ORIGINAL PAPER

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Juggling with volatiles: exposure of perfumes by displaying male orchid bees

Abstract Male orchid bees (Euglossini) pollinate 10% of the neotropical orchid flora while collecting floral scents, which they store and accumulate in hind tibial pouches. The purpose of these fragrances is unclear, as is the context, timing and mechanism of their possible exposure. Here we show for the first time that males expose and relocate their fragrances during courtship display. We present high-speed video analyses revealing an intricate and repetitive leg movement performed by displaying male *Euglossa cognata*. The behavior involves several morphological structures of hitherto unknown function and suggests transfer of substances from the hind tibia to a contralateral mid-tibial tuft of hairs. Body-side-specific fluorescent dye application and consecutive detection of signals on males after display confirmed this transfer. Deposited on the mid-tibial tufts, the fragrances are ideally placed in order to become ventilated by jugal combs on the wing bases, as previously suggested by Bembé (in Apidologie 35:288–291, 2004). Being clearly distinct from motor patterns involved in fragrance collection, the described movement is continuously performed by displaying males, suggesting an equally continuous exposure of volatiles. Although the findings strengthen, the view that the volatiles serve as attractants in the context of mating behavior, the signal addressee, conspecific males or females, has yet to be found.

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Introduction

Male orchid bees (Euglossini; five neotropical genera, 200+ species) are well known for their habit of collecting volatile chemicals (fragrances) from flowers and other fragrant sources in their habitat (Vogel 1966; Dodson et al. 1969; Janzen et al. 1982; Roberts et al. 1982; Ackerman 1989; Whitten et al. 1993; Roubik and Hanson 2004). The behavior has evolved more than 20 million years ago (Engel 1999; Cameron 2004) and given rise to an entire pollination syndrome, which is based on specific long-range attraction of male Euglossini and comprises of more than 700 species of orchids and many other neotropical plants (Janzen 1971; Williams and Dodson 1972; Dressler 1982; Williams 1982; Ackerman 1983). The collected fragrance compounds, mostly terpenoids and aromatics, are stored in cuticular pouches formed by the male bees' enlarged hind tibiae, where complex and perhaps specific blends accumulate (Eltz et al. 1999). The process of fragrance collection itself involves a broad range of biochemical, morphological and behavioral adaptations (Vogel 1966; 1984; Whitten et al. 1989): First, the bee lands on the fragrant surface and applies to it droplets of lipids secreted by its labial glands in which the nonpolar volatiles are dissolved and retained, analogous to the greasy extraction ('enfleurage') used in the perfume industry. Then the bee uses setal brushes on its fore-tarsi to absorb the mixture, hovers up, and quickly transfers the liquid to the hind tibiae by squeezing the fore-tarsal brushes through specialized combs on the mid basitarsi. Once deposited on a hair-filled groove on the hind tibiae, the liquid is drawn inside the cuticular pouches by what seem to be capillary forces.

The role of the collected chemicals in the male bees' lives and the ultimate causes of euglossine fragrance

collection were subject to much speculation but have remained a mystery. Proposed functions include exposure of fragrances during courtship for short- or long-range attraction of females (Vogel 1966; Schemske and Lande 1984), exposure for attraction of conspecific males in order to form leks (Dodson 1975), sequestration and metabolisation of fragrances as precursors of sex pheromones (Williams and Whitten 1983), chemical nuptial gifts to females which may use them for impregnating nests against microbial enemies, and use as an antipredator defense by the males themselves (Roubik 1989). None of these hypotheses has received direct observational or experimental support.

This lack of stringent testing is due to difficulties with observing euglossine mating behavior. Males of some species are known to perch on stems or branches in the vicinity of forest clearings, where matings are rarely but occasionally observed (Kimsey 1980; Stern 1991; Eltz et al. 2003). At these perch sites, the males perform a characteristic display behavior which involves lengthy series of hovering flights (genus *Euglossa*) or series of wing buzzes (genus *Eulaema*) interchangeably with erect standing on the perch. Depending on species and/or situation the males either perch alone or in interaction with a few to many conspecific males (Peruquetti 2000).

A key prerequisite to an understanding of euglossine fragrance biology is to know whether, when and how chemical substances are actually exposed from male hind tibiae during displaying. Here, we present the results of a detailed investigation of the display behavior of caged male *Euglossa cognata*. Using high-speed videography, we revealed a repetitive sequence of stereotype leg movements, which involves a set of morphological features with hitherto unknown function and which is clearly distinct from movements performed during fragrance collection. Fluorescent dye experiments demonstrate that this movement effects the relocation of substances from the hind tibial pouches and suggest that fragrances are continuously exposed during display bouts.

Methods

Adult males of *E. cognata* were captured at *p*-dimethoxy benzene baits along Pipeline Road (Parque Nacional de Soberania) in Central Panama in December 2003 and April 2004. For 3–7 days, the bees were kept in 50×50×60 cm insectaria at the Smithsonian Tropical Research Institutes' (STRI) Gamboa laboratory and were trained to feed from feeders holding a 40% honey–water solution.

High-speed videography

On 21 December 2003, 20 males were transferred to a 3.5×2.5×2 m mosquito mesh flight cage set up in a growing house at the University of Düsseldorf Botanical Gardens. The cage was fitted with a set of potential display perches (seven small potted woody plants of different species) as well as honey–water feeders. Supplemental to daylight the cage was artificially illuminated from 08:00 hours to 19:00 hours by two 500 W true-light reflectors. Air temperature varied between 24°C and 32°C, humidity varied between 70% and 90%. Eight males survived and were marked individually with enamel paint dots on the second tergite. Video recordings of display behavior were made from 2–7 January using a Redlake MotionPro 500 high-speed digital imaging camera connected to a desktop PC, which can record up to 500 frames per second. However, despite of additional lighting from two 2,500 W reflectors, limitation of light restricted recordings to 60, 100 or 200 frames per second, which corresponded to exposure times of 1/480, 1/800, and 1/1,600 s per frame. Image resolution was set to 1,280×1,024 pixels. The camera was placed on a tripod and fitted with an Olympus 80–200 mm zoom lens in combination with a range of extenders, allowing for almost format-filling shots from relatively long distances (∼60–100 cm). Sequences were saved as uncompressed AVI files of variable length for subsequent single-frame analysis.

Scanning electron microscopy (SEM)

For SEM, we used dried, frozen or hexane extracted specimens of *E. cognata* from the same locality in Panama. Legs were transferred to EtOH (70–98%) and acetone (99%), and finally subjected to critical point drying. SEM was conducted with a LEO 1430 VP.

Fluorescent dye experiment

In April 2004, the contents of the *right* hind tibial pouches of 25 males were artificially supplemented with a saturated solution of rhodamine B (Kremer, Aichstetten, Germany) in methyl salicylate. Rhodamine B is a fluorochrome that absorbs light in the UV and visible spectrum and emits strong red fluorescence (550–650 nm; Du et al. 1998). Methyl salicylate is a component of orchid floral scents and potent attractant of *E. cognata* and other Euglossini. It is known to occur naturally in tibial pouches of *E. cognata* (Eltz et al. 1999). Droplets of 0.25 µl of solution were applied to the distal part of the hair-filled tibial groove that forms the interface to the fragrance pouch using microcapillary tubes. Confirmed visually under a stereomicroscope, 0.25–2.5 µl of liquid were passively drawn into the pouch within minutes. Occasionally, the tibiae were gently squeezed with forceps in order to increase the speed of flow. After treatment, 15 males were introduced into a 2.2×2.0×2.0 m mosquito mesh flight cage placed in a half-shaded out-door site near the STRI laboratory in Gamboa, Panama, and fitted with display perches and

feeders. After 2–15 days later, the surviving nine males were captured after lengthy and undisturbed sequences of display behavior (5–15 min of perching/hovering, including many leg crossings; see Results), immediately immobilized on ice, placed in clean vials, and finally killed by freezing. As a non-displaying control, ten treated males were introduced into a small (50×50×60 cm) insectary and given the opportunity to fly, drink, rest, and groom. Limited space and lack of suitable perch sites precluded display. After 24 h, seven surviving males were immobilized and killed in the same manner as the displaying males.

For visualizing and quantifying rhodamine B residues on the bee exoskeleton, all males were pinned, mounted, and photographed in a special box, which was illuminated internally with two 4 W UV lamps (Lema, USA, DL-228) and connected to a Nikon Coolpix 4,500 digital camera. Photographs of dorsal views of whole bees were taken twice for each specimen (with opposite orientation). Secondly, photographs were taken from the inside of detached left and right hind legs. To reduce the effect of short-wavelength cuticular autofluorescence, we used a cut-off filter at 515 nm. For quantifying and comparing the 'redness' of tufts on left versus right mid tibiae, we analyzed 75×75 pixel squares cropped from the original RGB images, each covering the tuft plus its immediate surroundings. For these, we calculated a simple relative index of redness, $R = r/l$, with r representing the mean red value of all pixels (red channel in the RGB histogram), and l representing the mean total brightness of the same pixels (luminance in the RGB histogram). High values of R indicate a high proportion of red in the spectrum reflected by the photographed structure. It should be noted that this relative index is bound to underestimate left/right differences based on unequal rhodamine B distribution, because it ignores rhodamine-based absolute differences in brightness.

Results

Male perching and display

Four of eight surviving males were repeatedly observed to perch on cage vegetation in a way that was broadly similar to that described for wild *Euglossa imperialis* (Kimsey 1980) and caged *Euglossa hemichlora* (Eltz et al. 2003). Males perched on stems, branches as well as on the edges of leaves of treelets present in the cage, with a clear preference for an individual of *Coffea arabica* with a diameter at breast height of 0.8 cm. Here, the males performed a characteristic display: series of brief hovering flights (1–3 s) alternated with 'standing' on the perch. When standing, the mid legs were held close to the thorax and did not touch the perch; the wings were folded on top of the abdomen. In case that a given male remained undisturbed it sometimes continued displaying at the same perch site for up to 15 min before flying to the feeders, performing up to 8 hovering flights per minute.
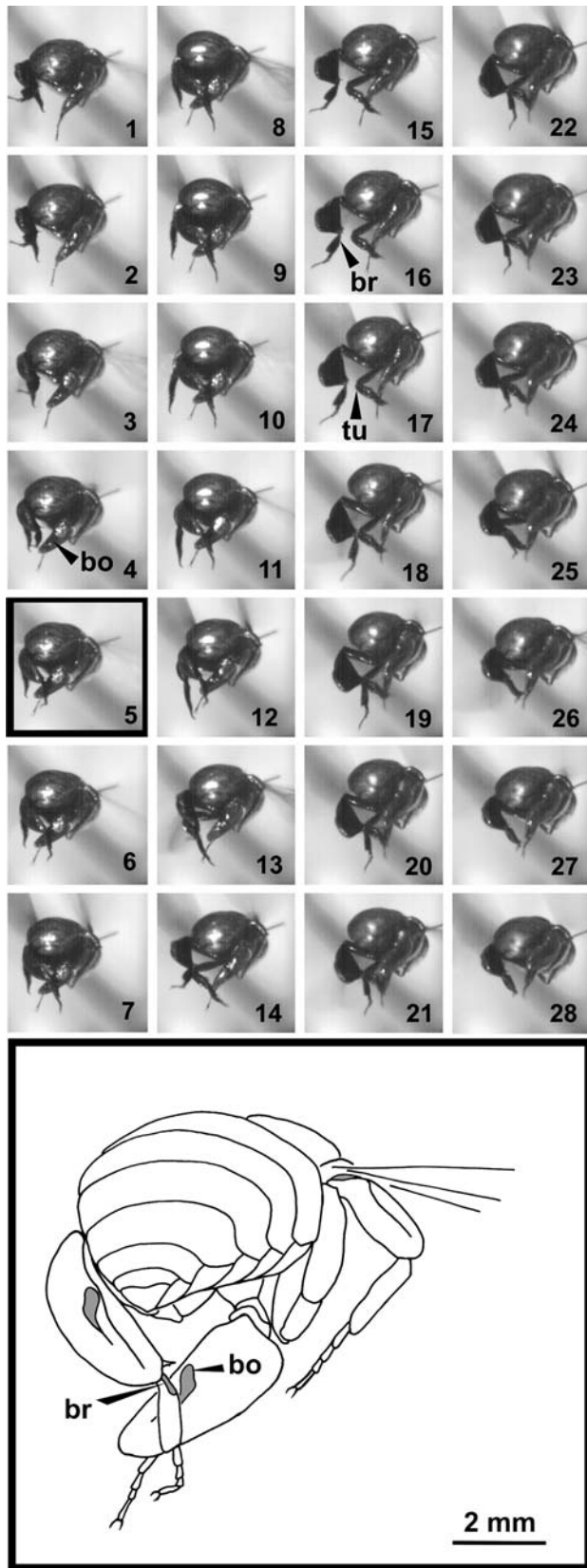
Leg movements during hovering flights

During approximately 50% of the hovering flights of displaying males (and only then), we observed rapid leg movements that were subsequently recorded using the high-speed digital imaging system. Single-frame analysis of recordings of 58 leg movements revealed highly stereotype details, which are best visualized by viewing the GIF animations provided as Electronic Supplementary Material in the online version (S1-4). Shortly after take-off from the perch the male swings forward one of its hind legs (the right one on Fig. 1, frames 1–4) underneath the abdomen. When it has reached its maximal position, the dorsal groove of the hind tibia comes to a rest almost directly underneath the centre of the abdomen, pointing distally away from the bee (Fig. 1, frame 4). Now, the ventral side of the second hind leg (the left one on Fig. 1) is moved against it in a way that leads to an almost rectangular crossing of the two hind legs (Fig. 1, frames 5–11). During this crossing the hind legs physically touch each other for an average of 58 ms (range: 17–200 ms; $N = 55$). Intimate contact is established between the dorsal groove of the first hind leg's tibia (right leg in Fig. 1) and the basal inner side of the second hind leg's basitarsus (left leg in Fig. 1). Although the morphological structures themselves are not clearly discernable on the video frames, the two areas of contact correspond exactly to the positions of (1) the 'bowl', the more dorsal part of the hair-filled groove on the hind tibia ('Napf' sensu, Vogel 1966; 'bo' on Figs. 1 and 3a), and (2) to a well defined ridge and dense brush of fine hairs on the basal end of the hind basitarsus('br' on Figs. 1 and 3b). After the contact between the hind legs is suspended, the mid leg which is ipsilateral to the second hind leg (the left on Fig. 1) swings backwards between the two hind legs until its tibia touches the same very area of the second hind legs' basitarsus, that was in contact with the first hind legs' tibial groove one tenth of a second earlier (Fig. 1, frames 14–18). The area of the mid tibia that contacts the hind basitarsus corresponds exactly to the position of the 'mid-tibial tuft' (sensu Dressler 1978; 'tu' in Fig. 2 and 3b), a conspicuous patch of branched hairs. Following this contact all legs return to their normal in-flight position.

One complete sequence of the described movements took an average of 386 ms (range: 317–833 ms; $n = 47$). Sometimes ($n = 7$) two or three complete sequences were performed in a row (involving alternately opposite sets of legs), or incomplete behaviors which lacked the mid leg movement ($n = 7$). After completion of the movements the mid-tibial tufts came to rest directly underneath the wing bases (Fig. 2).

The sequences that are shown in Figs. 1 and 2 correspond to those of the GIF animations S1 and S2 in the
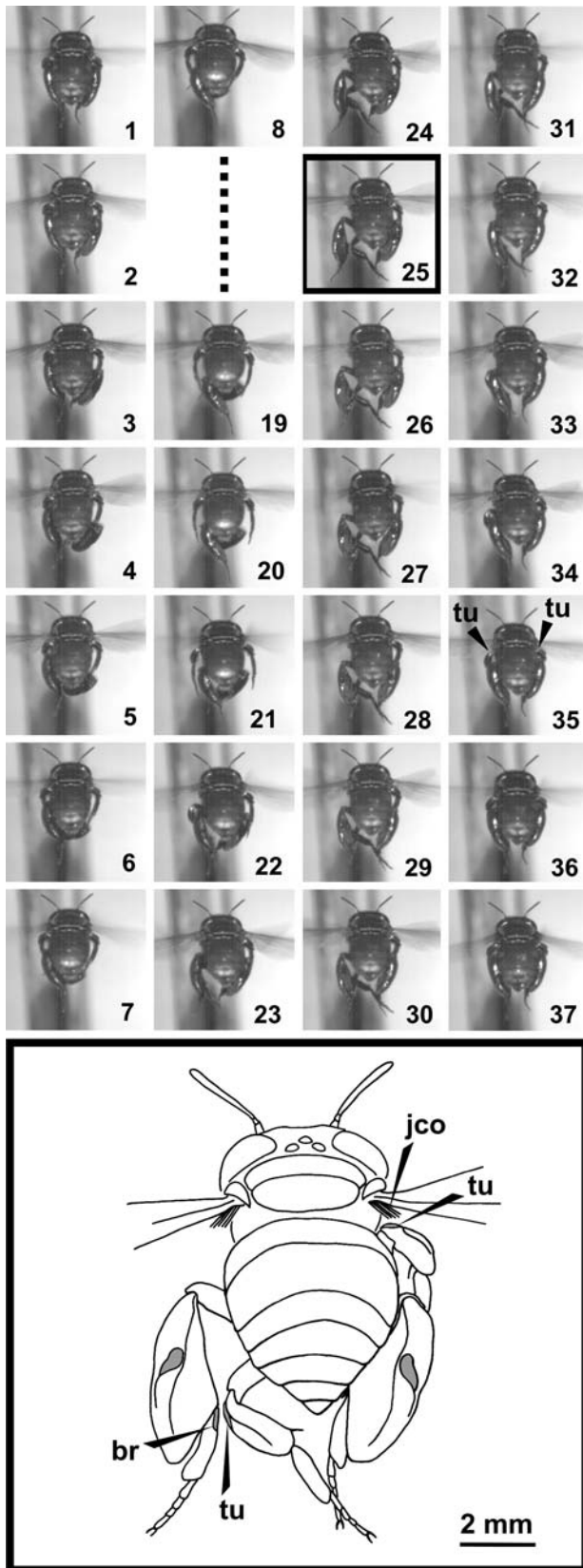
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Fig. 1 Single-frame video sequence of leg movements occurring during the hovering flight of a displaying male *Euglossa cognata*, captured at 100 frames per second (*top*), and schematic view of frame no. 5 of the same sequence, captured briefly before physical contact of the two hind legs (*bottom*). *Arrows* and *grey shadings* indicate the positions of morphological structures making contact during the movement. Legend: *bo* hind tibial 'bowl' (interface between fragrance container and outside world); *br* hind basitarsal 'brush'; *tu* mid-tibial 'tuft'. Further details in the text. The same sequence can be viewed as GIF animation in the online version (S1)

Fluorescent dye experiment

Visualization of rhodamine B fluorescence confirmed a transfer of substances from the hind tibial surface to the contralateral mid-tibia. Figure 4 shows a specimen that was treated with 2.5 µl of rhodamine B solution on its right hind tibia. After 2 days of repeated display, it showed clear red fluorescent signals on the right hind tibia (groove), on the left basitarsal brush, and on and around the left mid-tibial tuft. Clearly visible signals on left mid-tibial tufts were observed in six out of nine displaying males, but not in any of the control males. Quantitatively, left mid-tibial tufts of displaying males were redder than right mid-tibial tufts (T-test for paired samples: $n = 9$; $t = 2.64$; $P < 0.03$). There was no corresponding difference or even trend in control males ($N = 7$; $T = 0.74$; $P = 0.48$).

Discussion

High-speed videography has revealed an intricate and highly stereotype sequence of leg movements taking place during hovering flights of displaying male *E. cognata*. We suggest that the described motor pattern is the long-sought mechanism of and final proof for perfume exposure in displaying male orchid bees. Confirmed by fluorescent dye marks, the specific quality and the dynamics of the movement indicates transfer of substances from the hind tibial groove (the interface of the fragrance container with the outside world) to the surface of the contralateral mid tibia, with a brief stop-over on the contralateral basitarsus. The basitarsal brush as well as the mid-tibial tufts so far lacked a conclusive functional explanation, although both are generally present in male but not in female *Euglossa* (Dressler 1982; Roubik and Hanson 2004; T. Eltz, personal observation, see below). Especially the mid-tibial tufts and the associated 'velvet area', a dense carpet of short hairs apical to the tufts, are very prominent structures that have been used as diagnostic characters in euglossine taxonomy (Dressler 1978). Their function in the context of fragrance exposure has recently been postulated on the grounds of morphology (Bembé 2004). When hovering, both tufts and velvet area are directly underneath the base of the flapping wings and seem perfectly positioned as a perfume dispensor. The so-called 'jugal combs', another structural oddity characteristic for all euglossines, might also play a role. These

HTML version of this article. Two additional GIF animations (S3 and S4) show the leg movement from different angles.
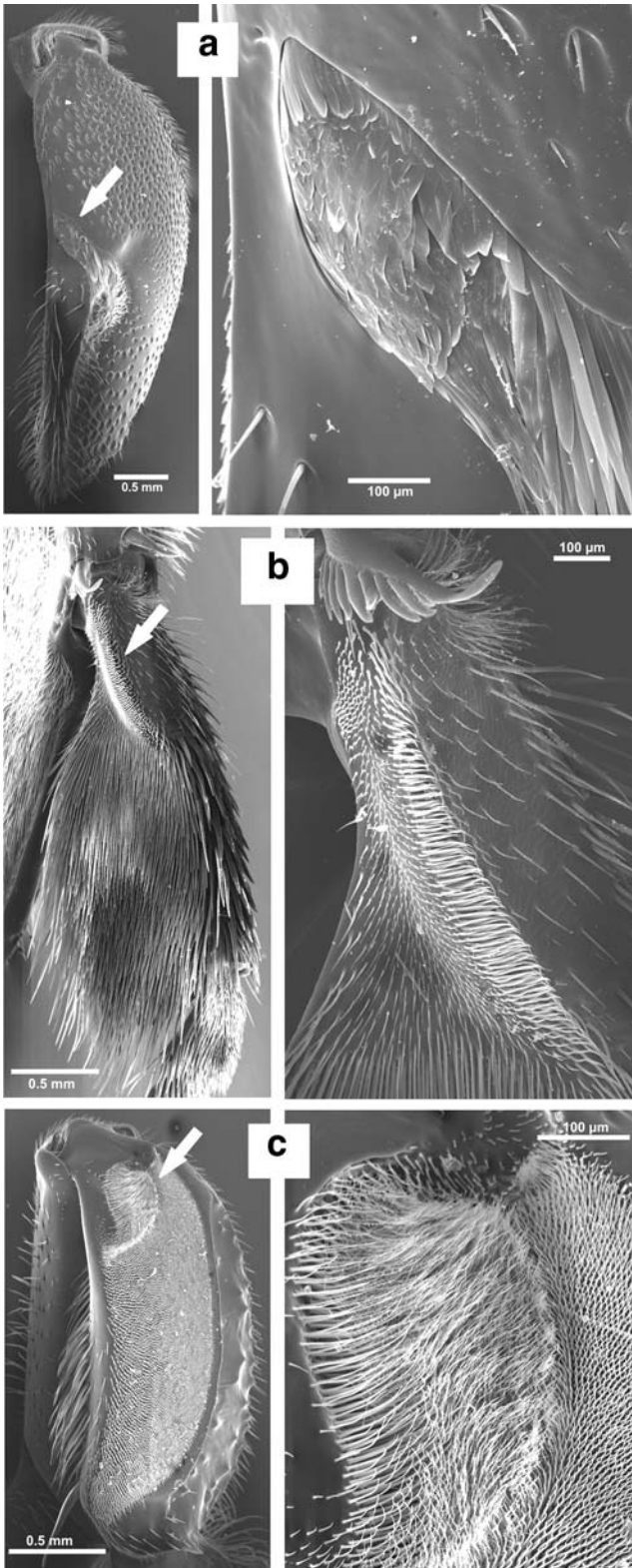
Fig. 2 Single-frame video sequence of leg movements occurring during the hovering flight of a displaying male *Euglossa cognata*, captured at 60 frames per second (*top*), and and schematic view of frame no. 25 of the same sequence, captured briefly before contact between the left hind basitarsus and the ipsilateral mid tibia (*bottom*). *Arrows* and *grey shadings* indicate the positions of morphological structures involved in the movement. Note that after the movement is completed the mid-tibial tufts come to rest in close vicinity to the wing base and the jugal combs. Legend: *br* hind basitarsal 'brush'; *tu* mid-tibial 'tuft'; *jco* 'jugal comb'. Further details in the text. The same sequence can be viewed as GIF animation in the online version (S2)

tufts, thereby aiding in the evaporation of volatiles ('spray ventilation', Bembé 2004). In our video sequences of hovering males, the jugal combs are indeed visible as 'flashes' of black shades appearing directly above the base of the mid tibia (see GIF animation S2 in the HTML version). Although a physical contact between comb and tuft remains hypothetical, Bembé's scenario represents an intriguing complementation to the observed leg movements.

The described leg movements are clearly not equivalent with behaviors reported by Peruquetti (2000), who observed male *E. cordata* transferring liquid from their hind tibiae to the leaves of the tree, partly leaving visible marks on them. The process by which this was achieved was described as being analogous to fragrance collection, only in reverse order (Peruquetti 2000). Our described leg movement has very little in common with fragrance collection. Obviously, it starts where the collection process ends, namely on the hind tibial groove that covers the opening to the fragrance container. But here the analogies end. All subsequent behaviors and the involved morphological structures serve no function during fragrance collection. Vice versa, the fore-tarsal brushes, which are used to absorb volatiles from fragrant surfaces, were not involved in the leg movements reported here. Also, we never observed visible traces of liquids on the surface of the perch.

The observed leg movements are probably not confined to *E. cognata*, but constitute a widespread and perhaps general behavioral feature of male Euglossini. In fact, a similar behavior was observed in caged males of *E. hemichlora*, but was originally interpreted as midleg thrusts aimed at the ventral side of the *contralateral* hind tibia (Eltz et al. 2003). This interpretation was based on regular low resolution video analysis and is considered incorrect in the light of the present observations. Two additional species, *E. tridentata* (displaying in a flight cage) and *Eulaema bombiformis* (displaying at a forest clearing on Barro Colorado Island, Panama), were also observed performing leg movements that conform to the motor pattern described here (T. Eltz, personal observations). The occurrence of the respective morphological features across orchid bee genera further suggests that the reported movements are common to if not all Euglossini. We have confirmed the presence of distinct hind basitarsal brushes similar to that shown in Fig. 3b on pinned males of all five genera, and

combs are sets of long parallel bristles projecting distally away from the base of the hind wing. During wing stroke, the jugal combs might brush through the tibial

Fig. 3 Scanning electron micrographs of entire leg segments (*left*) and details of cuticular structures involved in the described leg movement (*right*) of *Euglossa cognata*. **a** Right hind tibia with hair-filled groove. Detail showing layers of flattened setae covering the dorsal 'bowl'(*bo* in Figs. 1, 2). **b** Inner side of left hind basitarsus. Detail showing the basal 'brush' (*br* in Figs. 1, 2). **c** Proximal surface of left mid tibia covered by modified setae. Detail showing the basal 'tuft' (*tu* in Figs. 1, 2), consisting partly of branched setae, and adjacent parts of the 'velvet area' (*va* in Figs. 1, 2), consisting of simple, spiraled setae
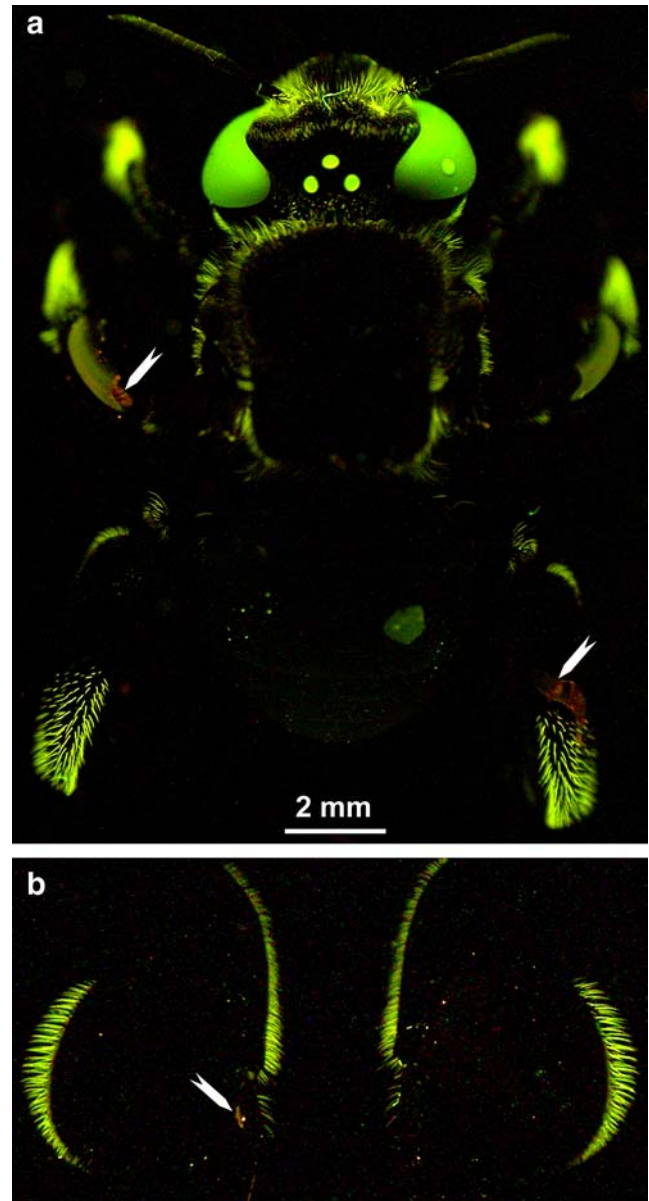
Fig. 4 Red fluorescence emission from the cuticle of a rhodamine B-treated male *E. cognata*, photographed under UV light directly following display. **a** Dorsal view of the complete specimen showing signals on the right hind tibial groove as well as on the contralateral (*left*) mid-tibial tuft (see *arrows*; corresponding structures in Fig. 3a, c). **b** Inside of removed left and right hind tibiae and basitarsi of the same specimen, showing a signal on the left basitarsal comb (see *arrow*; corresponding structure in Fig. 3b). Note green autofluorescence of the cuticle

on all of the inspected species (including 40 species of *Euglossa*, 5 *Eulaema*, 6 *Eufriesea*, 2 *Exaerete*, and *Aglae caerulea*). Equally, males of all genera have conspicuous hair fields on the outer surface of the mid tibiae, although well defined basal tufts are lacking in *Exaerete* and *Aglae*.

The ultimate purpose of the described behavior, as well as that of fragrance collection in general, remains to be clarified. From our findings, it seems likely that fragrance exposure represents an integral part of the display behavior that is more or less continuously promoted by perching males. This strengthens the view that the hind tibial contents have an attractant function in the context of mating behavior. Whether long or short range attraction is paramount, and whether the odor signal is aimed at males or females (or both) remains to be elucidated by behavioral experiments. In the meantime, accumulating evidence from chemical analyses suggest that the complex fragrance blends of males are species specific, even when samples from more distant localities are compared (Eltz et al. submitted). This specificity suggests a critical role in the context of species recognition and makes long-range attraction of conspecifics seem feasible.

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