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Enfleurage, lipid recycling and the origin of perfume collection in orchid bees

Thomas Eltz[1,*], Yvonne Zimmermann[1], Jenny Haftmann[2], Robert Twele[2], Wittko Francke[2], J. Javier G. Quezada-Euan[3] and Klaus Lunau[1]

[1]*Department of Neurobiology, University of Düsseldorf, Düsseldorf 40225, Germany*
[2]*Institute of Organic Chemistry, University of Hamburg, Hamburg 20146, Germany*
[3]*Departamento de Apicultura, Universidad Autónoma de Yucatán, Mérida, Yucatán 97100, Mexico*

Enfleurage, the extraction of elusive floral scents with the help of a lipophilic carrier (grease), is widely used in the perfume industry. Male neotropical orchid bees (Euglossini), which accumulate exogenous fragrances as pheromone analogues, use a similar technique. To collect fragrances, the bees apply large amounts of straight-chain lipids to odoriferous surfaces from their cephalic labial glands, which dissolve the volatiles, and the mixture is then transferred to voluminous hind-leg pockets. Here, we show that males do in fact operate a lipid conveyor belt to accumulate and concentrate their perfume. From the hind-leg pockets of caged male *Euglossa viridissima*, deuterated derivatives of carrier lipids were consecutively sequestered, shuttled back to the labial glands and reused on consecutive bouts of fragrance collection. Such lipid cycling is instrumental in creating complex perfume bouquets. Furthermore, we found that labial glands of male orchid bees are strikingly similar to those of scent-marking male bumblebees in terms of size, form and structure. This, and a prominent overlap in secretory products, led us to propose that perfume collection evolved from scent-marking in ancestral corbiculate bees.

Keywords: fragrance collection; scent-marking; labial gland; pheromone analogue; volatile sampling; bumble-bees

1. INTRODUCTION

Odours are an important means of intraspecific communication in bees, especially in the context of mate attraction and pre-mating behaviour. The relevant chemicals are produced by a variety of exocrine glands, from either males or females or from both (Ayasse *et al.* 2001). A common mate-finding strategy in bees is scent-marking, during which male bees deposit pheromonal secretions within territories or along regular flight paths, thereby attracting conspecific males and females (Kullenberg *et al.* 1973; Vinson *et al.* 1982; Bergman & Bergström 1997; Kindl *et al.* 1999). Male neotropical orchid bees (Euglossini: five neotropical genera, more than 200 species) are highly unusual in that they use environmental odours as chemical signals. Males of these bees collect volatile chemicals from flowers, fruits, faeces, decaying wood or tree wounds, which they do not ingest but accumulate outside their body in voluminous leg pockets (Vogel 1966; Dressler 1982; Roubik & Hanson 2004). In their quest for fragrance, male euglossines serve as specific pollinators of a great number of neotropical flowering plants; among them at least 650 species of orchids that exhibit some striking floral specializations (Dodson *et al.* 1969; Williams 1982). Although the exact role of the fragrances in euglossine mating biology remains uncertain, it has recently been shown that males actively expose and ventilate their perfume at territorial perch sites in the forest understorey, where mating occurs (Eltz *et al.* 2005*b*; Zimmermann *et al.* 2006). The stored fragrances of experienced males consist of complex and species-specific blends of terpenoids and aromatics, for the acquisition of which the bees must pay numerous visits to diverse sources (Eltz *et al.* 2005*a*).

The process of fragrance collection involves a series of leg manipulations and morphological as well as physiological adaptations. After landing on a fragrant surface, a male bee uses dense brushes of hair on its fore-tarsi to absorb the volatiles, then hovers up and quickly transfers the substances to cuticular pockets on the hind tibia, which are themselves densely populated with branched hairs and connected to the outside world only by a narrow canal (Vogel 1966; Kimsey 1984). Experiments by Whitten *et al.* (1989) have shown that males facilitate the uptake process through the application of fatty acid-derived secretions from their cephalic labial glands. These lipids, mostly straight-chain hydrocarbons, alcohols, esters, acetates and diacetates, are spat onto the fragrant surface and probably serve to dissolve and retain the volatiles (Whitten *et al.* 1989, 1993). Jointly, lipids and volatiles accumulate in hind-leg cavities where the lipids may represent a substantial proportion of the total content of solutes (Eltz 1997). As the bees may live and collect fragrances for several months, some mechanism should exist to remove the lipids from the leg pockets. Whitten *et al.* (1989) speculated that the labial-gland lipids are selectively taken up through the cuticular lining of the leg pocket and are either catabolized or shuttled back to the labial gland. In the present study, we tested these hypotheses by means of tracer experiments using

* Author for correspondence (eltz@uni-duesseldorf.de).

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deuterium-labelled compounds, which were directly applied to the hind-leg pockets of caged male *Euglossa viridissima*. In the first experiment we investigated whether (9*Z*)-eicosene-1,20-diyl diacetate, a major labial-gland lipid of *Euglossa*, is relocated over time from hind-leg pockets, whether or not it is transported to the labial glands and whether or not it is then reused for fragrance collection. In the second experiment we tested whether the relocation mechanism is selective, that is, whether it would discriminate between specific labial-gland lipids, unspecific lipids and an aromatic fragrance compound. Finally, we conducted a morphological investigation of *E. viridissima* cephalic labial glands which, in combination with data from the literature, inspired new insights regarding the origin of euglossine fragrance collection.

2. MATERIAL AND METHODS

Experimental males of *E. viridissima* were captured at scent baits near the Campus of Biological Sciences of the Autonomous University of Yucatán at Xmatkuil, México, and kept in 60×50×60 cm nylon mesh insectaries placed outside the laboratory, where they learned to forage at honey–water feeders.

(a) *Lipid relocation and reuse*

In the first experiment, 2 µl of deuterated solvent-free (9*Z*)-eicosene-1,20-diyl diacetate-d_6 (C20:1-DIAC-d_6) were applied directly to the hind-leg pockets of 68 individual males of *E. viridissima*. C20:1-DIAC is the most abundant compound in the cephalic labial-gland secretion of this and most other species of *Euglossa* (Williams & Whitten 1983). The liquid was deposited by microcapillary tubes onto a hair-filled groove surrounding the opening of the pocket (1 µl on each body side). As confirmed visually under a stereo microscope, the liquid was passively drawn inside within minutes. Occasionally, the tibiae were gently squeezed with forceps to increase the speed of uptake. Males were then either sampled directly for chemical analysis or put back into the cages for variable amounts of time (up to 13 days). Additionally, eight males were extracted without treatment ('before' in figure 1*a*). Individual heads (with labial gland), hind legs (with perfume pocket) and 'rest bodies' (thorax with fore- and middle legs, abdomen) were extracted separately in 0.5 ml *n*-hexane. The heads of 12 of the treated males (one sampled on each day of the experiment except the first) were dissected and the cephalic labial glands extracted separately from the rest of the head. During the experiment, males had no access to fragrance sources. Only on days 1, 2, 5, 7, 9 and 11 after the treatment, we briefly exposed a fragrant filter paper. After males had collected on the filter paper for several minutes, 4×4 cm pieces of paper were extracted in hexane.

(b) *Selectivity of the recycling process*

In the second experiment we applied to the hind legs of the males 2 µl of a mixture of six components: (9*Z*)-eicosene-1,20-diyl diacetate-d_6 (C20:1-DIAC-d_6); (11*Z*)-eicosene-1-yl acetate-d_3 (C20:1-AC-d_3); octadec-1-yl acetate-d_3 (C18-AC-d_3); eicos-1-yl acetate-d_3 (C20-AC-d_3); (9*Z*)-tricosene (C23:1); and methyl salicylate-d_3. Of those, the first four are deuterated derivatives of compounds naturally occurring in *E. viridissima* labial glands (figure 1*c*). (9*Z*)-Tricosene is a straight-chain hydrocarbon *not* naturally present in male *E. viridissima*, and methyl salicylate-d_3 is an aromatic compound attractive to many perfume-seeking *Euglossa* (Roubik & Hanson 2004), including *E. viridissima* (Skov & Wiley 2005). Individual males were sampled either 1 hour ($N=15$) or 3 days ($N=15$) after the treatment and processed as explained earlier.

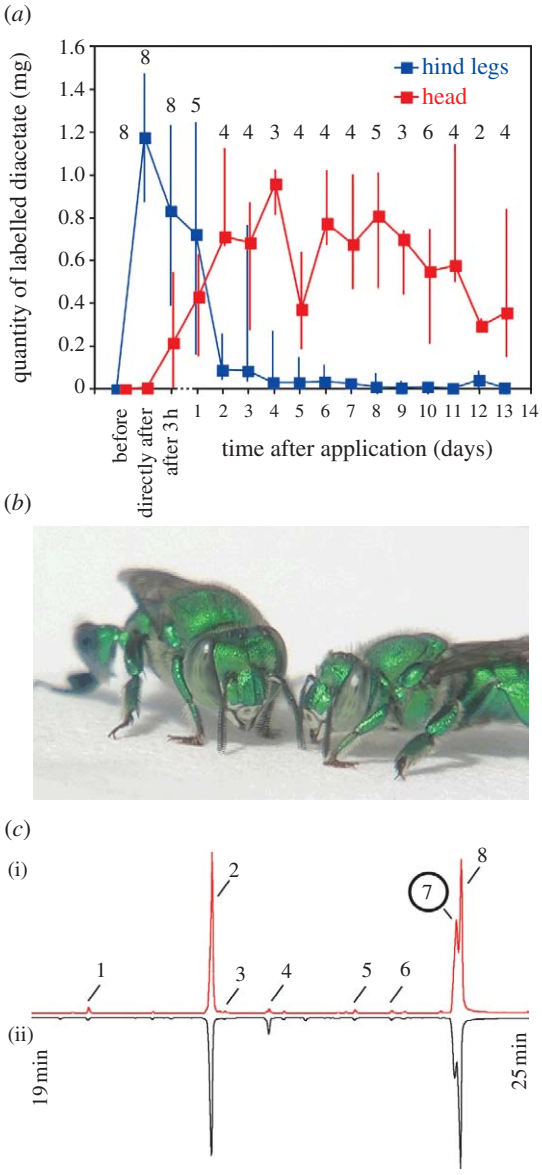

Figure 1. (*a*) Consecutive relocation of (9*Z*)-eicosene-1, 20-diyl diacetate-d_6 (C20: 1-DIAC-d_6) from hind legs to heads of *E. viridissima* following artificial delivery to the hind-leg pockets (median and data range; sample size). (*b*) Males collecting volatiles at filter paper with brushes on forelegs. Note half-open mandibles allowing deposition of labial-gland secretion (see also movie in the electronic supplementary material). (*c*) Overlaid total ion chromatograms of extracts of (i) an individual labial gland and (ii) a filter paper on which males had collected on day 9 of the experiment. Note the peak of deuterated C20: 1-DIAC-d_6 (7) on filter paper. 1, octadec-1-yl acetate (C18-AC); 2, (11*Z*)-eicosene-1-yl acetate (C20:1-AC); 3, eicos-1-yl acetate (C20-AC); 4, pentacosene (C25:1); 5, octacosa-1,18-diyl diacetate (C18-DIAC); 6, heptacosene (C27:1); 8, natural (9*Z*)-eicosene-1,20-diyl diacetate (C20:1-DIAC).

(c) *Deuterated compounds*

Deuterated derivatives of labial-gland acetates were synthesized starting from the corresponding long-chain alcohol

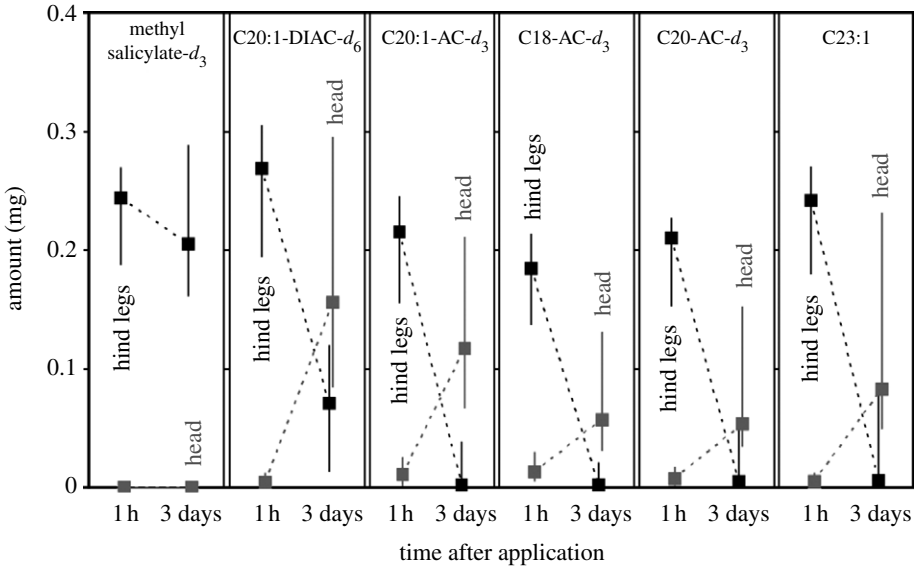

Figure 2. Selective relocation of compounds from hind legs to heads following artificial delivery to the hind-leg pockets. All five straight-chain lipids, including the non-specific (9*Z*)-tricosene (C23:1), significantly decreased in hind leg and increased in head extracts from 1 hour to 3 days after the treatment ($p<0.0001$ for all comparisons; Mann–Whitney *U*-test). In sharp contrast methyl salicylate-d_3 decreased only slightly ($p<0.05$) in hind-leg extracts and was not detected in head extracts after 3 days. Median and data ranges are shown; $N=15$ for each point in time.

and acetic anhydride-d_6, resulting in compounds that are 3 or 6 Da heavier than the natural compound. Deuterated methyl salicylate-d_3 was synthesized from salicylic acid and methanol-d_3.

(d) *Chemical analysis*

Gas chromatography/mass spectrometry (GC/MS) was performed on a HP 5890 II GC fitted with a 30 m DB-5 column and a HP 5972 mass selective detector. Injection was splitless and the oven was programmed from 60 to 300°C at 10°C min^{-1}. Quantification was done using external standards. In cases where the peaks of deuterated and natural derivatives were not sufficiently separated to allow individual peak integration, we integrated both peaks together and calculated the area of the deuterated derivative based on relative abundance of the heavy ion that represents protonated acetic acid originating from the acetate moiety *m*/*z* 64 (in relation to *m*/*z* 61). Tests with artificial mixtures demonstrated unequivocally that this procedure is reliable.

(e) *Microscopy*

Whole heads of male *E. viridissima* captured at fragrance baits were fixed in glutaraldehyde, postfixed in OsO_4 and embedded in epoxy resin. Semi-thin sections (0.5 μm) were stained with borax/toluidine blue. Ultra-thin sections were stained according to Reynolds and examined with a Zeiss transmission electron microscope (TEM).

3. RESULTS

(a) *Lipid relocation and reuse*

The amount of C20:1-DIAC-d_6 extracted from hind legs decreased dramatically over the first days after the treatment until, after day 4, it had dropped below 3% of the applied mass (figure 1*a*). Conversely, the amount of C20:1-DIAC-d_6 in heads increased until day 4, when it had reached a maximum of 80% of the mass applied to hind legs. Chemical analysis of dissected labial glands ($N=12$) confirmed that a large proportion of the labelled C20:1-DIAC-d_6 present in heads was located in the labial glands (figure 1*c*). GC/MS of all extracts of filter papers on which males had collected in the course of the experiment revealed lipid profiles very similar to those present in labial-gland extracts, including the deuterated C20:1-DIAC-d_6 (figure 1*c*).

(b) *Selectivity of the recycling process*

All five straight-chain lipids, including the unspecific (9*Z*)-tricosene, were relocated to heads over the 3 days of the experiment, albeit in different amounts (27 to 56% of the mass applied to the leg pockets). By sharp contrast, methyl salicylate-d_3 showed only minimal decrease in hind legs and was not detected in heads at all 3 days after the treatment (figure 2).

(c) *The labial gland*

Cephalic labial glands of male *E. viridissima* are large glands of grape-like shape with two lobes in each horizontal half of the head, filling much of the space around the eye and brain (figure 3*a–c*). The conglomerate of globular acini is very fragile and dissection often leads to visible spilling of an oily liquid. The structure of apical and basal regions of the secretory cells is shown in figure 3*d*,*e*. In its extraordinary size, bilobally paired form, structure and ultrastructure, the gland is very similar to cephalic labial glands of male bumble-bees (*Bombus* spp.), which have previously been studied in detail by Ågren *et al.* (1979).

4. DISCUSSION

Our results demonstrate that male *E. viridissima* relocate carrier lipids from their leg pockets, shuttle them back to the labial glands and reuse them on subsequent bouts of fragrance collection. While this is a highly economic way of using the carrier secretion, it also leads to an ever-increasing concentration of exogenous volatiles in the leg pockets. It enables males to use large amounts of carrier lipids to capture efficiently tiny quantities of volatiles, and

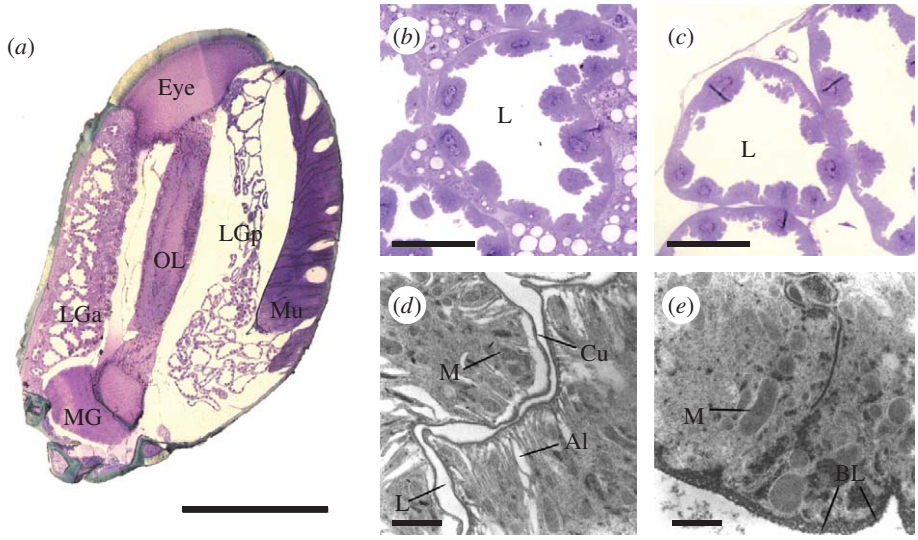

Figure 3. Position in the head and structure of the cephalic labial gland of a mature male *E. viridissima*. (*a*) Sagital semi-thin section of head. The gland fills much of the space around the brain and is divided into an anterior part under the malar area (LGa) and a posterior part (LGp) behind the eyes and optical lobe (OL). Both (*b*) LGa and (*c*) LGp consist of globular acini (60–200 μm in diameter) formed by a single layer of glandular cells, surrounded by fat body cells in the case of (*b*) LGa. Ultrastructure of (*d*) apical and (*e*) basal regions of a glandular cell showing high density of mitochondria (M) and infoldings of the apical cell membrane (AI), indicative of trans-epithelial transport activity. Apically, the cells are lined by a thin cuticle (Cu) that encloses the secretory lumen (L) of the gland, which opens at the base of the proboscis. BL, basal lamina; MG, mandibular gland; Mu, muscle. Scale bars, (*a*) 1 mm, (*b*,*c*) 50 μm and (*d*,*e*) 1 μm.

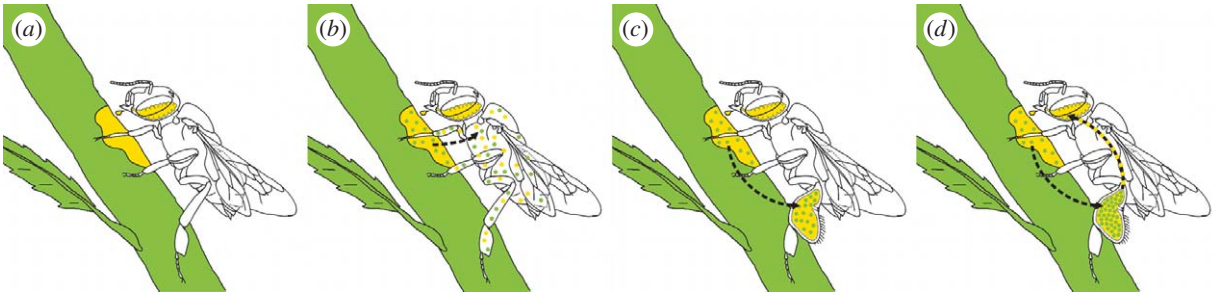

Figure 4. Hypothesized evolutionary transition from scent-marking to active fragrance collection in male orchid bees. (*a*) Ancestral male scent-marking a twig with lipoid labial-gland secretion (yellow). (*b*) Ancestral male after intimate contact with marked plant surface, with plant-derived volatiles (green spots) adsorbed on cuticle (retained in labial-gland lipids). (*c*) Active collection of plant-derived volatiles along with labial-gland lipids in specialized hind-leg pockets. (*d*) Enrichment of plant-derived volatiles in leg pockets by labial-gland lipid relocation and reuse.

to do so repeatedly from varied sources. Thus, lipid cycling is essential in creating the complex perfume bouquets which males emit at display sites (Eltz *et al.* 2005*b*).

In the haemolymph, the lipids are probably shuttled in association with lipoproteins (lipophorin), a common mechanism for lipid transport in insects (Blacklock & Ryan 1994). The observed relocation of (9*Z*)-tricosene, which is not naturally present in *E. viridissima*, is consistent with such a generalized transport mechanism. The mechanism responsible for selective removal of straight-chain lipids from the cuticular pockets is unknown. Both physical and biological filters are likely to contribute to the perfect separation of straight-chain lipids from volatiles. First, the cuticle of the leg pocket may represent a barrier for some of the most hydrophilic fragrance compounds, e.g. those with oxygen-containing functional groups. However, many fragrance compounds do not contain oxygen (or any electrophilic group) and seem unlikely to be separable from lipids on the grounds of polarity alone. We suspect that straight-chain compounds are selectively loaded onto lipophorin by membrane-associated processes in the epidermis. In this context, it should be emphasized that male euglossines are clearly outstanding in their capacity to seclude hazardous chemicals in their leg pockets, including some with high cuticle permeability, e.g. dichloro diphenyl trichloroethane (DDT) (Roberts *et al.* 1982). The euglossine fragrance leg pocket as a chemical filter clearly deserves further study.

The reuse of labial-gland lipids for perfume accumulation is just one of a series of adaptations that male euglossines have undergone for the acquisition of exogenous volatiles (Vogel 1966; Roubik & Hanson 2004). Among these, the application of labial-gland lipids to plant surfaces was not necessarily a novel trait of male orchid bees. Males of extant bumble-bees (Bombini spp.) deposit cephalic labial-gland secretions on plant parts (e.g. bark on tree trunks, branches, twigs, leaves, trampled grass; Kullenberg *et al.* 1973) along flight circuits on which males patrol, as originally described by Newman and Darwin (Newman 1851; Darwin 1886). The secretion is

smeared onto the objects with the help of mandibles, proboscis and the underside of the body (Kullenberg *et al.* 1973), and its odour attracts other males and presumably virgin females (Free 1987). Along with stingless bees (Meliponini) and honeybees (Apini), bumble-bees (Bombini) are the closest extant relatives of orchid bees (Euglossini), all four tribes forming the monophyletic clade of the corbiculate bees (Apinae, see below). Of those, males only of Euglossini and Bombini have well-developed cephalic labial glands (Duffield *et al.* 1984; Cruz-Landim *et al.* 2005), and the respective glands are similar in size, general shape and structure (figure 2; Ågren *et al.* 1979), indicative of a common origin. The chemistry of labial-gland contents of male bumble-bees has been studied intensively, covering a broad range of species from various localities (Bergström *et al.* 1981; Free 1987; Bergman & Bergström 1997; Kindl *et al.* 1999). Marking secretions have been found to be relatively simple blends consisting of compounds belonging to two distinct classes of chemicals: fatty acid-derived lipids occur in almost all species and are dominated by straight-chain hydrocarbons, alcohols, ethyl esters and acetates with similar or identical chain length as those produced by euglossine labial glands (Williams & Whitten 1983). In addition, some bumble-bee species produce terpenoids such as all-*trans*-farnesol or 2,3-dihydro-6-*trans*-farnesol (Bergström *et al.* 1981; Bergman & Bergström 1997). Terpenoids are conspicuously absent in labial-gland extracts of male euglossines (this study; Williams & Whitten 1983; Whitten *et al.* 1989). We propose that a common ancestor of bumble-bees and orchid bees used fatty acid-derived labial-gland secretions to scent-mark plant parts, and that active perfume collection in male orchid bees was preceded by accidental adsorption of plant secondary metabolites from the marked plant. Such involuntary contamination would have been aided by the marking lipids acting as solvent and carrier for plant volatiles, and by the intimate contact of males with the marked surface. If the smell of plant compounds conveyed mating advantage to those males (e.g. owing to preference for plant odours in choosy females, see Lunau (1992) and Roubik & Hanson (2004)), then it could have initiated the evolution of active perfume collection (figure 4). Presumably, the plant parts scent-marked (and sampled) by the hypothetical ancestor were stems or leaves, which are either inherently odoriferous or can be induced to secrete odoriferous exudates (sap, resin) by mechanical damage and/or microbial infection. Resin and exudates from tree wounds are fragrance sources of extant male orchid bees (Dressler 1982; Whitten *et al.* 1993).

Males of all species and genera of orchid bees possess enlarged hind tibiae with perfume pockets, suggesting that active volatile collection evolved only once and basally in the Euglossini. The dating of this event is uncertain, partly owing to the controversy concerning phylogenetic relationships among corbiculate bee tribes (Schultz *et al.* 1999). All recent phylogenies using morphological or behavioural characters, as well as simultaneous analyses of multiple datasets, support the tree originally proposed by C. D. Michener (1944): (Euglossini + (Bombini + (Apini + Meliponini))) (Ascher *et al.* 2001 and references therein). Taking into account biogeographic and corbiculate bee fossils (Engel 2001), active perfume collection originated at some point between the Late Cretaceous (80 Myr BP) and the Middle Miocene (45 Myr BP). The evolution of perfume collection from scent-marking is consistent with such an early origin because it is based on non-floral substrates (resin, sap) that undoubtedly existed at this time. It further fuels the view that euglossophilous orchids, being well-studied fragrance sources of extant Euglossini, have capitalized on a pre-existing behaviour (Dressler 1968; Ackerman 1983) and, although being highly specialized for euglossine pollination may have had little effect on the evolution of euglossine bees.

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