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— Short Communication —

INHIBITION OF BIOCHEMICAL TERPENE PATHWAYS IN *ACHILLEA MILLEFOLIUM* FLOWERS DIFFERENTLY AFFECTS THE BEHAVIOR OF BUMBLEBEES (*BOMBUS TERRESTRIS*) AND FLIES (*LUCILIA SERICATA*)

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Abstract—Floral scents serve multiple functions in the interactions with organisms. Flowers of *Achillea millefolium* (Asteraceae) emit scent bouquets dominated by terpenoids. These flowers are mainly visited by flies and beetles, whereas bumblebees, common visitors at other Asteraceae, are absent from *A. millefolium* flowers. In order to test how a reduced mono- and sesquiterpenoid emission affect insect behaviour we inhibited the biochemical pathways towards the production of terpenoids of *A. millefolium* plants and conducted behavioural choice tests. The inhibition resulted in reduced emission rates of most mono- and sesquiterpenes and thus altered the olfactory phenotype of the flowers. In a flight cage, flies usually chose flowers with a natural scent bouquet, bumblebees clearly preferred flowers treated with inhibitors. These findings confirm that floral scents play a pronounced role in foraging decisions of flower visiting insects and support the notion that responses towards scent are animal species-specific emphasising the role of scents as floral filters.

Keywords: flight cage, floral scent bouquet, Fosmidomycin, hydroponic plants, Lovastatin

INTRODUCTION

Plants emit floral scent bouquets to communicate with diverse flower visitors. Some floral volatiles act as attractants for mutualists and simultaneously repel antagonistic insects (Kessler et al. 2008; Junker & Blüthgen 2010; Schiestl 2010). Accordingly, floral scents have been shown to determine the presence, absence and frequency of flower visits of multiple insect species (Junker et al. 2010; Larue et al. 2016). *Achillea millefolium* (Asteraceae) flowers are mostly visited by syrphids, flies and beetles (Larue et al. 2016). Although bumblebees and honeybees are common flower visitors in other Asteraceae e.g. *Cirsium arvense*, they naturally do not visit flowers of *A. millefolium* (Larue et al. 2016). The floral scent bouquet of *A. millefolium* is dominated by mono- and sesquiterpenoids (Larue et al. 2016) that can serve defensive functions in floral scent bouquets (Junker & Blüthgen 2010; Schiestl 2010; Junker et al. 2011). The precursors of mono- and sesquiterpenoids, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) derive from two pathways. Monoterpenoids are formed in the plastidic methylerythritol phosphate (MEP) pathway whereas sesquiterpenoids are synthesized in the cytosolic mevalonate pathway (MVA) (Dudareva et al. 2005). In a previous study (Larue et al. 2016), we experimentally augmented the scent bouquets of *A. millefolium* with volatiles extracted from a plant species (*C. arvense*) that predominantly emits aromatic compounds. Following this augmentation, *A. millefolium* flowers were visited by honeybees and bumblebees. In order to discriminate between the attractive effect of aromatics and the repellent effect of terpenoids, in this study we inhibited the biochemical pathways of hydroponic *A. millefolium* plants involved in terpene-synthesis. Inhibited and control plants were used in flight cage choice tests with bumblebees (*Bombus terrestris*) and flies (*Lucilia sericata*) to study their behaviour towards terpenes.

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MATERIALS AND METHODS

Organisms

Achillea millefolium seeds originating from a single population near Hamburg, Germany (obtained from the Botanical Garden of the University of Hamburg, Germany), were placed on filter papers (Rundfilter, 5.5 cm Ø, Macherey-Nagel, Düren, Germany) in petri dishes and were treated with 250 µl of a watery gibberellic acid solution (1000 ppm, Sigma-Aldrich Handels GmbH, Vienna, Austria). After germination, seedlings were transplanted into 2 ml micro test tubes (Safe lock tubes, Eppendorf AG, Hamburg, Germany) filled with 1.2 ml agar grow medium (1.5 g agar dissolved in 120 ml water, Bacto-Agar, A. Hartenstein GmbH, Würzburg, Germany), which was enriched with a fertilizer combination suited for the nutritional requirements of young plants (180 µl of Grow, Bloom and Micro fertilizer, mixture ratio 2:1:1, Advanced Hydroponics, Vienna, Austria). In order to prevent dehydration, tubes were closed until the roots of the

seedlings were strong enough to supply plants with nutrients and water. At that time the lower tip of each of the tubes was removed using pincers and tubes were inserted into 55 L metal water basins filled with deionized water and fertilizer solution with the same fertilizer-water ratio as described above, allowing roots to grow into the solution. Fertilizer solution was changed every two weeks and the fertilizer mixture adjusted to the growth stage of the plants according to the manufacturer's instructions. For ventilation, the water fertilizer solution was aerated *via* a silicon tube leading directly into the water of each basin using an electrical pump (LP-100 air-pump, Resun, Shenzhen Xing Risheng Industrial Co., Ltd.; Shenzhen, China). Room temperature remained constantly at 18 °C and fluorescent tubes (Osram, T8 L 58W/865 LUMILUX Daylight G13, PK Beleuchtungstechnik, Hohenbrunn, Germany) as well as red/blue LED-lamps (LED Pflanzenlicht 14W, esmart, Pinnow, Germany) illuminated the plants for 11 hours a day. *Bombus terrestris* and *Lucilia sericata* used in experiments were purchased (biohelp, Vienna, Austria) and kept and reared in the lab. Insects were fed with sugar solution and flower pollen (Hochland Bio-Blütenpollen, Hoyer, Polling, Germany). The used insect species represented close relatives of common flower visitor species observed on flowers of *A. millefolium* or other Asteraceae in the field (Larue et al. 2016).

Inhibition of biochemical pathways

In order to inhibit the metabolic products of the methylerythritol phosphate (MEP) and mevalonate pathway from *A. millefolium* we used pathway-specific inhibitors. For best results, we used a combination of Fosmidomycin (blocking the MEP pathway) and Lovastatin (blocking the mevalonate pathway) as isopentenyl diphosphate (IPP) is exchanged via/through the plastid membrane between both pathways (Dudareva et al. 2005; Junker et al. 2011). For applying the specific inhibitors to hydroponic plants via/through the roots, inhibitors were dissolved in water. For the treatments, a 10 mM Fosmidomycin stock solution and a 1 mM Lovastatin stock solution were used. 25 mg of Fosmidomycin (Life technologies Austria, Vienna, Austria) were dissolved in 12.2 ml distilled H_2O. To increase the water solubility of Lovastatin (Sigma-Aldrich Handels GmbH, Vienna, Austria) a method analogous to that described in Kita et al. (1980) was implemented. To exclude any effects of solvents and additives (see Kita et al 1980), a control solution was prepared in the same way as done for the Lovastatin solution excluding both inhibitors. All solutions were stored in multiple aliquots at -20 °C until usage. Before starting the inhibition experiments, treatment and control hydroponics were taken out of the metal basins, root tips were cut to improve absorption of inhibitors and plants placed in plastic vases (Erlenmeyer flasks of PP, 100 ml, A. Hartenstein GmbH, Würzburg, Germany) containing one of the following solutions: Control plants received 91.75 ml nutrient solution and 8.25 ml control solution. Plants of the treatment group were supplied with a mixture of 91.75 ml nutrient solution and the pathway-specific inhibitors, i.e. 0.75 ml of the 10 mM Fosmidomycin stock solution and 7.5 ml of the 1 mM Lovastatin stock solution.

Floral volatile collection and analysis

Floral headspace of treatment (N = 10) and control (N= 3) plants were collected using the dynamic headspace sampling (compare Larue et al. 2016). Volatiles were sampled 12 hours after inhibition experiment started, as pre-trials showed that inhibitors worked best after that period of time. Flowers of the hydroponic plants were enclosed into scentless polyethylene tetraphthalate (PET) oven bags (Toppits® Cofresco Fischhalteprodukte GmbH & Co. KG, Minden, Germany), without picking them from the plant for 15 min and the enriched headspace were sampled for 2 min into volatile traps with a flow rate of 200 ml min^{-1}. Volatile traps were filled with a mixture of 1.5 mg Tenax-TA (mesh 60-80; Supelco, Germany) and 1.5 mg Carbotrap B (mesh 20-40; Supelco, Germany). Floral volatiles were analysed by using an automatic thermal desorption system (TD, model TD-20, Shimadzu, Japan) coupled with gas chromatograph/mass spectroscopy (GC-MS, model QP2010 Ultra EI, Shimadzu, Japan). GC was equipped with a ZB-5 column (Zebron ZB-5, 5% phenyl polysiloxane, length 60 m, inner diameter 0.25 mm, film thickness 0.25 µm, Phenomenex, Newport Beach, USA). The carrier gas (helium) had a flow of 1.5 ml min^{-1} and the oven temperature was hold for 1 min at 40 °C before it raised with 6 °C min^{-1} until the maximum of 250 °C. The MS interface was 260 °C and the ion trap worked at 200 °C. Floral compound identification was performed with a GCMSsolutions software (Version 2.72, Shimadzu Corporation), mass spectra of authentic standards as well as spectral libraries (ADAMS, ESSENTIALOILS-23P, FFNSC 2, W9N11) and Kovats indices generated using n-alkanes to identify floral volatiles.

To compare the floral scent bouquets from control and inhibited flowers 12 hours after inhibition, we calculated Euclidean distances and used them for non-metric multidimensional scaling (NMDS).

In order to directly compare the floral scent bouquets of experimental plants, we fitted control and inhibited plants onto an ordination (NMDS) based on Euclidean distances and tested whether the centroids of both groups significantly deviated using the R package vegan (Dixon 2003).

Flight cage choice tests

In order to compare the insects' behaviour towards control flowers of *A. millefolium* and those with an inhibited floral bouquet, bumblebees (N = 17) and flies (N = 11) were allowed to choose between flowers of a control and a treatment plant approximately 12 hours after inhibition. For behavioural bioassays, we used the same plants as for scent sampling. Bumblebees and flies were kept in flight cages in the laboratory at a temperature of 20 ± 2 °C. After capturing them from the flight cages, all insects were directly tested under daylight conditions (Sylvania Grolux T5, 54W, Havells Sylvania Germany GmbH, Vienna, Austria) at a temperature of 20 ± 2 °C and each individual was used only once. Up to three naïve insects per species were tested simultaneously. The flight cage where insects were tested was of pyramidal shape with 50 cm side length and 30 cm height. The upper part consists of mosquito nets and the

ground was made from PVC containing two holes 20 cm apart from each other. Flowers were attached to the holes (height from ground about 10 cm) and were supplied with water, nutrients and inhibitors from underneath the flight cage. Insects were released in a flight cage and the observation and recording started after 20 seconds of acclimation. Depending on the insects' activity the total observation time varied between 10 - 40 minutes. During this time, approaches to and landing on flowers were recorded. The number of corymbs per plant and number of flower per corymb was held similar in control and treatment plants. Insects' preferences for inhibited or control plants were expressed as the difference D of the proportional number of insects landing on inhibited flowers minus the proportion of insects landing on control flowers, i.e. positive values indicate a preference for inhibited flowers, negative values for control flowers. To take account for the nested design of our experiment (i.e. up to three insect individuals were included in one trial) we tested whether D significantly deviates from zero (against the null hypothesis ($D = 0$)) using linear mixed effect models (LME) with approaches or landings as dependent variable and experimental trials as a random factor. LME were conducted using the R package *nlme* (Pinheiro et al. 2015). All statistical analyses were performed in R (R Development Core Team 2013).

RESULTS

Floral volatiles emitted from inhibited and control plants

A. millefolium flowers treated with inhibitors 12 hours prior to volatile sampling emitted reduced amounts of individual monoterpenes and a sesquiterpene compared to control plants (Tab. 1). Unexpectedly, flower treated with inhibitors emitted higher amounts of limonene (not significant after Bonferroni correction; Tab. 1). As a consequence, floral scent bouquets from control and treatment plants were well separated in a NMDS based on Euclidean distances (Fig. 1) and centroids of control and inhibited plants deviated significantly ($R^2 = 0.56$; $P = 0.004$).

TABLE 1. Scent emission of *A. millefolium* flowers 12 hours after application of inhibitors to hydroponic plants. Shown are mean emission rates and standard deviation (SD) of each compound [ng hr^{-1} dry $weight^{-1}$] from control and treatment flowers. *P*-values for comparison of the emission of individual substances in control and treated flowers using Wilcoxon-test are also given. Scent compounds are listed in order of the Kovats' index within each class. Significant deviations after Bonferroni correction are indicated by asterisks.

Compounds		Control ($N = 3$)		Treatment ($N = 10$)		
[ng hr^{-1} dry $weight^{-1}$]	RI	Mean	SD	Mean	SD	*P*-values
Monoterpenes						
α-Thujene	926	193.02	89.39	1.66	2.54	0.005
α-Pinene	938	600.25	191.67	714.76	583.83	0.922
Camphene	956	147.46	77.34	56.93	42.56	0.002*
Sabinene	979	2731.83	491.82	106.05	67.65	0.002*
β-Pinene	982	221.69	83.58	271.34	307.74	0.846
α-Terpinene	1022	127.76	63.41	0.00	0.00	NA
ρ-Cymene	1030	459.98	186.46	99.94	103.79	0.002*
Limonene	1033	98.94	45.12	481.69	389.86	0.027
β-Phellandrene	1036	127.69	45.09	57.22	113.66	0.084
1,8-Cineole	1039	301.45	128.66	156.57	160.08	0.027
(E)-β-Ocimene	1049	82.04	29.50	7.03	9.87	NA
γ-Terpinene	1064	281.79	156.67	13.37	15.76	NA
α-Terpinolene	1095	59.41	43.68	0.00	0.00	NA
Allo-Ocimene	1132	10.62	10.19	0.08	0.28	NA
Camphor	1158	78.31	65.82	10.13	19.49	0.004
sum		349.97	652.53	123.64	281.57	0.025
Sesquiterpenes						
β-Caryophyllene	1445	27.16	31.87	14.68	14.94	0.049
Aromatics						
Benzaldehyde	963	209.07	42.99	192.95	93.28	0.432
Aliphatics						
(Z)-3-Hexen-1-ol	854	99.34	47.84	36.70	22.95	0.002*
(Z)-3-Hexenyl acetate	1005	507.31	168.70	20.96	12.70	0.002*
sum		303.32	238.72	28.83	20.14	0.333

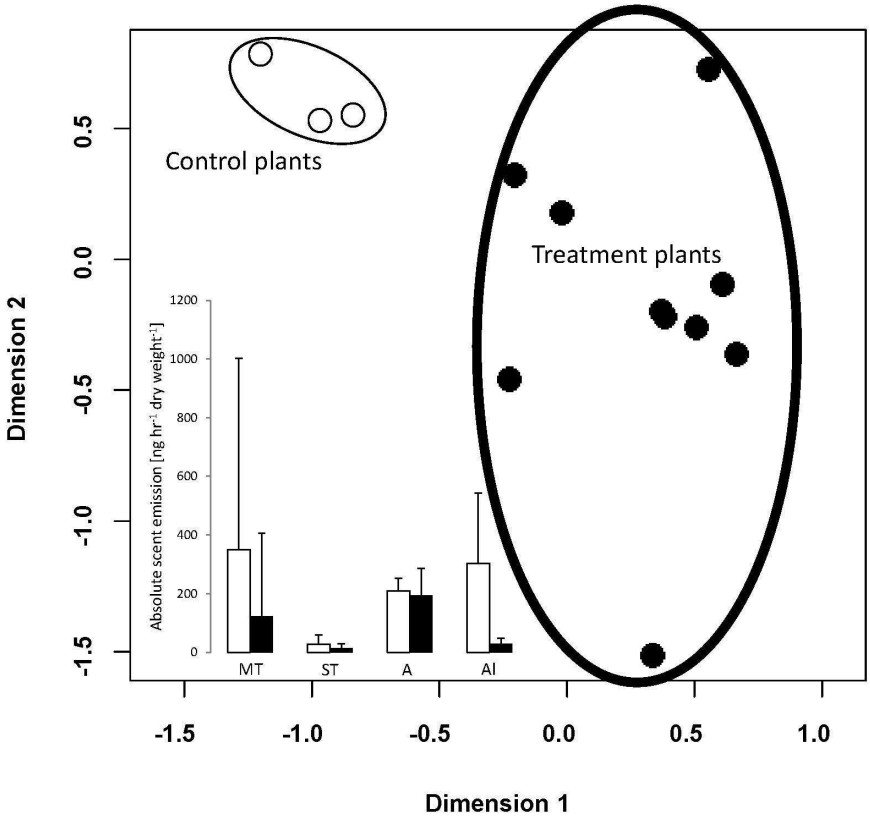

FIGURE 1. Nonmetric multidimensional scaling (NMDS) based on Euclidean distances of floral scent bouquets (proportional emission) from inhibited (treatment, filled circles) and control (open circles) *Achillea millefolium* plants 12 hours after starting the inhibition. The bar graph shows the absolute scent emission (mean ± SD) of monoterpenes (MT), sesquiterpenes (ST), aromatics (A) and aliphatic compounds (Al) from control (white bars) and inhibited (black bars) plants.

Flight cage choice tests

In choice tests where flies and bumblebees were allowed to choose between flowers of inhibited and control *A. millefolium* plants, flies showed a preference for control flowers in landing approaches (lme: $Df = 6$, F-value = 6.99, $P = 0.04$; Fig. 2) and landings (lme: $Df = 6$, F-value = 6.99, $P = 0.04$; Fig. 2). Bumblebees preferred to land on flowers with an inhibited scent bouquet (lme: $Df = 9$, F-value = 6.37, $P = 0.03$, Fig. 2) whereas they were indecisive in landing approaches but with a tendency towards inhibited flowers (lme: $Df = 9$, F-value = 1.50, $P = 0.25$; Fig. 2).

DISCUSSION

Pathway-specific inhibitors of the biochemical pathways towards mono- and sesquiterpenoid production affected the floral scent emission of *Achillea millefolium* plants. Nearly all scent compounds of the targeted biochemical pathways (mono- and sesquiterpenoids) were affected in a way as expected, i.e. their emission rates were reduced in inhibitor-treated plants compared to control plants. Thus, the scent bouquets of control and treatment plants were clearly different. According to the hypothesis that floral scents affect the behaviour of flower-visiting animals, bumblebees and flies discriminated between control and inhibited flowers of *A. millefolium* in choice tests in a flight cage. Bumblebees that usually do not visit *A. millefolium* flowers in the field (Larue et al. 2016) preferred inhibited flowers; flies, common visitors of *A. millefolium* flowers, preferred control flowers emitting a natural scent bouquet. Note that the species used in the experiments are not the same species as observed in the field study but represent close relatives. These results support the notion that floral scents are important traits in controlling the identity and frequency of flower visitors in interactions with plant species (Junker et al. 2010; Larue et al. 2016).

The inhibition of the pathways led to a significant reduction in the emission rates of many mono- and sesquiterpenes, but not to scent bouquets deprived of terpenoids. It is debated whether animals respond to whole bouquets (ratios of the compounds) or to key compounds that mediate specific functions such as attraction or repellence. It has been shown that in specialized and generalized pollination systems either individual key-compounds or a small number of volatiles mediate the attraction to flowers (Wright & Smith 2004; Schäffler et al. 2015). Likewise, floral scent bouquets may have a defensive function due to the emission of individual compounds (Junker et al. 2011). Other examples, however, show that whole bouquets of volatile compounds in specific ratios are used to locate host plants (Bruce et al. 2005). Although we observed clear preferences of both, bumblebees and flies, for treatment or control plants, respectively, our data do not allow to clearly discriminate between the mode of action of floral scent bouquets in our system. Potentially, terpene inhibition reduced the emission rate of a compound that functions as repellent for bumblebees or as attractant for flies. Alternatively, the inhibition clearly altered the ratio of emission rates of the compounds synthesized by *A. millefolium*, which may have resulted in modified behavioural responses. Future studies require additional experiments potentially using synthetic blends of volatiles to

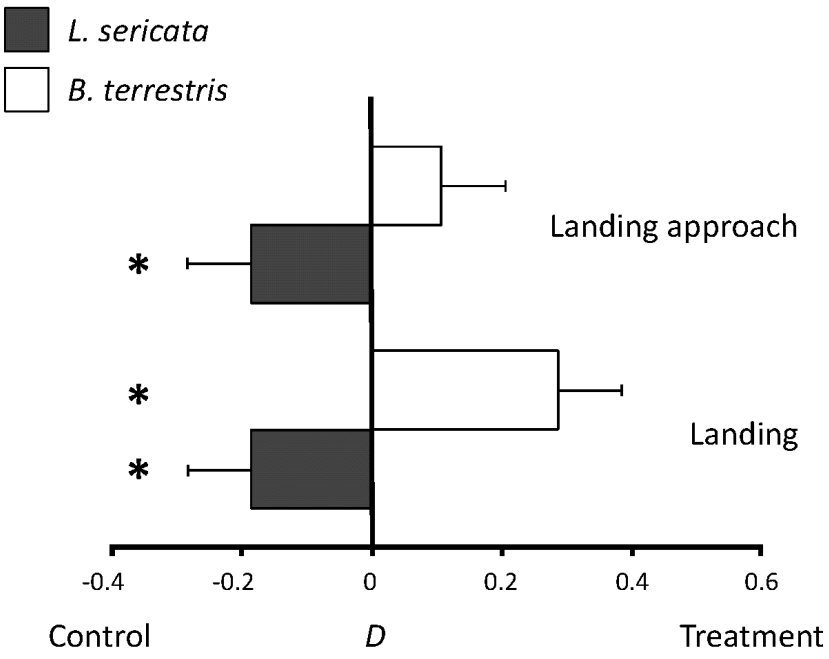

FIGURE 2. Results of flight cage choice tests: Responses of *Lucilia sericata* (dark grey bars, $N = 11$) and *Bombus terrestris* (white bars, $N = 17$, D, mean ± STD) to flowers of *Achillea millefolium* plants with natural scent bouquets (control) or scent bouquets with inhibited terpene-pathways (treatment). D is defined as the proportional number of insects visiting flowers of treatment plants minus the proportional part of insects visiting control flowers. To test whether responses (D) significantly deviate from zero we calculated linear mixed effect models (LME) and tested against the null hypothesis ($D = 0$). Significant deviations are indicated by asterisks * $P < 0.05$.

fully reveal the mode of action of attraction and repellence in generalized systems.

The experimental manipulation of floral scent bouquets is a powerful tool to study the relevance of volatiles in flower-visitor interactions. Our data confirm that both positive and negative responses by insects to volatiles contribute to the behaviour of flower visitors and thus visitation patterns. This allows flowers that do not have morphological barriers (such as *A. millefolium*) to select flower visitors from the locally available spectrum to facilitate the visitation by some animals but reduce or prevent the visitation of others. Therefore, floral scents are potent floral filters with strong effects on the behaviour of consumers of floral resources.

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REFERENCES

Adams RP (2007) Identification of essential oil components by gas chromatography / mass spectrometry, 4 edn. Allured Publishing Corporation, Carol Stream, Illinois.

Bruce TJA, Wadhams LJ, Woodcock CM (2005) Insect host location: a volatile situation. Trends in Plant Science 10:269-274.

Dixon P (2003) VEGAN, a package of R functions for community ecology. Journal of Vegetation Science 14:927-930.

Dudareva N, Andersson S, Orlova I, Gatto N, Reichelt M, Rhodes D, Boland W, Gershenzon J (2005) The nonmevalonate pathway supports both monoterpene and sesquiterpene formation in snapdragon flowers. Proceedings of the National Academy of Sciences of the United States of America 102:933-938.

Junker RR, Blüthgen N (2010) Floral scents repel facultative flower visitors, but attract obligate ones. Annals of Botany 105:777-782.

Junker RR, Gershenzon J, Unsicker SB (2011) Floral odour bouquet loses its ant repellent properties after inhibition of terpene biosynthesis. Journal of Chemical Ecology 37:1323–1331.

Junker RR, Höcherl N, Blüthgen N (2010) Responses to olfactory signals reflect network structure of flower-visitor interactions. Journal of Animal Ecology 79:818-823.

Kessler D, Gase K, Baldwin IT (2008) Field experiments with transformed plants reveal the sense of floral scents. Science 321:1200 - 1202.

Kita T, Brown MS, Goldstein JL (1980) Feedback-regulation of 3-hydroxy-3-methyglutaryl coenzyme a reductase in livers of mice treated with mevinolin, a competitive inhibitor of the reductase. Journal of Clinical Investigation 66:1094-1100.

Larue A-AC, Raguso RA, Junker RR (2016) Experimental manipulation of floral scent bouquets restructures flower–visitor interactions in the field. Journal of Animal Ecology 85:396-408.

Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team (2015) nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-120.http://CRAN.R-project.org/package=nlme.

R Development Core Team (2013) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna.

Schäffler I, Steiner KE, Haid M, van Berkel SS, Gerlach G, Johnson SD, Wessjohann L, Dötterl S (2015) Diacetin, a reliable cue and private communication channel in a specialized pollination system. Scientific Reports 5:12779.

Schiestl FP (2010) The evolution of floral scent and insect chemical communication. Ecology Letters 13:643-656.

Wright GA, Smith BH (2004) Variation in complex olfactory stimuli and its influence on odour recognition. Proceedings of the Royal Society of London Series B-Biological Sciences 271:147-152.