasonality. Full
343 results table of all compounds for each species is found on the OSF
344 (https://osf.io/rwxv6/?view_only=1851fc1b29de4b8eaaf1f0657d9ee876).

345 These trends are not just due to overall increases or decreases in collection throughout
346 the year. We found no evidence for seasonality in number or total amount of compounds

collected, except for the amount of compound collected by *E. tridentata* which peaked in June (Table S7). In addition, we did not find a correlation between compound abundance or frequency and seasonality. Seasonal compounds do not differ in their mean abundance relative to non-seasonal compounds (ANOVA: *E. flammea*, $F_{1,103}$=2.34, *p*=NS; *E. imperialis*, $F_{1,137}$=0.22, *p*=NS; *E. tridentata*, $F_{1,182}$=0, *p*=NS). Seasonal compounds also do not differ in their frequency relative to non-seasonal compounds (ANOVA: *E. flammea*, $F_{1,103}$=0.076, *p*=NS; *E. imperialis*, $F_{1,137}$=0.59, *p*=NS; *E. tridentata*, $F_{1,182}$=0.001, *p*=NS).

We found that all three species exhibited similar seasonality in their compound collection. We looked at the peak month for all compounds identified as seasonal in each species and found no differences in mean peak collection month (Fig. 4). The mean for *E. flammea* was found in late May (phase=5.8), while for *E. imperialis* and *E. tridentata*, the mean was mid-June (phase=6.5 and phase=6.3, respectively). While there was no difference between the mean peak month for compound seasonality in each species, violin plots show that the distribution differs. *E. imperialis* and *E. tridentata* have most peaks in the early-mid rainy season (Fig. 4), while *E. flammea* has a more even spread throughout the year. We found no difference in peak phases between absolute and relative analyses (Figure S8).

Discussion

The unique nature of orchid bee perfume collection makes it an excellent example to study the dynamics of chemical communication. Male orchid bees collect chemical compounds from a range of exogenous sources which they use as perfumes during courtship. These perfumes are important for mating and are suggested to contribute to reproductive isolation between orchid bee species. Here, we investigate the chemical ecology of three sympatric species of orchid bee, testing how species differ in their perfumes and whether these environmentally derived mating signals exhibit seasonality. We found that, as previously described, orchid bees exhibit high levels of species specificity in their perfumes.

We show that species differences are maintained over time with remarkable consistency throughout the year. Differentiation between species is maintained despite intraspecific variation, seasonality in compound collection, and potentially shared collection sources between species. Our results suggest an astounding robustness of orchid bee perfume chemical signals in the face of changing environmental conditions and available resources even though male bees rely exclusively on exogenous sources for perfume formation. This consistency and stability in perfumes supports the idea that perfumes are experiencing stabilizing selection, and that they are contributing to reproductive isolation between species.

Orchid bee perfumes exhibit remarkable species specificity and remain stable across a large geographic range (Zimmermann et al. 2006; Ramírez et al. 2010a; Weber et al. 2016). We find that this species specificity is also maintained through time, with species maintaining consistent differences throughout changing seasons. In comparison with species which biosynthesize their pheromones, such as *Heliconius* butterflies, we find more intraspecific variation in orchid bees (variation explained by species: *Heliconius,* 58%; orchid bees, 37%) (Darragh et al. 2017, 2019, 2020). Nonetheless, species identity is the best predictor of perfume divergence among orchid bees, with greater interspecific variation than intraspecific variation. The pattern of species-specificity and consistency detected suggests that orchid bee perfumes are under strong stabilizing selection, as predicted for signals important for reproductive isolation (Löfstedt 1993).

We find that most variation in orchid bee perfumes reflects species identity and not local resource availability. Orchids, and the majority of plants, have been described to flower during the dry season in the tropical forests of Central America (Fournier and Salas 1966; Janzen 1967; Croat 1969; Frankie et al. 1974; Ackerman 1983; Ramírez 2019). However, male bees do not only rely on floral sources alone for perfume collection and have been described to collect compounds from many types of sources, including rotten or fungus-

infected logs, exposed plant roots, leaves, and even walls sprayed with insecticide (Roberts et al. 1982; Whitten et al. 1993; Ramírez et al. 2002; Cappellari and Harter-Marques 2010). The consistency in perfumes across environments with different plant source, such as Florida which lacks scent orchids (Pemberton and Wheeler 2006; Ramírez et al. 2010a), as well as the attraction exhibited towards compound baits (Ramírez et al. 2002) suggests that male orchid bees search for chemical compounds rather than specific compounds sources. This means they could switch easily between sources throughout the seasons to fulfill their species-specific preferences. Furthermore, male bees have been proposed to exhibit learned avoidance through negative feedback whereby collection of a particular chemical compound reduces its attractiveness, preventing overcollection (Eltz et al. 2005a; Pokorny et al. 2013). A diversity of perfume sources, alongside a learning mechanism, could buffer orchid bee perfumes from changing due to seasonal conditions.

Many chemical compounds are collected by an individual male orchid bee, making it difficult to determine which compounds are used as perfume and collected purposefully and which are "noise" compounds (Ramírez et al. 2010a). This is a limitation of our study, as we do not distinguish between compounds which are of behavioural importance and those which are not. The required behavioural experiments are time-consuming and difficult in orchid bees. However, by studying phenotypic patterns, both temporally as in our study, and geographically as has been done previously in *Heliconius* butterflies, we can predict which compounds are most likely to be biologically relevant (Darragh et al. 2020).

In general, only one or a few compounds are collected in high abundance by each species (Eltz et al. 1999; Zimmermann et al. 2009a). In this study, the perfumes of *E. flammea* and *E. imperialis* are dominated by a small number of compounds, while *E. tridentata* has a less clear dominance pattern. We found that many more compounds were found at a high frequency than at high abundance. These compounds may be target

compounds for the bees explaining their high frequency, or alternatively could be compounds produced by the perfume sources of the bees, collected as by-products. While neither abundance nor frequency alone can be assumed to translate to biological importance, we propose that by combining this data with information on the geographic and temporal consistency of compound presence in a species we can predict which compounds are likely to be important for mating and reproductive isolation. In support of this approach, some of the indicator compounds we identify exhibit antennal responses from the corresponding species for which they are indicators (Hexahydrofarnesylacetone in *E. imperialis*, (*E*)-β-ocimene and 2,3-epoxygernaylacetate in *E. tridentata*)(Eltz et al. 2006; Brandt et al. 2021). We propose that these, as well as the other indicators which have not been tested using electroantennography, are excellent candidates for behavioral trials.

Individual male orchid bees form complex perfumes by collecting compounds from a variety of different sources. It has been suggested that this results in subsets of compounds ("motifs") which are derived from the same source and are intercorrelated (Zimmermann et al. 2009a). We find some overlap with previously identified motifs. We find a motif of short chain acetates previously identified in *E. imperialis* (Zimmermann et al. 2009a). However, we do not detect a hexahydrofarnesyl acetone motif, perhaps expected as a widespread compound like this is likely collected from different sources throughout the year, eroding any correlations found in a certain season (Zimmermann et al. 2009a). Interestingly, we find shared sesquiterpene and acetate motifs between the closely related *E. imperialis* and *E. flammea*. This could indicate the use of shared compound sources, implying that closely related species can maintain species-specific perfume blends despite sharing compound sources. However, it could also be related to compound synthesis, with compounds originating from the same biosynthetic pathway more likely to be correlated, irrespective of the compound source. Many correlations are between biosynthetically similar compounds

such as aromatics, acetates, sesquiterpenes, or even isomers. This might suggest that species have shared motifs due to biosynthetic constraints rather than shared compound sources.

Our study reveals the remarkable robustness of an environmentally-acquired signal in the face of changing seasonal resources. Our data revealed strong phenotypic differences between species that remain consistent throughout the seasons, as well as the presence of species-specific compounds. These findings support the idea that perfumes are important for reproductive isolation as species-specificity is maintained despite potential changes in resource availability through the seasons. This therefore ensures that species differences could prevent interspecific mating year-round. The temporal consistency in each species' perfume also suggests that orchid bee perfumes are experiencing stabilizing selection towards a species mean. Furthermore, we find evidence for intraspecific variation and seasonality in the collection of some compounds, perhaps to some extent due to changing compound availability through the seasons. Behavioural testing of the large number of compounds presented in the study is currently not feasible and we hope that the species-specific compounds identified in this study provide candidates for future behavioral and functional experiments.

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Figures

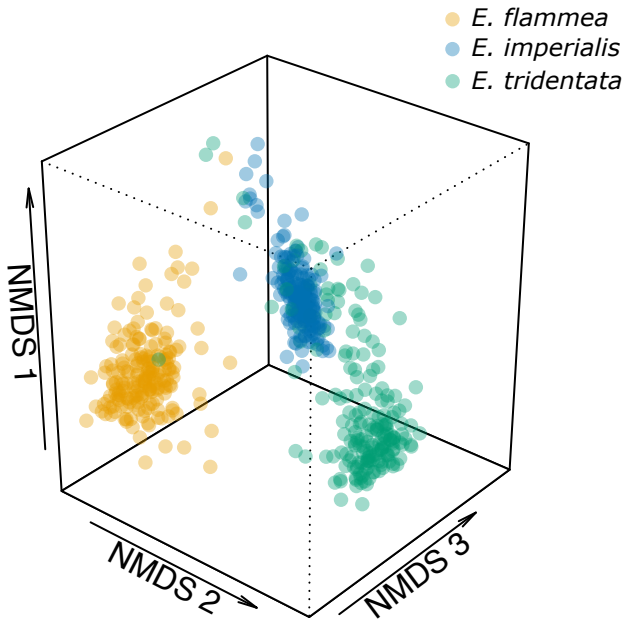
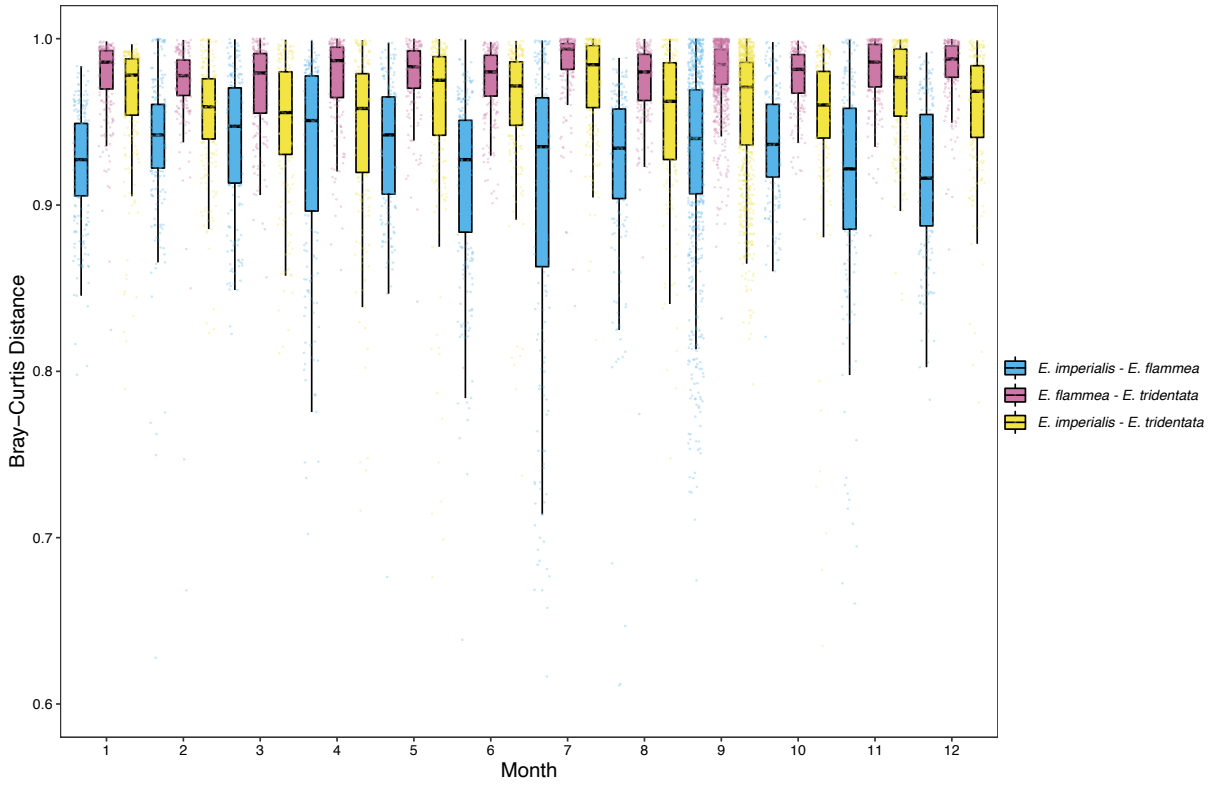


FIGURE 1. NMDS (nonmetric multidimensional scaling) plot illustrating in three dimensions the variation in the perfumes of males of three *Euglossa* species: *E. flammea*, *E. imperialis,* and *E. tridentata*. Stress=0.09.



682

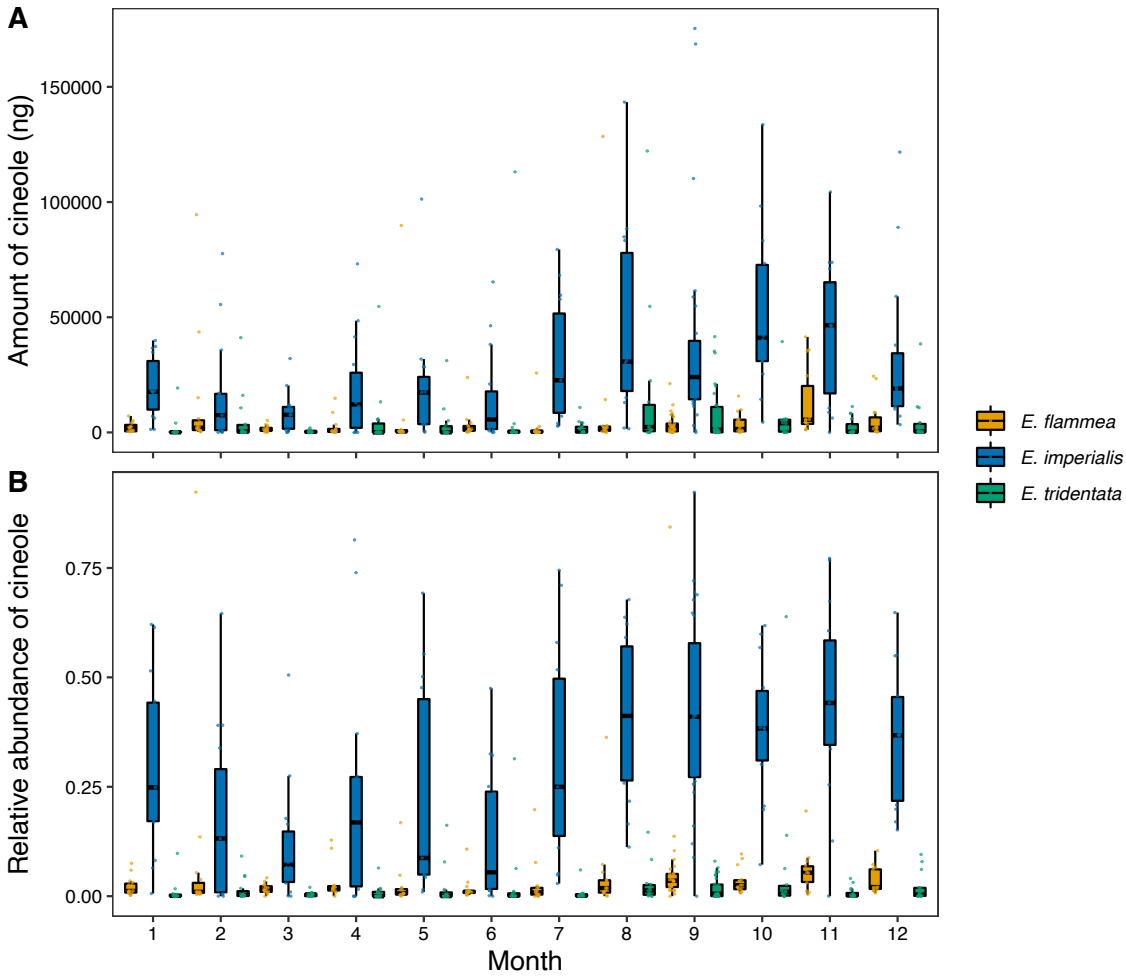
683 FIGURE 2. Pairwise Bray-Curtis distances between *E. imperialis*, *E. flammea* and *E.*

684 *tridentata* for each month of the year. More data points are included in Month 9 as this month

685 was sampled in two different years at the start and end of sampling. For the x-axis, 1 is

686 January and 12 is December. 23 outlier comparisons were removed with low Bray-Curtis

687 distances.



688

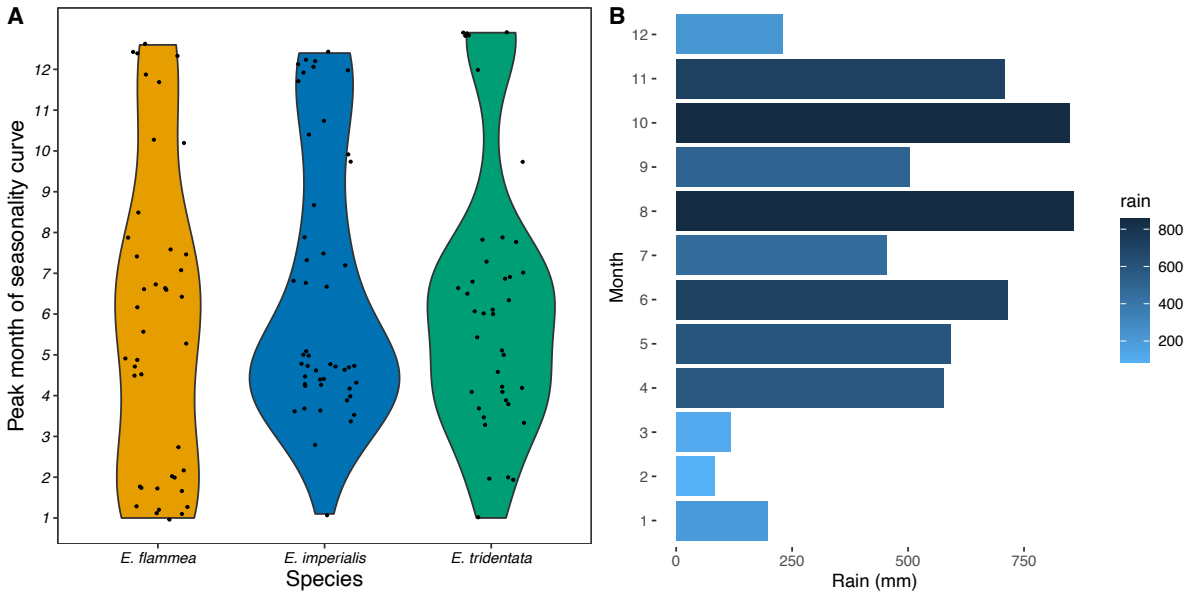
689 Figure 3. (a) Absolute amount of cineole in male *E. imperialis*, *E. flammea* and *E. tridentata*

690 for each month of the year. (b) Relative abundance of cineole in male *E. imperialis*, *E.*

691 *flammea* and *E. tridentata* for each month of the year. More data points are included in

692 Month 9 as this month was sampled in two different years at the start and end of sampling.

693 For the x-axis, 1 is January and 12 is December.



694

695 FIGURE 4. (a) Violin plot illustrating the variation in the peak month of the seasonality
696 curve for compounds of each species. Only compounds which were determined to exhibit
697 seasonality were included. Species did not differ in their peak month of seasonality (Kruskal
698 Wallis, df=2, H test statistic=0.83, p=NS). (b) Rain data from La Gamba field station in the
699 years 2015-2016. Data from September 2015 and 2016 was combined and the average taken.
700 For the y-axes, 1 is January and 12 is December.

701

702

703

Tables

Table 1. Five most abundant compounds in each orchid bee species (percentage of total perfume).

E. flammea	*E. imperialis*	*E. tridentata*
(*Z*)-carvone oxide 52%	cineole 30%	(*E*)-β-ocimene 12.7%
carvone 5.4%	germacrene d 16.6%	2,3-epoxygernaylacetate 7.7%
2-methylformalinide 4.3%	hexahydrofarnesylacetone 13.3%	eugenol 7.3%
(*E*)-limonene oxide 4.3%	nerolidol 4.8%	4-methoxycinnamicalcohol 6.3%
cineole 4.1%	α-phellandrene 2.7%	geranylgeraniol 6.1%

Table 2. Five compounds most frequently identified in each orchid bee species (percentage of bees containing compound)

E. flammea	*E. imperialis*	*E. tridentata*
(*Z*)-carvone oxide 99%	hexahydrofarnesylacetone 97%	(*Z*)-linalool oxide 91%
2-methylformalinide 99%	cineole 95%	2,3-epoxygernaylacetate 90%
carvone 97%	germacrene d 95%	(*E*)-β-ocimene 83%
cineole 96%	nerolidol 94%	unknown RI=1318.1 79%
nerolidol 95%	δ-cadinene 92%	1,4-dimethoxybenzene 79%

Table 3. Compounds which are the best indicators of species identity.

Species/compound	A (specificity)	B (coverage)	sqrtIV
Euglossa flammea			
2-methylformalinide	0.994	0.989	0.992
(*Z*)-carvone oxide	0.992	0.989	0.990
carvone	0.992	0.968	0.980
Euglossa imperialis			
hexahydrofarnesylacetone	0.979	0.968	0.973
Unknown (RI=1803.6)	0.994	0.852	0.920
Unknown (RI=2242.8)	0.993	0.841	0.914
Euglossa tridentata			
(*E*)-linalool oxide	0.994	0.912	0.952
2,3-epoxy geranyl acetate	0.999	0.897	0.947
(*E*)-β-ocimene	0.998	0.830	0.910

Note: A is a measure of species specificity of the compounds, B is a measure of species coverage, and sqrtIV combines A and B to form an indicator value. sqrtIV ranges from 0 (compound not present in any individuals of that species) to 1 (compound only present in that species, and present in all individuals).