Correlates of display activity and perch residency of male orchid bees in cage experiments

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Males of the neotropical orchid bees collect volatile chemicals from various environmental sources, including leaves, fungi and flowers, to accumulate species-specific blends in hind tibial pouches. Subsequently, male bees expose perfumes during display at perches in the forest understory. While there is as yet no direct proof that females are attracted to male perfumes, conspecific males often approach perch holders in the natural habitat and are known to respond to tibial perfumes when those are presented at perch sites in bioassays. Here we ask whether the tibial perfume phenotype of male *Euglossa imperialis* is related to display activity or the success in holding a perch following intrusions of conspecific males in cage experiments. Neither the amount nor the complexity of tibial perfume correlated with display activity or perch residency in field-caught males observed for four days in a $9 \times 9 \times 3$ m flight cage. Instead, we found a positive relationship between display activity and the amount of labial gland lipids in hind tibial pouches, suggesting that the most actively displaying males had more recently collected volatiles. The success of males in holding a display perch against intruding males was also unrelated to tibial perfume phenotype. Instead, males that had previously occupied a given perch were more likely to win a contest over that perch than males that had not. Overall, our results suggest that stored perfume is not a key determinant of male display intensity or perch residency. These findings are in broad agreement with results from a field study conducted at La Gamba in which males caught during display had perfume compositions similar to those of males caught at chemical baits.

POKORNY T., MILLAHN E., SCHLÜTTING P., RAMÍREZ S.R. & Thomas ELTZ T., 2019: Korrelation von Balzaktivität und Balzplatz-Besetzung männlicher Prachtbienen in Käfigversuchen.
Die Männchen der neotropischen Prachtbienen (Euglossini) sammeln Umweltdüfte und kombinieren diese in speziellen Taschen auf ihren Hintertibien zu einem komplexen „Parfüm", das sie während ihres Balzverhaltens im Unterwuchs des Waldes freisetzen. Eine Lockwirkung auf Weibchen ist bislang nicht bewiesen, aber Männchen scheinen auf Parfümsignale von Artgenossen zu reagieren, wenn sie in deren Balzterritorien eindringen und mit ihnen interagieren. Wir untersuchen hier die Frage, ob der Parfüm-Phänotyp eines Männchens mit seiner Balzaktivität bzw. seinem Erfolg, Balzplätze zu behaupten, korreliert ist. Weder die Menge noch die Komplexität des Parfüms von Köder-gefangenen Männchen waren mit der mittleren Balzaktivität oder der Anzahl von Balzplätzen (vertikale Sitzwarten) korreliert, die diese in einem $9 \times 9 \times 3$ m großen Flugkäfig im Garten der La Gamba-Forschungsstation besetzten. Stattdessen gab es einen positiven Zusammenhang zwischen der individuellen Balzaktivität und der in den Tibien enthaltenen Menge von Labialdrüsenlipiden, was darauf hindeutet, dass stark balzende Männchen kürzlich Düfte gesammelt haben. Der Erfolg beim Verteidigen von Balzplätzen zeigte ebenfalls keinen Zusammenhang mit dem gespeicherten Parfüm. Stattdessen wurden Balzplätze hauptsächlich dann erfolgreich behauptet, wenn das balzende Männchen bereits in der Vergangenheit an der jeweiligen Sitzwarte aktiv war. Insgesamt stimmen unsere Ergebnisse mit denen anderer Käfig- und Freilandstudien überein und verstärken den Eindruck, dass Menge und Komplexität der gespeicherten Parfüme keine starken Einflussfaktoren des Balzverhaltens von Prachtbienenmännchen sind.

Keywords: Euglossini, courtship, territory, intrasexual conflict, residency effect.

Introduction

The males of neotropical orchid bees (Euglossini) are characterised by their use of volatiles collected from their environment for intraspecific signalling. These volatiles, derived from flowers, resin, sap or decaying substrates, are not ingested. Instead, they are stored in specialized sponge-like cavities on the hind legs, where complex and broadly species-specific blends of "perfume" accumulate (Vogel 1966, Zimmermann et al. 2009). Individual males found in nature vary markedly in the amount and the number of compounds collected, with few individuals possessing many compounds and some having stored only small quantities of few compounds. While the reasons behind perfume marking remain uncertain, it has been shown that males expose the chemicals in a sexual context, i.e. during a characteristic male display behaviour at the places where mating occurs. These display sites are usually vertical structures (trees) in the forest understory on which the males perch and assume a typical posture (Kimsey 1980, Stern 1991, Eltz et al. 2003, Pokorny et al. 2017 and see Fig. 1).

In the genus *Euglossa*, males display by performing repetitive short hovering flights and somewhat more extensive patrolling flights intermittent with perching behaviour. It is during the hovering flights that males expose perfume from their hind leg pockets by stereotypic leg movements. Display has been observed to continue for lengthy periods of time, and both the general location of the perch (exposed to the wind on hills and ridges) as well as the angular orientation of the male on the perch (almost always exactly downwind) suggest that perfume dispersion is the central function of male display (Pokorny et al. 2017).

However, the potential recipient(s) of the perfume signal is (are) uncertain. While females have been (rarely) observed to copulate with displaying males on the perch (Kimsey 1980, Eltz et al. 2003, Pokorny et al. 2017, Zimmermann et al. 2006), direct bioassay evidence for attraction of females to male perfumes has not been forthcoming. On the other hand, conspecific males arrive quite regularly and engage in interactions with the resident male (Dodson 1966, Kimsey 1980, Stern 1991, Eltz et al. 2003, Zimmermann et al. 2006). This behaviour appears to be mediated by perfume stimuli: Zimmermann et al. (2006) exposed hind leg extracts of two closely related sympatric species of *Eulaema* at their respective active perch trees in Panama and French Guiana, and found that males arrived quickly at the 'correct' (conspecific) extracts, behaving as if encountering conspecific displaying males. In general, the behaviour of interacting euglossine males at display sites appears competitive rather than mutualistic, with males engaging in ritualized zig-zag or sustained circling flights near the perch. The interactions can last from a few seconds up to dozens of minutes (Kimsey 1980, Stern 1991, Roubik & Hanson 2004, T. Pokorny, pers. obs.), and in almost all cases end with one of the males leaving the site and the other resuming display (see Stern 1991).

For the experiments presented here we assumed that the success of male display, be it for intersexual or intrasexual communication or both, depends on the male perfume phenotype. Therefore, we predicted that the general likelihood of a male to occupy perches and perform display behaviour should increase with perfume amount and complexity. Furthermore, we assumed that perfume communication is used among males to settle conflicts and predicted that the likelihood of defending or taking over a perch increases with perfume amount and complexity. To test these predictions we conducted two behavioural experiments in a large flight cage.

Materials and methods

Two experiments were carried out in the gardens of the La Gamba Tropical Station, Puntarenas, Costa Rica. The Display Activity experiment took place in March/April 2014 and the Interaction experiment in March/April 2018. For both experiments, males of *Euglossa imperialis* were captured in the nearby forest at chemical baits of 1,8-cineole, and placed individually in Eppendorf tubes with breathing holes for transport. Back at the field station they were released into small insectaries containing branches with leaves and artificial flowers providing sugar water (40 % sugar). All artificial flowers were refilled with sugar water every morning, and bees soon learned to drink from them.

Display Activity experiment

After having habituated for two days in the small insectaries, 16 males were marked individually with one coloured bee tag (star symbols; Graze, Endersbach, Germany) on the scutum, plus a combination of three coloured paint dots (enamel paint, Revell) on the second metasomal tergite (Fig. 1b). They were then introduced into a large experimental cage (9 × 9 x 3 m, see Fig. 1a) containing flowering plants (*Calathea lutea*, *Stachytarpheta* sp.), artificial flowers mounted on corner poles, and 21 numbered stems of forest trees with diameters (3.1 to 5.4 cm; all branches removed) preferred for display by *E. imperialis* spread evenly throughout the cage. The first display was observed on the 4th of April 2014, five days after the males had been introduced into the experimental cage. During the next four days (5th to 8th of April 2014), individual display activity and perch occupancy was quantified by doing rounds of observations of all perches between 0600 and 0930 hours, the time of peak display activity on sunny days. During each round the perches were observed in a pre-defined sequence with each perch being observed only once for a few seconds. In case a bee was seen to be displaying at a given perch it was identified and the round was continued. Thus, an individual bee was never counted twice on a given perch per round. An observation round took between 5 and 7 minutes, and 15 to 20 rounds were completed per day, totalling 66 over the four days.

We calculated an index of daily display activity by dividing the number of observations per individual by the number of rounds of observation on that day. Daily activity was averaged over four days to estimate the overall display activity per individual. Perch occupancy was calculated as the mean of the number of different perches used by an individual per day. After completion of observations on the 8th of April 2014, all 16 males were caught, killed by freezing, and both their hind legs were extracted by submersing them permanently in 0.5 ml of n-hexane for GC/MS analysis (in Bochum). The bees were pinned and dried for subsequent size measurements (head width) using callipers. For each bee, two measurements were taken, and the average value for each individual was used in the downstream statistical analysis.

Interaction experiment

In addition to the small insectary and the large experimental cage, this setup included a medium-sized mesh cage (2 × 2 x 2 m) that was equipped in the same manner as the small insectary. Display opportunities in the large experimental cage were provided by the same plants as during the Display Activity experiment, but this time only three additional tree stem perches were installed.
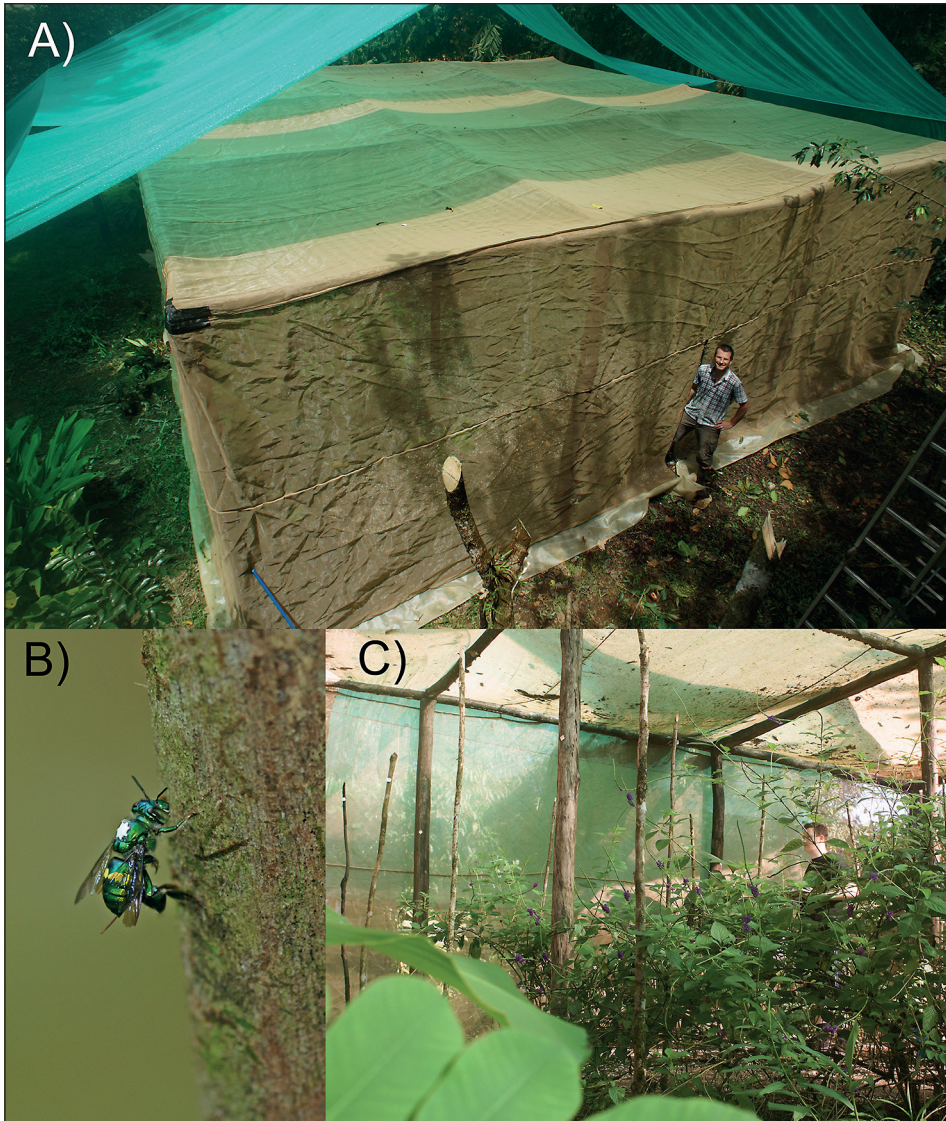
Fig. 1: A) Experimental cage, viewed from the top. B) Marked *Euglossa imperialis* male in the typical posture during display at a perch. C) Researcher observing display behaviour in the cage. – Abb. 1: A) Der Versuchskäfig, Ansicht von oben. B) Markiertes *Euglossa imperialis*-Männchen in der typischen Display-Haltung an der Ansitzwarte. C) Forscher beim Beobachten des Displayverhaltens im Käfig.

One day after capture and acclimatization to the small insectary, bees were marked on scutum, scutellum and abdominal tergites using four different colours of enamel paint (Revell). Paint was applied in small dots using the tip of a wooden toothpick, and after a short drying time each bee was released back into the small insectary. The next day, about two thirds of the marked bees were released into the large experimental cage. These bees

were defined as 'experienced', i.e. they had had time to familiarize themselves with the experimental environment, while the remaining bees remained in the small insectary. After 24 h, the bees in the experimental cage were recaptured and transferred to the medium-sized mesh cage, which was positioned directly adjacent to the experimental cage. Trials on perch residency, i.e. defence or take-over of a perch tree after a male-male interaction, commenced the following day, with observations conducted between 0600 and 1500 hours, depending on weather conditions. After one week, new bees were captured, and the procedure was repeated.

For each trial, a group of four males (either all four experienced or two experienced males and two males that had remained in the small flight cage) was released into the large experimental cage and observed for three hours for display activity and male-male interactions at perch trees. Display activity was documented, including the identity of the displaying male and the perch. Once an interaction took place, its duration was noted, as well as its outcome, i.e. the male that returned to the contested perch for display was considered the winner of the interaction. Both interacting males were then captured, singly transferred to clean Eppendorf vials and placed in a freezer (-20°C) for later sample preparation. The remaining two males were observed for up to three more hours or until they too interacted, after which they were captured and frozen. Subsequently, a new group of males was released into the experimental cage and observed in the same manner. When the bees became inactive or it was past 1500 hours before the allotted observation time had expired, observations were stopped and continued the next morning after onset of bee activity. Extractions of hind legs for chemical analysis and measurements of head widths were performed as described above.

Chemical analyses

Hind leg extracts were analysed using coupled gas chromatography (GC: HP 5890 II) and mass spectrometry (MS: HP 5972) in splitless mode and with a sample volume of 1 µl. The GC was fitted with a non-polar DB-5MS column (30 m x 0.25 mm x 0.25 µm). The oven program ran from 60°C to 300°C at 10°C per min, with the final temperature held for an additional 15 min. Based on previous experience with *E. imperialis* tibial samples (Zimmermann et al. 2009; Weber et al. 2016), we sorted peaks into two groups of compounds: exogenous volatiles (perfume compounds, before 20.5 min) and endogenous labial gland lipids (straight chain saturated and unsaturated acetates, diacetates and alcohols, after 20.5 min). For each sample, we assessed the total amount of perfume compounds (summed peak areas < 20.5 min), perfume complexity (number of different perfume compounds), the total amount of labial gland lipids (summed peak areas > 20.5 min), and labial gland lipid complexity (number of different lipid compounds).

Statistical analyses

Data of the Display Activity experiment were analysed using Spearman rank correlation in Statistica v13 (Statsoft, Tulsa, Oklahoma, USA). Data of the Interaction experiment was analysed in R 3.3.3 (R Development Core Team 2017), testing for the impact of the different factors that might influence the outcome or duration of male-male interactions. These were: perfume amount, perfume complexity, body size (via the proxy head width), experience in the experimental cage and previous occupation of the contested perch.

Results

Display Activity experiment

Thirteen of the 16 males displayed during the four-day observation period, with 9 to 12 males displaying per day. The mean individual likelihood to be observed displaying during an observation round varied from 0 to 39.2 % (mean 13.5 %) over all four days (Fig. 2), peaking on the first observation day (16.3 %), with a slight decline towards the last day of the experiment (12.1 %). The amount and complexity of exogenous perfumes in hind leg extracts taken at the end of the experiment varied over approximately half an order of magnitude among the 16 individuals. However, neither the amount nor the complexity of tibial perfume was correlated with display activity or perch occupancy (Tab. 1, Fig. 2). The only significant correlates were a positive relationship of mean display activity with the amount of labial gland lipids, and a negative relationship between mean display activity and the ratio in the amount of perfume/lipids (on day 4 and overall). There was also a positive but non-significant relationship (P < 0.1) between head width and display activity.
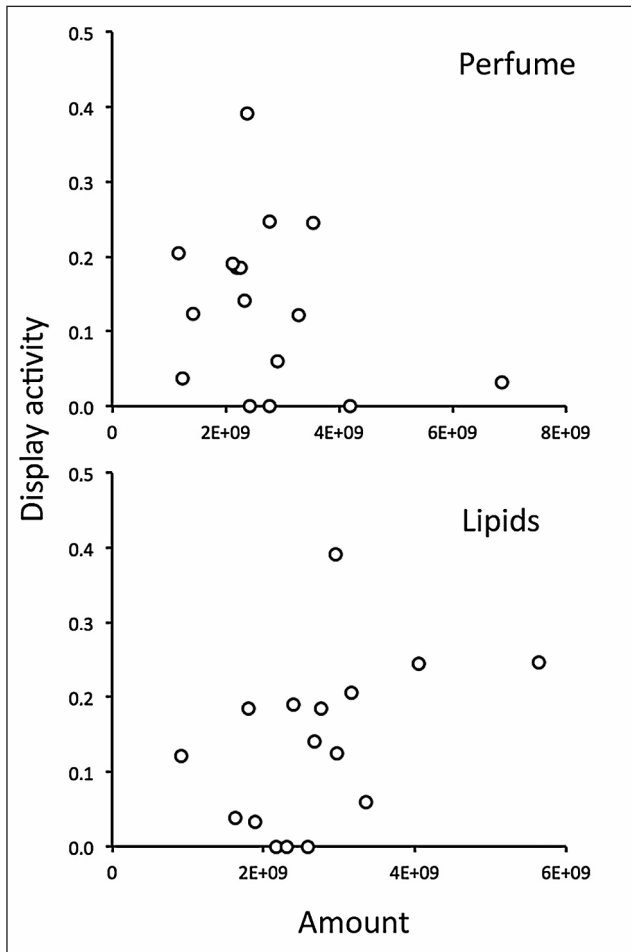

Fig. 2: Relationship between the amount of exogenous perfume (top) and the amount of endogenous labial gland lipids (bottom) in hind leg extracts and the display activity of male *Euglossa imperialis* (N = 16) summed over four days of the Display Activity experiment. See text and Tab. 1 for statistics. – Abb. 2: Korrelation der Menge exogenen Parfüms (oben) beziehungsweise der Menge endogener Labialdrüsenlipide (unten) von Hinterbeinextrakten und der Displayaktivität, aufsummiert über vier Tage, von männlichen *Euglossa imperialis* (N = 16) im Experiment zur Displayaktivität. Siehe Text und Tabelle 1 für die Ergebnisse der statistischen Analyse.

Tab. 1: Results of Spearman rank correlation tests between chemical/morphological (variable 1) and behavioural (variable 2) traits of male *Euglossa imperialis* participating in the Display Activity experiment. – Tab. 1: Ergebnisse der Spearman Rangkorrelationstests zwischen chemischer/morphologischer Eigenschaften (Variable 1) und dem Verhalten (Variable 2) von *Euglossa imperialis* Männchen im Experiment zur Displayaktivität.

Variable 1	Variable 2	N	Rs	t(N-2)	p
Amount of perfume	Mean display activity	16	-0,330	-1,310	0,211
	Mean no. of perches	16	-0,342	-1,361	0,195
No. of perfume compounds	Mean display activity	16	-0,050	-0,188	0,853
	Mean no. of perches	16	-0,081	-0,303	0,766
Amount of labial gland lipids	Mean display activity	16	0,555	2,494	0,026
	Mean no. of perches	16	0,401	1,637	0,124
No. of labial gland compounds	Mean display activity	16	0,178	0,678	0,509
	Mean no. of perches	16	0,058	0,219	0,830
Ratio amount of perfume/lipids	Mean display activity	16	-0,552	-2,475	0,027
	Mean no. of perches	16	-0,478	-2,035	0,061
Head width	Mean display activity	16	0,479	2,039	0,061
	Mean no. of perches	16	0,283	1,105	0,288

Interaction experiment

A total of 23 interactions were documented during 15 of the 17 trials. Variation in perfume amount and complexity spanned more than an order of magnitude in this experiment. Neither perfume amount nor perfume complexity correlated with male body size (Spearman rank correlations, n.s.). Males winning interactions did not differ from the losing males in the amount and complexity of perfumes or labial gland lipids (Wilcoxon paired tests, n.s.) or body size (paired t-test, n.s.). Males with experience in the experimental cage did not win interactions more often than naïve males (Chi² test, n.s.); however, males that
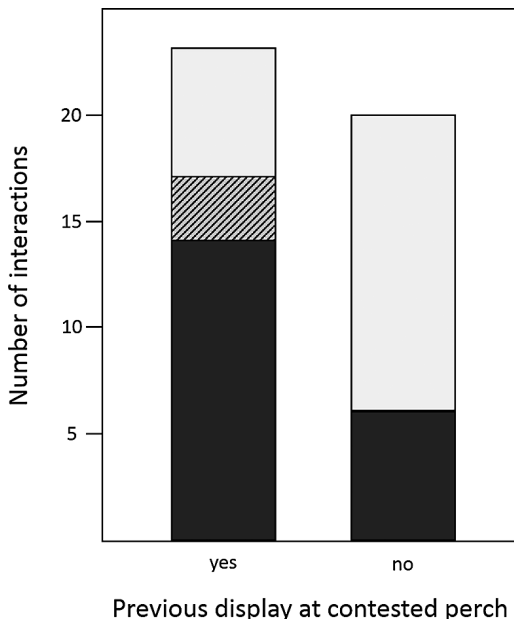

Fig. 3: Number of male-male interactions won (black) and lost (grey) in relation to whether a male had previously shown display behaviour at the contested perch or not. In three cases (hatched area), both interacting males had previously shown display at the contested perch. In all three cases, the male that had held the perch first, but not directly before the interaction, won against the newly displaying male. See text for statistics. – Abb. 3: Anzahl der Interaktionen zwischen Männchen, die gewonnen (schwarz) und verloren (grau) wurden, in Bezug darauf, ob ein Männchen bereits an der umkämpften Ansitzwarte Displayverhalten gezeigt hatte oder nicht. In drei Fällen (schraffierte Fläche) hatten beide interagierenden Männchen bereits an der entsprechenden Ansitzwarte Display gezeigt. In allen drei Fällen gewann das Männchen, welches als Erstes, aber nicht direkt vor der Interaktion, an dieser Ansitzwarte Displayverhalten gezeigt hatte. Statistik siehe Text.

had previously shown display activity at the contested perch won significantly more often than intruding males (Chi² test, chi² = 4.33, P < 0.05, see Fig. 3). In three cases, both of the interacting males had previously shown display at the contested perch. In all of these cases, the male that had displayed at the perch first won the interaction against the currently displaying male.

The duration of the interaction was not correlated to absolute differences in perfume amount or perfume complexity between the contestants. However, interactions were concluded faster when the males exhibited pronounced body size differences (Spearman rank correlation, r = -0.43, P < 0.05, Fig. 4), irrespective of whether the smaller or the larger male won the interaction.

Fig. 4: Relationship between the size differences (head width) between pairs of males interacting at a perch and the duration of the interaction (N = 23). Spearman rank correlation, r = -0.43, P < 0.05. – Abb. 4: Korrelation der Differenz der Körpergrößen (Kopfbreite) zwischen den jeweils interagierenden Männchen und der Dauer der Interaktion (N = 23). Spearman Rangkorrelation, r = -0.43, P < 0.05.
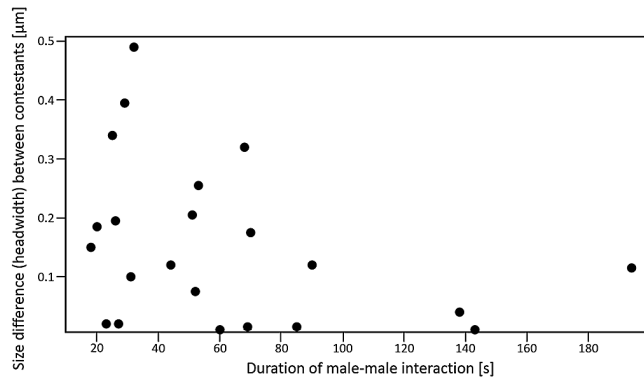
Discussion

Our experiments suggest that the perfumes stored by individual male orchid bees are a poor predictor of their display activity, the number of perches they occupied for display, or the likelihood of a male winning an interaction contest over display sites.

There are a number of possible explanations for this lack of effects, some experimental, others biological. First, it is possible that the circumstances in our experiments were too confined or too artificial in abiotic conditions (temperature, wind), preventing males from displaying in accordance with the status of their perfume signal. Considering that males were caught in the wild, it is possible that the short timeframe of observation, perhaps in combination with individual differences in how fast bees habituate to the cage situation, was insufficient to demonstrate perfume effects. While this is difficult to rule out completely, there is evidence from other studies suggesting that stored perfumes are indeed not strongly linked to display activity or the onset of display. For example, the longer-term (14 days) display activity of caged male *Euglossa hemichlora* varied among individuals but was also unrelated to perfumes extracted at the end of the observation period (Eltz et al. 2003). In the field, the amount of perfume stored by male *E. imperialis* captured while displaying along forest trails at La Gamba, Costa Rica, did not differ from that of control males captured at chemical baits (Pokorny et al. 2017). This suggests that there is no perfume-dependent shift in male orchid bee behaviour, with younger (or perfume-poor) individuals engaging in volatile collection and older (or well-stocked) males switching to display. Instead, display appears to be a rather stereotyped behaviour that is controlled by endog-

enous mechanisms independent of volatile uptake, a notion that was recently supported by the observation of display occurring in very young, cage-reared *Euglossa dilemma*, which had not had the opportunity to collect volatiles at all (J. Henske, pers obs.).

It should be emphasized that the lack of a stored perfume-effect on the onset and undertaking of display does not preclude perfume effects on downstream sexual success, i.e. mate attraction and mating. Second, it is possible that stored perfumes do affect display, but we have not measured the relevant perfume trait. The amount and complexity of individual perfumes are highly variable and easy to quantify, but the relative contribution of certain (unknown) compounds to the blend or the overall chemical composition (template match) could be more important in determining behaviour. Third, display activity might be stimulated by recent collection of compounds rather than the overall amount and composition of accumulated volatiles. This would be in agreement with our finding of a positive correlation between display activity and the amount of labial gland lipids (both in absolute terms and in proportion to exogenous perfume).

Labial gland lipids serve as carriers during perfume collection (Whitten et al. 1989), and it has been shown that they are specifically sequestered from the hind leg pouches to be subsequently reused (Eltz et al. 2007). A high concentration of labial gland lipids in the hind leg pouches thus indicates a recent collection event, which might even have occurred in the experimental cage (e.g., from damaged and possibly fungus-infected leaves; T. Eltz, pers. obs.). Positive feedback from recent perfume collection might lead to higher intrinsic motivation to show display activity, or reduce the chance of interrupting or terminating display behaviour in comparison to males that have not experienced a recent collection opportunity.

Recent volatile collection has previously been implicated to stimulate display in *E. imperialis* (Schemske & Lande 1984). When these authors allowed caged males to collect synthetic 1,8-cineole from filter pads, there were more displaying males and a higher number of occupied perches than in a second cage without 1,8-cineole. Finally, the lack of a stored perfume-effect on display activity could be due to the possibility that the possession of perfume does not play a role in the acquisition of mates in male orchid bees. While perfume exposure during male display is strongly suggested by specific stereotypic leg movements, associated specific morphological structures and fluorescent tracer experiments (Eltz et al. 2005), there is still no direct evidence that females use chemical information contained in the perfumes exposed by males (see discussion in Pokorny et al. 2017). Carefully controlled mating experiments are required to fill this knowledge gap.

While female attraction to male perfumes remains uncertain, it seems likely that males respond to exposed perfumes when they approach and interact with conspecific perchholders (see Zimmermann et al. 2006). Intrasexual conflicts can occur when competing for mates or for mating territories through which mates can be acquired (e.g. Wickman 1985). In orchid bees, all hitherto observed matings took place at or near the perch tree and with the current perch holder (Kimsey 1980, Eltz et al. 2003, Pokorny et al. 2017), and the only correlate of euglossine male mating success to date is the amount of time spent displaying (in a cage experiment, see Eltz et al. 2003).

While display itself is likely a stereotypic behaviour, success in occupying and holding a perch territory (and thus, presumably, mating success) might be dependent on display experience. This notion is in part corroborated by our finding that neither perfume phenotype nor body size were related to the outcome of male-male interactions at perch trees,

while previous occupancy of the contested display site led to males winning a contest significantly more often than males that intruded upon a territory.

In insects, many territorial contest situations are won by the owner of the territory ('residency effect', see Takeuchi & Honda 2009 and Kemp & Wiklund 2001) and this had also been observed for interactions in *Eulaema bombiformis* (Stern 1991). The outcome of territorial disputes can be influenced by individual motivation based on previous experience (Takeuchi 2006, Bergman et al. 2010) or invested resources. Studies on the wood butterfly *Pararge aegeria* suggested that residency effects might in part be due to inherently aggressive males dominating the available territories (Kemp & Wiklund 2004). Interestingly, we found that contests between interacting male *E. imperialis* were concluded faster when the difference in head width (i.e. body size) between opponents was more pronounced. In fact, the average contest duration for cases in which the opponents differed by less than the average size difference was twice as long (13 cases, average duration of the interaction: 80 s) as that of the cases in which size differences were larger than average (10 cases, average duration of the interaction: 39 s). These results could be explained by self-assessment of body size in relation to that of the opponent in combination with the opponent's apparent aggressiveness/motivation.

While it is unclear which additional factors could lead to a fast conclusion of a contest with either a clearly larger male or a clearly smaller male, it seems plausible that contestants interact for a longer period of time the more similar they are in body size before the contest is resolved. Escalation of contests, i.e. a longer duration, was also observed in *Melanitis leda* when the combatants were more similar in age, although in this case the parameter 'age' influenced the final outcome as well (Kemp 2003).

Further controlled experiments including other potentially influential parameters for contest outcome apart from prior residence might shed light on the factors leading to successful perch residency in orchid bees.

Acknowledgments

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