



# Stored perfume dynamics and consequences for signal development in male orchid bees

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## Abstract

Male orchid bees (*Euglossini*) collect volatiles from their environment to concoct species-specific “perfumes”, which are later emitted at mating sites. Intensity, complexity or composition of perfumes may encode age (survival) of a male, but how the individual perfume develops over time needs to be clarified. We investigated chemical changes during storage in leg pockets. We injected a mixture of eight perfume compounds into pockets of *Euglossa imperialis* and only the two most volatile compounds decreased over 12 days. Using a different approach we found significant shifts in quantities of naturally occurring perfume compounds of *Euglossa championi* over 10 days, with the strongest decreases (up to 70% peak area) in highly volatile minor compounds, e.g. monoterpenes, and noteworthy increases (up to 40%) in some sesquiterpenoids. Corresponding shifts were observed in legs of dried bees, suggesting that no metabolic activity is required for the observed changes to occur. Our results confirm that male orchid bees are generally good at preserving collected perfumes. However, subtle shifts towards heavier compounds in blends may occur over the lifetime of individual bees, e.g. due to evaporation or in-pocket chemical reaction, with old males acquiring a more pronounced base note in their seasoned perfumes.

**Keywords** Euglossini · Fragrance · Chemical communication · Pheromone analogue · Scent

## Introduction

Orchid bees (*Euglossini*) have unique relationships with flowering plants and other odoriferous substrates in their neotropical forest habitat (Dressler 1982; Williams and Whitten 1983; Ramirez 2009). Males are attracted to specific odorants over long distances and harvest the volatiles using specialized behaviors and morphological structures for uptake and storage (Vogel 1966; Ackerman 1989). While collecting the substances from flowers they act as specific pollinators of flowering plants, including many orchids and aroids, which in turn have evolved specialized floral chemistry and intricate flower morphologies to lure and manipulate

males of certain species of bees (Dressler 1968; Williams and Dressler 1976; Ramírez et al. 2011).

While the benefits for plants are straightforward (specific pollination), the purpose of the collected substances for the bees is still not fully understood. Male bees can be attracted in large numbers to synthetic chemicals, and mark-recapture baiting assays have shown that they engage in volatile collection over weeks and possibly over much of their adult lives (Ackerman and Montalvo 1985; Eltz et al. 1999). Chemical analyses of hind leg pockets, the organs where volatiles are placed during collection (see below), have shown both substantial variability in quantity and complexity of volatiles among individuals (Eltz et al. 1999; Ramírez et al. 2010), but also specificity, i.e. the composition of acquired “perfumes” is broadly species-specific even across localities and regions (Zimmermann et al. 2009; Weber et al. 2016). The males expose their perfume using specific behavior and leg structures during a characteristic display behavior, for which they perch on vertical stems in the forest understory (Eltz et al. 2005b). Matings occur at the same sites, but a direct proof of female attraction, or indeed any response of females, to male perfumes, is yet lacking (Kimsey 1980; Eltz et al. 2003). The current best theory is that perfumes

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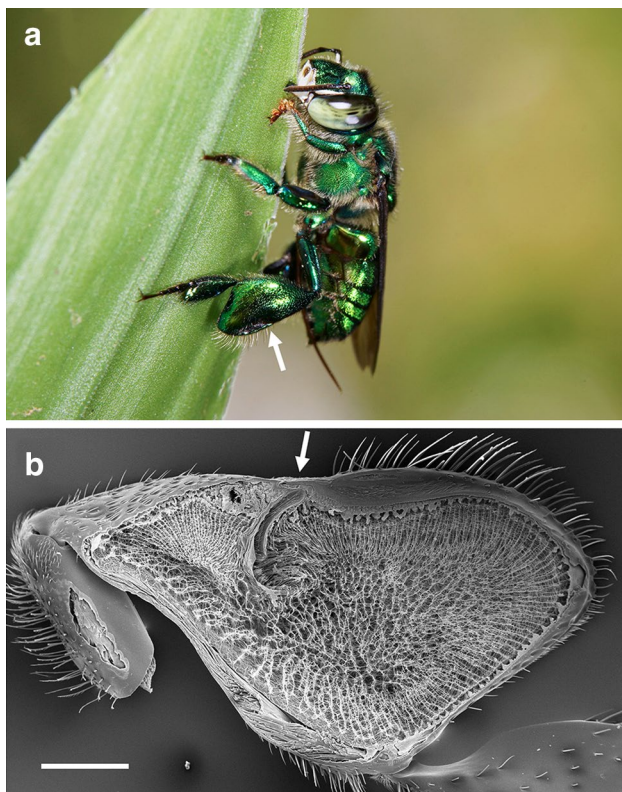
serve as intraspecific signals, either among or within sexes, and that they evolve by intra- or/and intersexual selection (Pokorny et al. 2017).

For volatile collection, males land on fragrant substrates and apply mixtures of long-chain aliphatic compounds (“lipids”, mostly acetates and diacetates) from their enlarged cephalic labial glands (Whitten et al. 1989). These lipids, along with the volatiles dissolved in them, are then absorbed with the help of fore-tarsal brushes and transferred to specialized pockets (containers, pouches, “organs”) on the hind tibiae (see Vogel 1966 and; Kimsey 1984). These pockets are invaginations of the cuticle that fill almost the entire hind tibial capsule, which is conspicuously swollen in male orchid bees (Fig. 1). The pocket communicates with the dorsal surface of the tibia via a narrow canal that widens near the surface to form a shallow depression functioning as an interface for volatile uptake (Vogel 1966) and exposure (Eltz et al. 2005b). Vogel (1966) concluded that volatile uptake works

through capillary forces exerted by the huge surface area of branched and densely interwoven hairs that populate the pocket, giving it a sponge-like structure. Although in some species there are what appear to be glandular epithelia of variable sizes lining the pocket, canal, and interface (Cruz-Landim et al. 1965; Vogel 1966; Eltz 1997), there is so far no evidence that additional volatiles are actively secreted into the pocket. The only endogenous compounds that are regularly found in extracts of male orchid bee hind tibiae are labial gland lipids, which are deposited in the pockets during volatile uptake (Williams and Whitten 1983; Whitten et al. 1989), and straight chain cuticular hydrocarbons (CHC), which are secreted onto the entire cuticular surface (dos Santos and do Nascimento 2015; Pokorny et al. 2015). The labial gland lipids have been shown to be selectively reabsorbed from the pockets, and shuttled back to the labial glands, for reuse during consecutive bouts of volatile collection (Eltz et al. 2007) (Fig. 2).

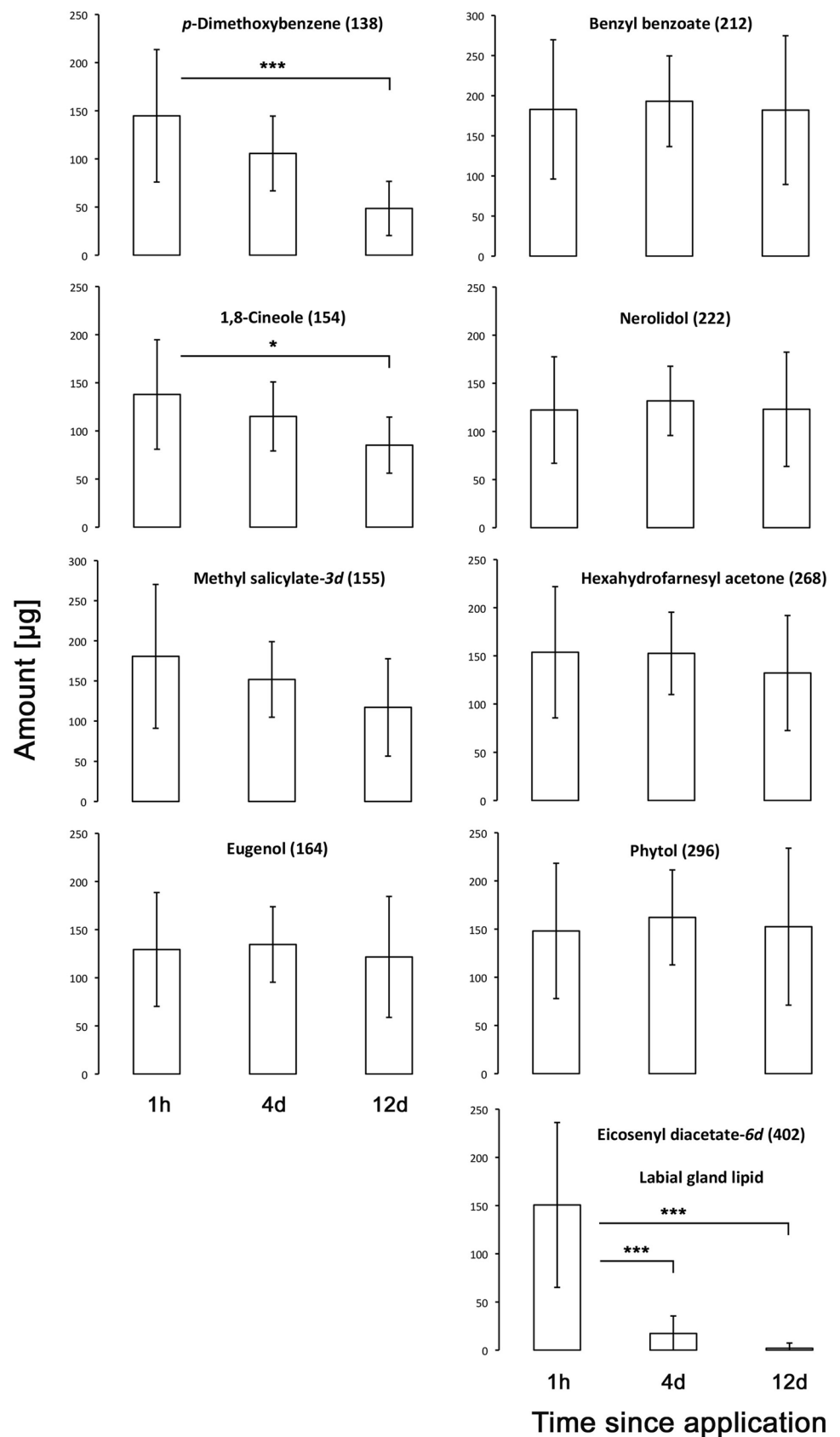
Previous evidence points to the hind-leg pockets being essentially storage devices. In a cage experiment, Eltz et al. (1999) analyzed contents of hind legs of male *E. imperialis* that were sampled after 0, 5, 10, and 15 days of captivity without access to odor sources and found no change in the total amount of tibial perfume over time. However, the experiment had relatively low power of detecting changes because it was based on natural contents, quantities of which vary dramatically among individuals (Eltz et al. 1999). The experiments presented here measured changes in quantities of chemicals that were applied in known concentrations into leg pockets or were based on comparisons of contents between left and right legs of individual males; both methods are substantially more powerful to detect changes over time both in overall quantity as well as in quantity of individual compounds.

Good storage capacity would be a prerequisite for volatiles to accumulate linearly over time, which in turn is a precondition for perfumes to reliably indicate the age of a signaling male. Age has been suggested to reflect fitness because old individuals have proven their ability to survive, and age-related expression of secondary sexual traits in males may allow females to choose genetically superior mates (Brooks and Kemp 2001; Manning 1985). Orchid bee perfumes, by their cumulative nature, seem perfectly suited to communicate male age or other age-related qualities, e.g. the diversity of foraged resources or the size of the area foraged from. Current evidence for perfumes being related to male age is circumstantial: the quantity and complexity of perfume blends was found to be positively related to a widely used age correlate of flying insects, wing wear, in some species (Eltz et al. 1999, 2015). However, the relationships were extremely noisy in all cases, and signs of non-linearity (saturation or hump-backed shapes of the relationship) cast doubt on a simple linear perfume accumulation over time. Thus,



**Fig. 1** **a** Male *Euglossa imperialis* collecting odorants from a leaf, sporting dense brushes of hair on fore tarsi for volatile absorption as well as swollen hind tibia containing the perfume pocket. **b** Lateral view in the left hind tibia of a male *Euglossa tridentata* cut open sagittally with a microtome. Scanning electron micrograph; scale bar 0.5 mm. Note that the perfume pocket, a cuticular invagination originating from a dorsal depression on the leg surface, fills almost the entire leg capsule and is densely populated by branched hairs. The arrows in **a** and **b** indicate the position of the dorsal opening to the pocket

**Fig. 2** Effect of time since application on quantities of nine chemical compounds in right hindlegs of *Euglossa imperialis* in the volatile application experiment. Means and standard deviations are plotted, with sample sizes being 8, 11 and 9 for 1 h, 4 and 12 days, respectively. Molecular weight is given in brackets behind compound names. See text for statistics, \* $p < 0.05$ , \*\*\* $p < 0.001$



the exact development of the perfume signal over individual male life-times is yet uncertain. It will essentially depend on three factors: (1) the dynamics of volatile collection over the adult life of individuals, (2) the development of display/volatile expenditure over the adult life of individuals, and (3) the extent of quantitative or compositional change that happens to stored perfume over time through evaporation and/or chemical reaction. The present study was specifically aimed at measuring (3), using experiments with caged male orchid bees in Costa Rica.

## Methods

The experiments took place in the months March and April of 2016 and 2018 at the La Gamba Tropical Station, Puntarenas, Golfo Dulce region, Costa Rica, which is located adjoining the Parque Nacional Piedras Blancas in the Pacific south of the country. Male orchid bees were caught in the nearby forest using chemical baits and introduced into flexible mesh cages (40 × 40 × 60 cm, bioform, Germany) equipped with sugar-water feeders and cuttings of forest shrubs to provide support for resting bees. Feeders were made from 1.5 ml Eppendorf cups fitted with colored plastic corollas and filled with 40% (volume) sugar water. Bees learned to drink from these feeders and slept with their mandibles attached to the mesh ceiling or the veins of leaves. No sources of volatiles were present in the cages, and we have never observed males to display in cages of that size (TE, pers. obs.). Two sets of experiments were conducted.

### Volatile-application experiment

After they had habituated to the cage situation we supplemented the right tibial pocket of 31 male *Euglossa imperialis* with a mixture containing equal amounts of the following chemicals: 1,4-dimethoxybenzene, 1,8-cineole, methyl salicylate-3*d*, eugenol, benzyl benzoate, nerolidol, hexahydrofarnesyl acetone, phytol, and eicos-9-enyl-1,20-diacetate-6*d*. The first eight compounds are volatiles of a range of molecular weights known from male euglossine perfumes, with four of them (1,8-cineole, methyl salicylate, hexahydrofarnesyl acetone and nerolidol) being present in variable quantity in those of *E. imperialis* (Eltz et al. 1999). The ninth compound, eicos-9-enyl-1,20-diacetate, is the major compound of cephalic labial gland secretions of most species of *Euglossa*, including *Euglossa imperialis* (Williams and Whitten 1983). Its deuterated version was synthesized by W. Francke, Hamburg (see Eltz et al. 2007). The deuterated Methyl salicylate-3*d* was synthesized by TE. Using deuterated compounds allowed us to distinguish the applied compounds from natural variants potentially present in hind tibiae. Hexahydrofarnesyl acetone was synthesized in the

lab of E. Hedenström, Sundsvall (Eltz et al. 2010), while all other compounds were procured from Sigma-Aldrich.

2 µl of the mixture were applied by pipetting droplets to the interface of the right hind tibial pocket using microcapillary tubes (Hirschmann, Germany). The uptake of liquid, which was observed under the stereomicroscope, was occasionally enhanced by gently squeezing the tibial capsule using forceps. This blows out air and results in liquid being sucked into the pocket more quickly. The application of 2 µl took approximately 5–10 min per leg and filled approximately 40% of the pocket volume, a level only rarely attained in wild males (T. Eltz, pers. obs.). After application, the bees were put back into the mesh cages and allowed to groom themselves. We sampled bees randomly from the cages after 1 h, 4 and 12 days. Bees were killed by freezing and individual right and left hindlegs were extracted separately in 0.5 ml of *n*-hexane (p.a., Carl Roth, Karlsruhe, Germany) for subsequent chemical analysis (see below). We also extracted and analyzed contents of left hind legs of the same individuals to (1) evaluate the side-specificity of the application procedure and (2) to assess the ‘natural background’ of compound quantities, i.e. the amounts collected from natural sources before capture.

### Amputation experiment series

This series of experiments was conducted with male *Euglossa championi* and based on comparing the natural contents of right and left hind tibial pockets of individual bees to allow for observation of finer scale changes. In the main experiment, called the “10-day alive” experiment ( $N=45$  bees), we amputated (at femoral–tibial joint) and extracted the right hind leg of bees at the beginning of the experiment, sealed the stump with honeybee wax, and put the males back in the mesh cages. After 10 days the bees were killed by freezing and their left hind legs were also sampled. Single hind legs were extracted in 0.5 ml of *n*-hexane (p.a.) containing 0.1 g/ml of 2-undecanone as an internal standard (ISTD).

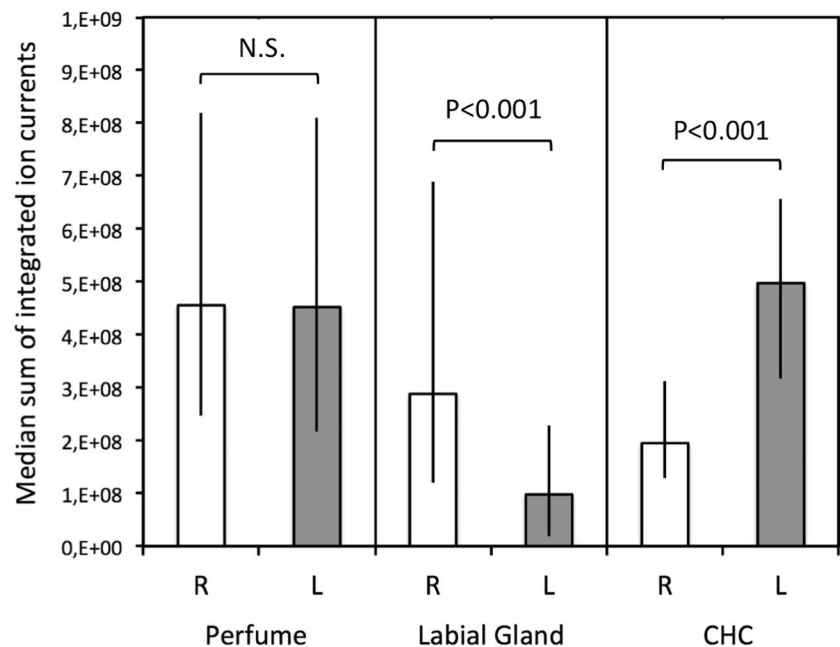
To test the assumption that one hind tibial pocket may serve as a control for observing changes in the other, we also sampled both hind legs of individuals ( $N=17$ ) at the same time, i.e. directly after bait capture. Furthermore, we carried out another control experiment to evaluate whether any changes potentially observed in the main experiment were based on metabolic processes. For this, the bees were killed directly by freezing following bait capture, and the right leg was cut and extracted. The specimens ( $N=17$ ) were then pinned using custom insect pins and left drying in a mesh-covered box placed on the bench of the air-conditioned laboratory. Particular care was taken not to touch the remaining hind leg to avoid contaminations. After 10 days, the remaining left hindleg was cut from the specimen and extracted

as above. All extracts were stored at  $-20\text{ }^{\circ}\text{C}$  previous to chemical analysis in Germany.

## Chemical analysis

Chemical analysis took place in Bochum, Germany. Samples were analyzed using a HP5890II gas chromatograph coupled to a HP5972 mass spectrometer (GC-MS, Hewlett–Packard, Palo Alto, California, USA), equipped with a DB-5MS column (30 m,  $0.25\text{ }\mu\text{m}$  film thickness,  $0.25\text{ mm}$  ID), with a splitless injection of  $1\text{ }\mu\text{l}$  aliquots of raw extracts. The GC oven was programmed from  $60$  to  $300\text{ }^{\circ}\text{C}$  at  $10^{\circ}\text{C}/\text{min}$  followed by  $15\text{ min}$  at  $300\text{ }^{\circ}\text{C}$ . We used manual peak integration targeted at the nine applied compounds (volatile application experiment) or automated integration with peak calling based on standardized integration thresholds (amputation experiment series) using ChemStation Software (Agilent Technologies, Santa Clara, California, USA). Absolute quantities were calculated from integrated ion currents based on analysis of dilution series of reference standards in the volatile application experiment. In the amputation experiment series, we used integrated ion currents (called “peak areas” from here on) directly for further analysis. We inferred compound structure by comparing spectra and retention times (Kovats indices) to entries in commercial spectral libraries or to those of existing user databases (see also Eltz et al. 2005a; Zimmermann et al. 2009). Compounds were grouped in three classes: Exogenous volatiles (= perfume; mostly terpenoids, aromatics and short-chain aldehydes), endogenous labial gland lipids (unsaturated straight chain acetates), and endogenous cuticular hydrocarbons (CHC; alkanes, alkenes and alkadienes), see also Fig. 3.

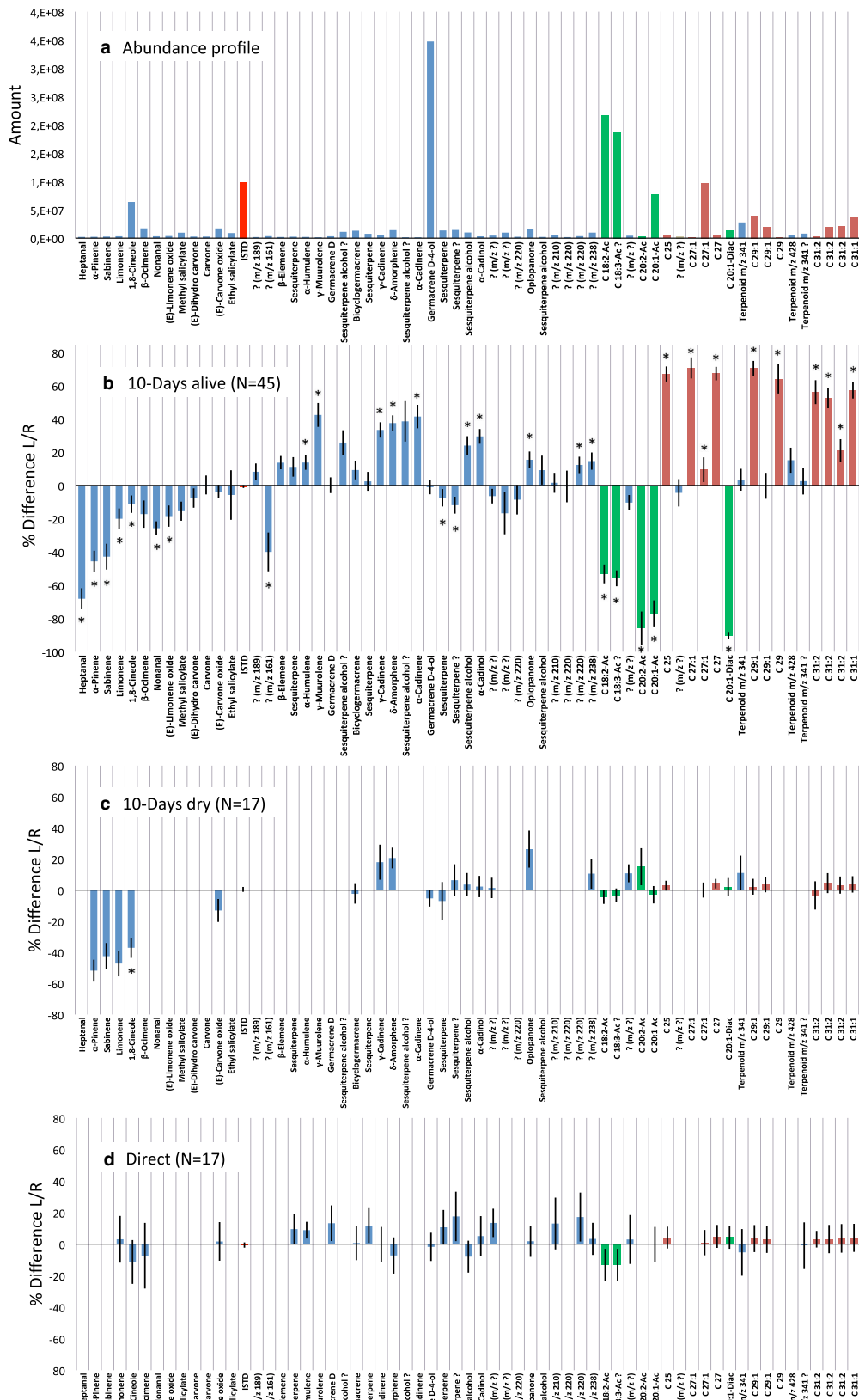
**Fig. 3** Median quantities (sums of peak areas) of three classes of chemicals in right and left hind legs of *Euglossa championi* in the 10-day alive experiment. Right legs were extracted right at the beginning, whereas left legs remained on live bees until after 10 days of captivity. Error bars are quartile ranges.  $p$  values are from Wilcoxon-matched pairs tests (Table 1)



## Statistical analysis

**Volatile application experiment** We tested for effects of time since application on quantities of each of the nine chemicals using Student’s  $T$  test between samples taken 1 h and 4 days, and 1 h and 12 days after application, respectively, with Statistica v12 (Statsoft, Tulsa, Oklahoma, USA).

**Amputation experiment series** All pairwise statistics were done with absolute peak areas of individuals (right and left hind leg) using the Wilcoxon-matched pairs test in Statistica v12. Compounds were analyzed individually (see Fig. 4) or summed within compound class (Fig. 3). For the individual compound analysis, we included only compounds that were detected in both legs of a minimum of five individuals per experiment. To visualize the changes of the entire perfume blend over time in the 10-day alive experiment we conducted a population-level similarity analysis using non-parametric Multidimensional Scaling (n-MDS) and associated techniques in Primer v.6. For this, the peak areas of all exogenous volatiles (perfume) were square-root transformed and standardized to represent % contribution to the sample blend. Bray–Curtis similarities were calculated between all samples and plotted in two dimensions using n-MDS. ANOSIM was used to test whether the body side of the leg ( $R/L$ ) had an influence on the rank order of similarities between samples, also in Primer v.6.



**Fig. 4** Results of GC–MS analysis of hind leg extracts of male *Euglossa championi* in the amputation experiment series; compounds sorted from left to right by increasing GC retention time (DB-5), with column colors indicating compound class (see text): exogenous volatiles (blue), endogenous labial gland lipids (green), endogenous cuticular hydrocarbons (dark red), and internal standard (bright red). **a** Mean quantities (integrated ion currents=peak areas) of compounds in right hindlegs of the 10-day alive experiment ( $N=45$ ). Right hindlegs were sampled at the beginning of the experiment. **b** Percentage change per compound between right and left hind leg in the 10-day alive experiment. Left hindlegs were sampled after 10 days of captivity. **c** Percentage change per compound between right and left hind leg in the 10-day dry experiment. Left hindlegs were sampled after dead (pinned) bees were left drying for 10 days in the laboratory. **d** Percentage difference between right and left legs when both were sampled at the same time directly after capturing the bees. Asterisks represent significant differences between in Wilcoxon-matched pairs tests (see text). Overall  $N$  of bees is given, but may be lower for individual compound comparisons (minimum of 5, see ESM Table 1)

## Results

### Volatile application experiment

The application procedure was successful in delivering the chemical mix almost exclusively to the right hind tibial pocket. This was evidenced by substantial quantities of the two deuterated compounds in right legs 1 h after the application (Fig. 2), whereas left legs contained only traces of methyl salicylate-*3d* ( $<0.2 \mu\text{g}$ ) and no eicos-9-enyl-1,20-diacetate-*6d* at all. In addition, judging from left leg extracts, the average amount of the other (non-deuterated) volatiles acquired from natural sources prior to application varied among compounds: For 1,4-dimethoxybenzene, eugenol and phytol it was very low (traces, all  $<1 \mu\text{g}$ ), whereas nerolidol ( $5.9 \mu\text{g}$ ), and especially hexahydrofarnesyl acetone ( $22.1 \mu\text{g}$ ) and 1,8-cineole ( $29.8 \mu\text{g}$ ) had substantial natural backgrounds. However, these backgrounds were still substantially below the applied quantities, i.e. did not strongly alter the overall variability among samples, and thus presented no obstacle for meaningful comparisons of contents among groups of individuals. Changes in compound quantities since applications are presented in Fig. 2. There were no significant differences in any of the eight volatiles between the 1 h and 4 days groups ( $T$  tests:  $N=8$  and  $11$ ; all non-significant), whereas two compounds were significantly reduced in quantity after 12 days [ $N=8$  and  $9$ ;  $P<0.001$  for 1,4-dimethoxybenzene ( $-66.5\%$ ) and  $P<0.05$  for 1,8-cineole ( $-38.2\%$ )]. Overall, there was a negative correlation between the percentage change between 1 h and 12 days and the molecular weight of compounds (Spearman rank correlation:  $N=8$ ;  $R_s=0.86$ ;  $p<0.01$ ), i.e. greatest losses were significantly associated with small molecular weight.

The deuterated version of the labial gland lipid, eicos-9-enyl-1,20-diacetate-*6d* showed a strong decrease

between 1 h and 4 days, and 1 h and 12 days respectively (both  $p<0.0001$ ), with almost complete loss over 12 days ( $-98.6\%$ , Fig. 2).

### Amputation experiment series

Right and left leg pockets were found to contain very similar quantities of chemicals when sampled directly from bait-captured individuals, suggesting that one body side represents an excellent control for changes happening to the other (Table 1; Fig. 4). There were no significant differences between left and right peak areas when compounds were summed within compound classes in the direct comparison (Table 1), nor were there differences for any single compound (Fig. 4). The predictive value of a peak area on the right body side on its counterpart on the left side was high (Linear regression:  $N=520$  pairs of peak areas;  $R^2=0.91$ ;  $p<0.0001$ ).

In the 10-day alive experiment, there were also no significant differences in the summed peak area of perfume volatile between right and left legs, i.e. the total amount of perfume remained essentially unchanged over 10 days of cage captivity (Fig. 3; Table 1). In contrast, the sum of labial gland lipids decreased and the sum of cuticular hydrocarbons increased significantly (Fig. 3; Table 1). When compounds were analyzed individually, the results partly mirrored those for sums, at least in labial gland lipids (all decreased) and cuticular hydrocarbons (all but one increased). In perfume volatiles, however, there was a mixed pattern: 20 out of 46 perfume volatiles showed significant changes in peak area, with 10 of them decreasing and ten increasing (Fig. 4, see ESM Table 1 for a complete list of compounds including relevant per-compound sample sizes). The strongest relative decreases (up to 70% of mean area) were found in highly volatile compounds such as short-chain aldehydes and cyclic as well acyclic monoterpenes. Other compounds, including several sesquiterpenes, sesquiterpene alcohols and a sesquiterpene ketone (Oplopanone), showed mean relative increases of up to 40% in peak area. The significant changes were almost all confined to minor compounds (see Fig. 4), whereas the most dominant major perfume compound, Germacrene D-4-ol, remained essentially unchanged.

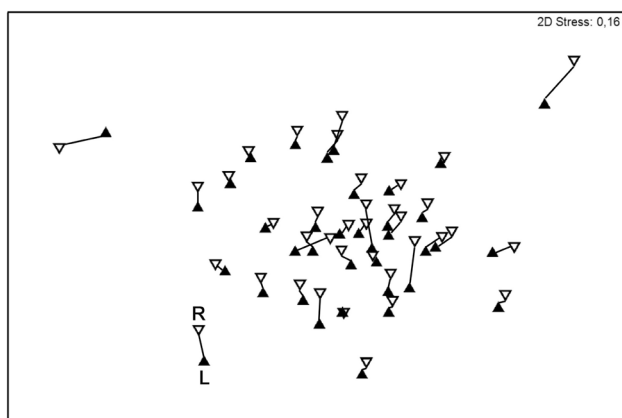
Corresponding trends of peak areas of perfume volatiles were also observed in the dried bees of the 10-day dry experiment ( $N=17$ ), although the changes were mostly not significant (exception: the significant decrease of 1,8-cineole). Here, no changes were observed in any labial gland lipid or cuticular hydrocarbon.

The observed shifts in relative abundances of compounds observed in the 10-day alive experiment did result in consistent shifts in perfume blend composition when plotted in two

**Table 1** Results of Wilcoxon-matched pairs tests (Statistica v12) on the effects of body side (right versus left) on the amounts of chemicals belonging to three different compound classes present in leg pockets of *Euglossa championi* in the amputation experiment series

Experiment/Substance class	<i>N</i>	<i>P</i>
<i>10-Day alive experiment</i>		
Perfume	45	0.38
Labial gland lipids	44	<0.0001
Cuticular hydrocarbons (CHC)	45	<0.0001
Internal standard	45	0.28
<i>10-Day dry experiment</i>		
Perfume	11	0.15
Labial gland lipids	16	0.8
Cuticular hydrocarbons (CHC)	17	0.72
Internal standard	17	0.83
<i>Direct comparison</i>		
Perfume	17	0.43
Labial gland lipids	16	0.44
Cuticular hydrocarbons (CHC)	17	0.69
Internal standard	17	0.55

Results for the 10-Day alive experiment (top) correspond to Fig. 2. *N* Sample size, *P* two-sided error probability



**Fig. 5** Two-dimensional representation (stress=0.16) of differences in chemical composition between tibial perfumes in the 10-day alive experiment plotted by multi-dimensional scaling (MDS). Distances between symbols approximate the overall difference in chemical composition calculated between pairs of samples. Right and left legs of the same individual are connected with lines. Right legs (open triangles) were sampled directly, left legs (filled triangles) after 10 days of captivity. Five outlying individuals were removed to scale-up the graph and improve detail at the core

MDS dimensions (Fig. 5). However, these individual shifts were not strong enough to result in population level (non-paired) differences between right and left legs (ANOSIM:  $N = 45$ ,  $R = -0.015$ ,  $p = 0.95$ ).

## Discussion

The present study has used more powerful techniques for detecting a chemical change in hind tibial pockets over time than previous investigations. Using one body side to control for changes occurring to the other over ten days of captivity, we found consistent changes in quantity in more than half of the compounds naturally occurring in hindlegs of our study species, *E. championi*. With regard to absolute quantity, the most substantial changes occurred in long-chain acetates and diacetates (53–90% reduction), which are known to be the major compounds in cephalic labial gland secretions of *Euglossa* spp. These secretions serve as a carrier for exogenous volatiles during the process of volatile uptake, much similar to the use of lard during ‘enfleurage’ in the traditional perfume industry (Whitten et al. 1989). It has been shown in previous experiments that at least some of these acetates, i.e. C20:1-Ac, C20:1-DIA, C18-Ac, and C20-Ac, were reabsorbed nearly quantitatively from the pockets and relocated back to the labial glands (lipid recycling, Eltz et al. 2007). The present results corroborate these findings and suggest that the pocket boundary is highly permeable for long-chain acetates and diacetates, including homologues with more than one double bond. Furthermore, since it did not occur in pinned (dead) individuals, our experiments provide direct evidence that labial gland lipid removal from the pockets is an active process.

The present study found significant increases over time in almost all long-chain cuticular hydrocarbons (CHC), i.e. alkanes, alkenes and alkadienes of chain lengths between 25 and 33 carbon atoms. The same set of CHCs were also found on the wing surface of male *E. championi* (Pokorny et al. 2015), and are likely present on the entire cuticle surface. Their increase over time in live bees in the present study could be a consequence of damage to the cuticle occurring during the experiment, e.g. in form of the leg amputation (large scale injury) or through abrasion (small-scale scratches) caused by contact with the cage material or co-inhabitants (see Johnson and Gibbs 2004). Both forms of damage may have stimulated CHC synthesis directly or, indirectly, through increased water stress in the affected individuals. Heat and water stress are known to lead to changes in CHC profile composition or overall CHC quantity in other arthropods (Gefen et al. 2015). As with labial gland lipids, the observed changes in cuticular hydrocarbons did not occur in pinned individuals, i.e. were clearly caused by metabolic processes.

With regard to exogenous volatiles our results provided mixed insights. First, both experiments confirm that male euglossine leg pockets are generally good perfume storage containers. In contrast to labial gland lipids and cuticular



hydrocarbons the overall amount of exogenous volatiles in leg pockets in the amputation series experiments did not change over 10 days, neither in live nor dead bees. This is in broad agreement with findings of Eltz et al. (1999) in *E. imperialis*. The generally good volatile retention appears to be mostly based on the intricate pocket morphology, i.e. its narrow opening and the sponge-like structure of its inner surface (Vogel 1966; Eltz 1997). It remains unclear how the pocket wall can be an efficient barrier for containing exogenous perfume volatiles while at the same time being highly permeable for endogenous labial gland lipids, i.e. long-chain acetates (see discussion in Eltz et al. 2007).

Second, it became clear that changes in quantity did occur in a surprisingly large proportion of exogenous volatiles in the 10-day alive experiment, with decreases in monoterpenes and short-chain aldehydes (“losers” in the following) and increases of some sesquiterpenes and their oxygenated derivatives (“winners”). We will first discuss the possible causes of peak area decreases. Based on all evidence, these decreases seem best explained by simple evaporation. In the volatile application experiment, the relative magnitude of decrease in quantity was inversely related to molecular weight. In the 10-day alive experiment, we could not test directly for an effect of molecular weight because it was unknown for several unidentified compounds. However, the relative magnitude of losses was inversely related to GC retention time on DB-5, with early eluting compounds showing the strongest decreases. Since molecular weight and GC retention time are closely linked in compounds sharing the same overall chemistry, this relationship supports evaporation as the prime agent of compound peak area decreases. However, other mechanisms may also have contributed, in particular oxidation. Oxidative reactions, if they occurred, could have at the same time contributed to increasing the amounts of some of the oxygenated winners in the blend, especially sesquiterpene alcohols. However, the involved reaction pathways are not obvious and would have to be disentangled by careful experimentation. Some putative products of oxidation, e.g. heptanoic acid forming from heptanal, may have gone unnoticed in the 10-day alive experiment due to poor power for detection of those compounds by the GC methods used. It should be emphasized that most of the sesquiterpene winners in the 10-day alive experiment are non-oxygenated compounds and can, therefore, not be the products of oxidation.

Unfortunately, we cannot at present offer a straightforward explanation for the relative increases in sesquiterpenes and sesquiterpene alcohols during the 10-day alive experiment. Although the capacity for specialized terpene biosynthesis in insects is limited (but see, e.g., Lancaster et al. 2018), it cannot be excluded that these increases were caused by de novo glandular synthesis in the bees.

This seems unlikely, however, considering the variability of perfume composition in males of the same species of orchid bee. Although there is a certain degree of species specificity in perfume blend composition (Eltz et al. 2005a; Zimmermann et al. 2009), there is also substantial variation in the presence/absence of individual compounds among conspecific males ((Ramírez et al. 2010; Pokorný et al. 2013). No perfume compound, not even the most characteristic major semivolatile, is present in all individuals (e.g. Eltz et al. 1999), as would be expected in the case of de-novo synthesis from simple metabolic precursors. This is also true for the most conspicuous winners among sesquiterpenes in *E. championi*, delta-amorphene and gamma-cadinene, which were present in many but not all individuals at the end of the experiment. As an alternative to de novo synthesis from metabolic precursors, it is conceivable that enzymes in epithelia lining the leg pocket could have catalyzed chemical reactions among terpenoids in the pocket, thus producing shifts from losers to winners. Clearly, careful experimentation involving labeled compounds and freshly hatched (empty) males would be required to clarify this issue.

Regardless of what mechanism has caused the observed changes in perfume blends, it is possible that those shifts convey relevant information in a behavioral context. Assuming that females select mates based on their perfume phenotype, the proportion of winner compounds in the male blend may be an indicator of its age and, thus, of its survival capacity. In the 10-day alive experiment, there was a predictable shift in overall blend composition in male *E. championi* as evidenced by the Multidimensional Scaling analysis. This shift could be informative for a female euglossine sensorium even if it was undetectable for population-level statistics (ANOSIM). ANOSIM is impartial in the weight that it assigns to the different chemical compounds in the blend, whereas females may have a sensory or neural bias in favor of age-indicative compounds.

Generally, our results do not dispute the idea of near-linear, age-dependent accumulation of perfumes over time. Instead, the observed in-pocket shift towards heavier compounds suggests that yet another factor is contributing to perfume dynamics. In addition to being modified by continuing collection and exposure, perfume phenotypes seem also to “ripen”, resulting in old males to sport a stronger base note in their seasoned perfumes.

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