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# Floral scent terpenoids deter the facultative florivore *Metrioptera bicolor* (Ensifera, Tettigoniidae, Decticinae)

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## Abstract

Non-pollinating florivores (animals feeding on floral resources) represent severe antagonists that have negative impacts on the plants' reproduction. Plants would thus benefit by excluding them from their flowers. In this study, we tested whether floral scent compounds that are attractive to many pollinators, also have the potential to prevent facultatively flower-feeding herbivores from consuming flowers. For feeding trials, we chose the bush cricket *Metrioptera bicolor*, a species that mainly consumes grasses, but also feeds occasionally on flowers. Linalool and  $\beta$ -caryophyllene (mono- and sesquiterpenoid, respectively) turned out to be effective antifeedants, while other floral scent compounds had no effect. Furthermore, bush crickets completely rejected flowers of *Convolvulus arvensis* (Convolvulaceae) and *Melilotus alba* (Fabaceae), while preferring flowers of *Echium vulgare* (Boraginaceae) over leaves. In addition to feeding experiments, excrement of bush crickets and other orthopterans were searched for pollen. Most individual bush crickets had pollen in their faeces, largely from Poaceae and Gymnosperms, suggesting accidental ingestion of wind-dispersed pollen, rather than targeted consumption of floral pollen. Our results support the hypothesis of a dual role of floral scents in attraction and defence.

## Key words

$\beta$ -caryophyllene, flower defence, herbivory, linalool, Orthoptera, pollen, terpenoids

## Introduction

Recently, it was demonstrated that an undescribed orthopteran species of the family Gryllacrididae functions as an effective, and probably exclusive, pollinator of the orchid *Angraecum cadetii* on the islands of Mauritius and Reunion (Micheneau *et al.* 2010). This tight mutualism perhaps represents an exceptional system: usually, orthopterans in more generalized systems do not contribute to pollination and although they are frequent-flower visitors to some plants, have negative effects on these. The consumption of flowers by herbivores can have detrimental effects on the plants' reproduction (McCall & Irwin 2006), either through the destruction of anthers and stigmas (Kerner 1879) or by the reduction of flower attractivity by feeding on petals (Krupnick & Weis 1999, Krupnick *et al.* 1999).

It is hypothesized that the reproductive fitness of plants increases if floral traits are simultaneously attractive for mutualists and defensive against antagonists (Brown 2002, Irwin *et al.* 2004, Junker & Blüthgen 2010a). Accordingly, floral resources should often be toxic, unpalatable or unreachable for exploiters that would otherwise consume nectar, pollen or petals without transferring pollen from one plant individual to another (Dobson & Bergstrom 2000, Johnson *et al.* 2008, Galen & Cuba 2001).

Different mechanisms involving floral scents that have the potential to exclude certain taxa from florivory have been proposed: 1) Euler and Baldwin (1996) and Kessler *et al.* (2008) demonstrated that certain floral secondary metabolites are produced at different locations in *Nicotiana attenuata* (Solanaceae) and that these interact with different types of flower visitors: the defensive nicotine at a basal part and the attractive benzyl acetone at the outer corolla. Thus, different infochemicals affect flying and crawling flower visitors. 2) It has been proposed that diurnal scent emission rhythms correspond to activity patterns of mutualists, but not antagonists (Theis *et al.* 2007). The emission of floral scents that would attract both pollinators and antagonists may be reduced at times when the latter are most active (Euler & Baldwin 1996, Theis *et al.* 2007). 3) The same floral scent compounds may serve both functions together: attract pollinators and repel antagonists. For instance, linalool attracts bees (Harrewijn *et al.* 1995) and butterflies, but also efficiently repels ants from stealing nectar (Junker & Blüthgen 2008), suggesting a dual function of this floral scent compound (Junker *et al.* 2010).

Junker and Blüthgen (2010a) propose that the dependency on floral resources determines whether an animal is attracted or repelled by floral scents. Obligate flower visitors that depend on floral resources usually are attracted to floral scents; facultative flower visitors that have a broad dietary spectrum, are often repelled or deterred by secondary metabolites produced by flowers and thus may predominantly feed on nonfloral resources (Junker & Blüthgen 2010a, b).

In this study, we tested whether floral scent compounds that are attractive to common pollinators have the potential to prevent an orthopteran herbivore from consuming petals, pollen or nectar. We chose *Metrioptera bicolor* (Ensifera, Tettigoniidae, Decticinae), a species that feeds on grass, various herbaceous plants and small insects, but also occasionally on the flowers of some species (Ingrisch & Kohler 1998). Despite this highly generalist diet, *M. bicolor* does not fully develop without grass as a principal food (Ingrisch 1976) and thus represents a facultative consumer of floral resources. This bush cricket was furthermore allowed to choose between leaves and flowers of three plant species to reveal potential preferences. In addition, by searching for pollen in excrements of different species of bush crickets and grasshoppers, including *M. bicolor*, we quantified the natural utilisation of floral resources by various other Orthoptera.

## Materials and Methods

**Organisms.**—For laboratory trials, we used the two-colored bush cricket *Metrioptera bicolor* (Philippi 1830), a medium-sized (body

length: 15 - 18 mm), thermo- and xerophilic bush cricket. *M. bicolor* mainly inhabits semiarid grassland, but can also be found on juniper heath or poor and sandy grasslands (Detzel 1998). Individuals of *M. bicolor* were sampled at four sites in semi-arid grasslands in Northern Bavaria, Germany (nature reserve "Hohe Wann", and in Würzburg) and kept separately in gauze cages (20×20×29 cm). All bush crickets were kept in a climate chamber under long-day conditions (day/night: 14h/10h, 26°C/19°C) with a constant humidity at 50%. Water was sprayed two times daily at one side of the cages to provide drinkable water for the animals.

**Dual-Choice Tests.**—We explored the responses of *M. bicolor* to five different floral scent compounds. Selected substances represent widespread and dominant floral scent compounds from a broad spectrum of plant species, e.g., linalool occurs in 70% of all plant species sampled so far (Knudsen *et al.* 2006). Each component was offered within an artificial food as substrate (wafers: Hoch Oblaten-Bäckerei, Miltenberg, Germany,  $\phi = 44$  mm; ingredients: wheat flour, starch) and compared against an untreated wafer in a dual-choice test. For this purpose, substances were diluted in acetone (p.a.) and 666  $\mu$ l of the solution were applied on a wafer ( $\sim 330$  mg) for the treatment; pure acetone was applied for the control. After acetone entirely evaporated, a section of the treatment and control wafer (1/4 of the circumference) was placed in the cages in an upright position. Substances were applied in different concentrations, ranging from 1 to 100 mMol kg<sup>-1</sup> of wafer. For linalool, for example, this means that 1285.4 to 12.9 ng were offered per quarter of a wafer, which is within the range of mean hourly production of this substance by individual flowers (see e.g., Andersson *et al.* 2002). In addition to the floral scent compounds, we also tested the effect of a flavonoid (quercetin dihydrate), a floral pigment, on the feeding behavior of the bush crickets.

In a second test series, three insect pollinated plant species were used that occur in the same habitat as the bush crickets: *Convolvulus arvensis* L. (Convolvulaceae), *Echium vulgare* L. (Boraginaceae) and *Melilotus alba* Desr. (Fabaceae). All of these species are visited and pollinated by several insect taxa (Waddington 1976, Rademaker *et al.* 1999, unpub. obs.). Approximately equal amounts of leaves and flowers were offered to *M. bicolor* in a dual-choice test, in order to compare the bush crickets' consumption of vegetative and reproductive plant parts of the plant species. Each individual was fed with leaves and flowers from the same plant individual. Leaves and flowers were provided in an upright position in wet foam blocks to maintain their moisture during the trial.

In a third experiment, extracts of flowers and leaves provided on wafers, were also offered in dual-choice tests. Extracts were prepared and applied in the following way: oven-dried plant material (60°C for at least 3 d) was ground in a mortar into a fine powder. This powder was extracted with hexane and subsequently with acetone for 24 h each (2 ml solvent 100 mg<sup>-1</sup> powder) and shaken several times. Supernatants were stored in a freezer (-18°C) until use. Hexane and acetone fractions were concentrated to 8 ml using an air stream and were both applied subsequently to the same wafer. The amount of extract applied to each wafer was chosen to represent the natural concentration of substances in leaves and flowers, respectively (*i.e.*, the mass of plant material extracted corresponded to the wafer mass offered). After both solvents entirely evaporated, a section of each wafer (1/8 of the circumference) was placed in the cages in an upright position.

Each experiment lasted for 24 h. Most individuals were used in several consecutive trials, but not repeatedly for the same treatment.

Between subsequent trials, bush crickets were fed with grass seeds, fish food and fresh plant material for at least 24 h. Before and after each trial, plant material or wafer pieces were scanned digitally to acquire respective areas (pixels) and consumed area was obtained by subtraction from the original area. Dry mass consumption was calculated from consumed area using specific dry weight (mg pixel<sup>-1</sup>) for which 10 to 20 leaves, flowers and wafers were oven-dried at 60°C for at least 3 d and weighed. Individuals that did not feed at all in an experiment were excluded from the statistical analysis of the choice tests.

**Statistical analysis of dual-choice tests.**—Proportions of the consumed biomass of the treated wafers or flowers to the total consumption (treatment + control wafer or flower + leaf) were calculated for each replicate. Therefore, values larger than 0.5 indicate a preference for flowers or treatment, and values below 0.5 a preference for leaves or control. We used generalized linear models (GLM with binomial error distribution) with these proportions as response variable. For the trials with chemical compounds, we used substance, concentration of substance (mMol) and sex of bush crickets as explanatory variables. In the second analysis, plant species, treatment (fresh plant material or wafers treated with extracts) and sex of bush crickets were chosen as explanatory variables.

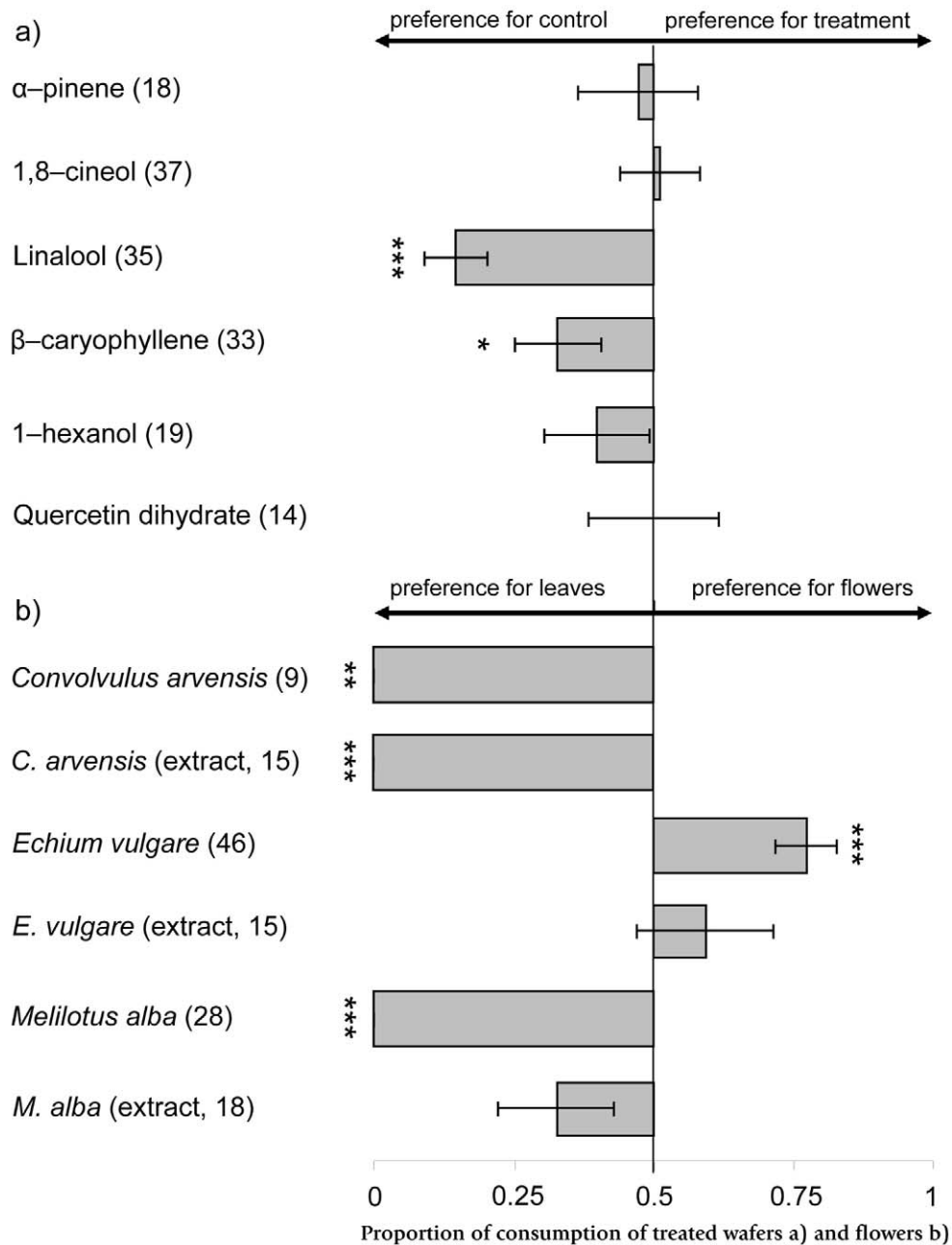
Beginning with the full model containing all explanatory variables, the models were reduced stepwise and each reduced model was compared with the previous one with a *chi*<sup>2</sup>-test (Crawley 2005). Additionally, proportions of flower or treatment consumption were individually tested against the null hypothesis (assuming equal consumption of flower and leaves/treatment and control, *i.e.*, proportion = 0.5) with a Wilcoxon test. Significant values were corrected for multiple tests by false discovery rate (FDR, Benjamini & Hochberg 1995). All statistical analyses were performed using R 2.4.0 (R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria).

**Excrement analysis.**—In order to quantify the importance of floral resources for orthopterans in their natural habitat, excrement of 40 *M. bicolor* individuals and 16 other orthopterans from 5 species (*Chorthippus biguttulus* L., 6 samples; *Chorthippus dorsatus* Zetterstedt, 5; *Conocephalus discolor* Thunberg, 1; *Gomphocerippus rufus* L., 1; *Phaneroptera falcata* Poda, 3) were scanned for pollen. Animals were caught on a flower-rich fallow field in Würzburg (*M. bicolor* in May / June 2008; the others in July 2007), and placed individually in containers. After two days, excrement pellets were collected and stored in a freezer (-18°C) until inspection for pollen. Two faecal pellets per orthopteran were pooled and dissolved in 100  $\mu$ l of water using ultrasound. One aliquot of this solution was placed on an object slide and the amount of pollen estimated using a light microscope.

## Results

**Dual-choice tests.**—Linalool as well as  $\beta$ -caryophyllene significantly deterred the bush crickets from feeding upon the wafers, while all other substances tested ( $\alpha$ -pinene, 1,8-cineol, hexanol and quercetin dihydrate) did not evoke any significant deterrence (Fig. 1a). The different substances affected the bush crickets' food choice, while the other factors, including the concentration of substances (mMol), had no effect on feeding decision (Table 1a).

Overall, in a high proportion (43.1%) of all trials, individual bush crickets failed completely to feed on the food items offered. In trials



**Fig. 1.** Feeding preferences of bush crickets in dual-choice tests. Preferences were measured as the proportion of dry mass consumption of wafers treated with compounds or of flower extracts to the total consumption (treated plus control wafers), or flowers, respectively. Shown are mean and standard error. In the upper part (a), effects of single floral-scent compounds on consumption by *M. bicolor* are shown. Floral scent compounds and one flavonoid (quercetin dehydrate) were tested against untreated wafers. Below (b), preferences between fresh flowers and leaves and flower extracts versus leaf extracts are shown. Significant deviation from an equal consumption of flowers vs leaves, or treatment vs control indicated by asterisks according to paired Wilcoxon rank sum test (proportion was tested against 0.5, FDR corrected) are given (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

where wafers were offered, rejection rate did not vary significantly between the different substance treatments (mean  $\pm$  SE = 29.2  $\pm$  5.1 %,  $Chi^2 = 3.3$ ,  $p = 0.65$ ) and extracts (mean  $\pm$  SE = 67.7  $\pm$  3.4 %,  $Chi^2 = 0.5$ ,  $p = 0.77$ ). In the trials where fresh leaves and flowers were offered, the rejection was highest in trials with *Convolvulus arvensis* (88.75%), followed by *Melilotus alba* (50%) and *Echium vulgare* (0 %;  $Chi^2 = 27.0$ ,  $p < 0.001$ ). However, bush crickets that fed on food items showed a strong and highly significant prefer-

ence for leaves over flowers in *C. arvensis* and *M. alba*, while they significantly preferred flowers over leaves in *E. vulgare* (Fig. 1b). In trials with extracts of *C. arvensis* the preference for leaves was confirmed. Wafers treated with flower extracts from *E. vulgare* and *M. alba* were not significantly more or significantly less consumed than wafers treated with leaf extracts (Fig. 1b). Plant species and the interaction term plant  $\times$  treatment had a significant influence on the feeding behavior, while sex, treatment and the other interaction

**Table 1.** Results from generalized linear model (GLM with binomial error distribution) analysis on data of the proportional consumption of wafers treated with individual substances (a) and on consumption of flowers or wafers treated with extracts (b) by *Metricoptera bicolor*. Starting with the full model containing all explanatory parameters, each reduced model was compared with the previous one with a  $\chi^2$  test resulting in deviance, degree of freedom ( $df$ ) and significance ( $p$ ) for each parameter.

a) Parameter	Deviance	$df$	$p$
Substance $\times$ mMol $\times$ Sex	16.89	11	0.11
Sex	0.34	1	0.56
mMol $\times$ Substance	4.23	5	0.52
mMol	1.64	1	0.2
Substance	13.91	5	<b>0.02</b>
Residual error	124.95		
Total	161.95		

b) Parameter	Deviance	$df$	$p$
Plant $\times$ Treatment $\times$ Sex	7.39	5	0.193
Sex	0.02	1	0.876
Plant $\times$ Treatment	12.45	2	<b>0.002</b>
Treatment	0.51	1	0.477
Plant	44.65	2	<b>&lt;0.001</b>
Residual error	25.84		
Total	90.85		

term, did not influence the choices of the bush crickets (Table 1b). Sample sizes and mean consumption [mg dry weight] in all trials are shown in Table 2.

**Excrement analysis.**—Pollen was found in 85% of all faecal samples from *M. bicolor* in various amounts. More than the half (58%) of the samples contained pollen from Poaceae, 28% from gymnosperms, 20% angiosperms (including Asteraceae, *Galium* sp., *Knautia* sp. and *Plantago lanceolata*; percentages add to more than 100% because some samples contained two different types of pollen). Half of the faecal samples from the other orthopterans (eight out of 16 individuals) did not contain pollen (three of six *Chorthippus biguttulus* specimen and all five *Chorthippus dorsatus*). Samples from four individuals (three *C. biguttulus* and one *Gomphocerippus rufus*) contained low amounts of pollen, suggesting that they accidentally ingested pollen rather than specifically fed on flowers. Only four samples (all three *Phaneroptera falcata* and one *Conocephalus discolor*) contained abundant pollen grains. Two individuals of *P. falcata* were caught on flowers (*Daucus carota*, Apiaceae and *Picris hieracioides*, Asteraceae) while consuming pollen or other flower tissues. Excrement of two *P. falcata* contained pollen, one from Asteraceae, the other individual a mixture of Asteraceae and Apiaceae pollen. Faeces of *C. discolor* contained pollen from Apiaceae.

## Discussion

Most orthopterans feed mainly on grasses, herbaceous plants and insects and occasionally on flowers (Ingrisch & Kohler 1998, Schuster 1974). Nymphs and adults of *Phaneroptera falcata* can be occasionally observed feeding on some Asteraceae, Apiaceae and Ranunculaceae flowers (pers. obs.). Florivory by the orthopterans seems to be restricted to a narrow taxonomic range, as suggested by the pollen analysis in faecal pellets, the feeding trials of this study and also by data in the literature (Ingrisch & Kohler 1998, Schuster 1974).

Although faecal samples of *M. bicolor* often contained large

**Table 2.** Mean consumption [mg dry weight] and standard deviation of (a) wafers treated with six substances<sup>1</sup> and untreated wafers or (b) flowers and leaves of three plant species<sup>2</sup>. Concentrations [mMol kg<sup>-1</sup>] of applied substances are given in brackets. Note that concentrations did not influence the decision made by the bush crickets (Table 1b).  $n$  = sample size of each trial.

a) Substance	n	treatment	control
$\alpha$ - pinene (1, 20, 50)	18	4.0 $\pm$ 7.0	4.2 $\pm$ 5.2
1,8 - cineol (1, 20, 50)	37	5.1 $\pm$ 5.9	4.1 $\pm$ 4.7
Linalool (1, 10, 50, 100)	35	1.3 $\pm$ 4.3	6.2 $\pm$ 5.0
( $\beta$ - caryophyllene 1, 5, 10, 20, 50)	33	2.8 $\pm$ 4.6	6.2 $\pm$ 5.4
1 - hexanol (1, 10, 20)	19	5.7 $\pm$ 6.5	7.7 $\pm$ 5.8
Quercetin dihydrate (1, 20, 50)	14	4.0 $\pm$ 4.5	5.4 $\pm$ 6.3

b) Plant species	n	flowers	leaves
<i>Convolvulus arvensis</i>	9	0 $\pm$ 0	2.0 $\pm$ 2.0
<i>Convolvulus arvensis</i> (extract)	15	0 $\pm$ 0	1.3 $\pm$ 0.9
<i>Echium vulgare</i>	24	3.9 $\pm$ 2.3	2.3 $\pm$ 5.1
<i>Echium vulgare</i> (extract)	7	5.8 $\pm$ 6.1	4.3 $\pm$ 4.8
<i>Melilotus alba</i>	12	0 $\pm$ 0	3.8 $\pm$ 1.7
<i>Melilotus alba</i> (extract)	9	7.2 $\pm$ 7.8	5.1 $\pm$ 5.1

<sup>1</sup> Substances belong to four chemical classes: Monoterpenoids ( $\alpha$  - pinene, 1,8 - cineol and linalool), sesquiterpenoid ( $\beta$  - caryophyllene), aliphate (1-hexanol), flavonoid (quercetin dehydrate).

<sup>2</sup> Plant species belong to three plant families: Convolvulaceae (*C. arvensis*), Boraginaceae (*E. vulgare*), Fabaceae (*M. alba*).

amounts of pollen from grasses and gymnosperms, this species cannot be regarded as palynivorous. Pollen from such wind-pollinated plants is often scattered on leaf surfaces and is unlikely to be harvested directly from flowers. These kinds of pollen may have been either accidentally ingested while consuming leaves or perhaps grazed from the leaf surface; this is also suggested by the fact that no gymnosperms were present close by the field site where the orthopterans were caught. Pollen from insect-pollinated angiosperms was rare in *M. bicolor* faeces, but more frequent in some other orthopterans.

The taxonomically restricted occurrence of florivory suggests that flowers of other taxa are either less palatable to orthopterans or defended against them. Floral adaptations as protection against nonpollinating florivores have been proposed by some authors (*e.g.*, Frame 2003, Dobson & Bergstrom 2000), but examined in a few case studies only. It has been suggested that the function of floral scents is not restricted to pollinator attraction (Raguso *et al.* 2003, Raguso 2008, Junker & Bluthgen 2010a), but also includes herbivore repellence (Pellmyr *et al.* 1991, De Moraes *et al.* 2001), which may have been the primary function during the early diversification of angiosperms in the Cretaceous (Frame 2003).

In feeding trials with wafers treated with floral scent compounds we tested the hypothesis that pollinator-attracting substances also have deterrent/repellent effects on *M. bicolor* that may be detrimental for the plants when feeding on flowers with no pollination service (Schuster 1974, Kerner 1879). We chose floral-scent compounds that are produced by a large number of flowering species (Knudsen *et al.* 2006) and/or are assumed to attract pollinators. For example, attraction is evoked by  $\alpha$ -pinene in moths (Cunningham *et al.* 2004), 1,8-cineol in moths and euglossine bees (Schiestl & Roubik 2003, Raguso & Light 1998), linalool in bees and butterflies (Andersson *et al.* 2002, Laloi *et al.* 2000), and  $\beta$ -caryophyllene in butterflies (Andersson *et al.* 2002); 1-hexanol, which is also a green-leaf volatile, attracts herbivores (Reinecke *et al.* 2002). Terpenoids are often the

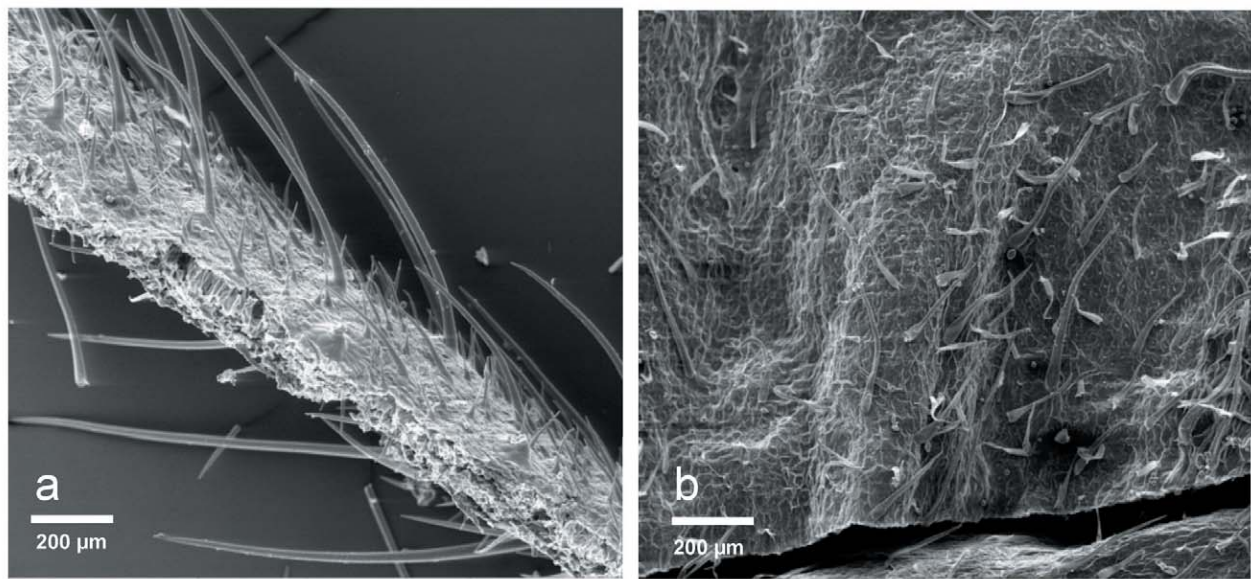


Fig. 2. Scanning electron microscope photographs of leaves (a) and petals (b) of *E. vulgare*. Leaves and flowers were oven dried (30°C, 2 d) prior to preparation for SEM.

dominant chemical class in floral scent compositions (Knudsen *et al.* 2006) and are known to have toxic, deterrent and antimicrobial functions in plant defences (Gershenzon & Dudareva 2007).

Our results demonstrate that monoterpenoids (linalool) as well as sesquiterpenoids ( $\beta$ -caryophyllene) may serve as an antifeedant against herbivorous insects. Two other monoterpenoids ( $\alpha$ -pinene and 1,8-cineol) and one aliphate (1-hexanol) did not affect the consumption by the bush crickets.

However, this outcome may be explained by the different volatility of floral scent compounds used for the tests. Since dual-choice tests ran for 24 h, substances with a relatively high boiling point (linalool and  $\beta$ -caryophyllene) were more likely to retain their effective dose during the whole period of time. On the other hand, the applied quantity of substances did not affect the choices of *M. bicolor*, suggesting that even very low amounts (*i.e.*, 1 mMol kg<sup>-1</sup>) of certain floral scent compounds have deterrent/repellent properties. Thus floral resources may be unpalatable for orthopterans due to the emission of floral scents that are either adapted to attract pollinators or as a defence against antagonists.

In contrast, the Gryllacrididae species that pollinates the orchid *Angraecum cadetii* feeds on the floral nectar, but does not destroy reproductive plant parts. Sequentially, the pollinia of the orchid are attached on the mouthparts of the cricket and the animals reliably transport the pollen to conspecific orchids indicated by the high percentage of fruit set (Micheneau *et al.* 2010). The authors of this study also report that these orchids emit a monoterpene-dominated bouquet, but in low quantities only (Micheneau *et al.* 2010).

Only a very few studies compare the palatability of flowers and leaves for phytophagous animals. *Pieris brassicae* (Lepidoptera) caterpillars, representing specialized consumers of Brassicaceae, prefer flowers of *Brassica nigra* over leaves of the same species. On flowers, these caterpillars achieved a higher growth rate, although flowers contained more defensive glucosinolates than leaves (Smallegange *et al.* 2007). However, results from such a highly specialized herbivore may not reflect the outcome in more generalized systems such as the one in our study.

We focused on a bush cricket (*M. bicolor*) with a generalized diet, a species known to occasionally feed on flowers besides vegetative

plant parts (Ingrisch & Kohler 1998). This species represents a potential, *i.e.*, unspecialized and facultative, consumer of floral tissues. The trials in which herbivores had the choice between flowers and leaves showed conflicting results: *M. bicolor* completely rejected flowers (fresh material) of *Convolvulus arvensis* and *Melilotus alba*. Extracts of flowers used in this bioassay are likely to contain both floral volatiles and other substances extracted from floral tissues. The chemical defences of flowers may thus include various kinds of deterrents, and some highly volatile components may have been missing from the extracts due to the oven-drying of plant material. This may also explain why *M. alba* had repellent flowers, yet the extract had no effect on the feeding choice.

Flowers of *Echium vulgare* (largely scentless, S. Dötterl, unpub. data) were preferred over leaves of the same plant species. In this case, mechanical defences may have played a major role in the decision of the bush crickets between leaves and flowers. Leaves of *E. vulgare* possess rigid trichomes, which are known as protective structures against herbivores in general (Valverde *et al.* 2001). The density of such trichomes was much higher on leaves than on flowers (Fig. 2). The other plant species used in this study do not feature any potentially defensive structures like trichomes.

Overall, our results add evidence to support the hypothesis that secondary floral metabolites serve as defensive traits against herbivorous animals that would otherwise have negative impacts on the plants' reproduction—and not only as attractive signals to pollinators.

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