

## Are eavesdroppers multimodal? Sensory exploitation of floral signals by a non-native cockroach *Blatta orientalis*

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**Abstract** The study of multi-modal communication has only recently been extended to innate and learned interactions between flowers and their animal visitors, and usually only to pollinators. Here we studied the relevance of floral scent and visual display of a night blooming, putatively hawkmoth-pollinated plant *Oenothera acaulis* (Onagraceae) in the attraction of non-native cockroaches *Blatta orientalis* (Blattodea: Blattidae), which function as facultative floral larcenists in coastal habitats of central Chile. We experimentally decoupled visual (corolla) and olfactory (fragrance) stimuli by presenting paper corollas and green mesh bags, with or without a freshly-picked natural flower inside. We then contrasted the behavioral responses of roaches in these treatments with those to the natural combination of traits in actual flowers and their respective control treatments, measuring the roaches' frequency of first visits, mean and total residence time spent in each treatment. The roaches primarily used olfactory cues when approaching *O. acaulis* flowers at two biologically relevant spatial scales. In addition, the presence of conspecific roaches had a strong influence on recruitment to the experimental arena, increasing the statistical differences among treatments. Our results suggest a primacy of floral fragrance over visual stimuli in the foraging responses of *B. orientalis*. Olfactory cues were necessary and sufficient to attract the roaches, and the visual cues presented in our manipulations only marginally increased their attraction within a 20 cm diameter of the stimulus. The full spectrum of floral visitation behavior was not elicited by the artificial flowers, suggesting the need for additional tactile or contact chemosensory stimuli not provided by paper. Although the nitrogenous scent compounds that we found in *O. acaulis* flowers are almost exclusively found in hawkmoth-pollinated flowers, the attractiveness of these compounds to a non-native, facultative flower-visiting insect indicates that they do not function as pollinator-specific signals for hawkmoth attraction [*Current Zoology* 57 (2): 162–174, 2011].

**Keywords** Flower fragrance, Corolla visual display, Larcenists, Night-blooming plants

Flowering plants use several sensory channels (olfaction, vision, mechanoreception, among others) to mediate reciprocally beneficial interactions with their animal pollinators (Raguso, 2004). Most studies of the sensory aspects of plant-pollinator interactions have been limited to single modalities of floral communication, usually visual cues (e.g., floral color, size, form), and less frequently chemosensory cues (e.g. floral scent, nectar chemistry and non-volatile surface chemistry) (rev. by Raguso, 2008a, 2008b). Despite this single-modality bias, there is growing support for the view that many animals, particularly insects, respond to integrated information from multiple sensory channels during foraging and host-finding (Steele, 2001; Henneman et al.,

2002; Schmidt et al., 2004; Blackmer and Can, 2005). Multimodal signaling has emerged as a major theme in the field of animal communication, with a recent review establishing a conceptual framework for distinguishing between alternative hypotheses concerning signal content, efficacy and interactions (Hebets and Papaj, 2005). Such hypotheses are just beginning to be explored in the context of flower-animal interactions (Kunze and Gumbert, 2001; Gegear and Lavery, 2005; Ōmura and Honda, 2005; Goyret et al., 2007). Several of the recent hypotheses developed by Hebets and Papaj (2005) are likely to be relevant to flower-visitor interactions. For example, among the content-based hypotheses outlined by these authors, we are likely to find that floral signals

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encode multiple messages, such that fragrance is learned as a species-specific aid to constancy (rev. by Wright and Schiestl, 2009) whereas flower size is positively correlated with nectar or pollen rewards (rev. by Strauss and Whittall, 2006). In this study, we will focus on more proximate aspects of multimodal signaling, including aspects that address signal detection in a natural environment and the potential for inter-signal interactions at a mechanistic ethological level of analysis (sensu Tinbergen, 1963).

The interactions between flowering plants and their visitors (such as pollinators, larcenists and parasites) are dynamic. Floral stimuli and the nutritious rewards sought by flower-foraging animals vary spatially and temporally (Chittka and Thomson, 2001) due to intrinsic (e.g., vegetation structure and individual plant size) and extrinsic (e.g., temperature, wind) factors, which together influence the effective expression of such attributes. This kind of background could be expected to lead to the integration of multiple flower-derived stimuli, providing a kind of contingency situation (Hebets and Papaj's "efficacy backup" hypothesis) for flower foraging animals. For example, in a windy, turbulent environment, where it is difficult for an animal to follow an odor at close range, enhanced reliance on visual signals can be expected to occur. Interactions between signals in different sensory channels may dramatically modify the behavioral responses of flower visitors, by raising the conspicuousness of the signals themselves (or the receiver's attention to them; Kunze and Gumbert, 2001) or by generating the appropriate context for a foraging decision (Goyret et al., 2008), and thereby enhancing the probability of resource location (see Hebets and Papaj, 2005). Through such mechanisms, floral scents and colors could have additive, redundant or synergistic effects on flower-visiting animals (Raguso and Willis, 2002; Goyret et al., 2008). However, despite of its potential impact, the application of animal communication theory to plant-pollinator interactions is still in its infancy (e.g. Schaefer et al., 2004).

This multimodal plant-animal signaling scenario could be even more complex if we now consider that most flowers are visited not just by pollinators, but by intricate assemblages of animals with diverse nutritional and reproductive imperatives. Floral nectar and/or pollen and oils may constitute their sole source of nutrition for obligate floral visitors (e.g., oligolectic bees; Gathmann and Tschardt, 2002), whereas facultative flower visitors (e.g., ants, crickets) may opportunistically supplement more generalized diets with flower parts or

products (Junker and Blüthgen, 2010). Furthermore, flowers often attract ambush predators, including mantids, assassin bugs and spiders (Dukas, 2001). These dynamic visitor spectra constitute miniature food webs of flowering plants visited by animals from different trophic levels, which may directly or indirectly affect a plant's reproductive success (e.g., Lara and Ornelas 2002; Gonçalves-Souza et al., 2008).

Here we explore the relationship between visual and olfactory signals produced by a night-blooming, putatively hawkmoth-pollinated plant *Oenothera acaulis* (Onagraceae) and the attraction of a nocturnal facultative flower-visiting insect *Blatta orientalis* (Blattaria: Blattidae). The South American evening primrose *O. acaulis* is a suitable candidate for testing ideas about multimodal plant-insect communication because it produces conspicuous visual and olfactory cues (see below) under variable photic and thermal conditions. The flowers open during the evening (around 1945 h) and remain turgid and with pollen and nectar until late morning (about 10 : 15 h) on the following day. The large size of *O. acaulis* flowers, coupled with their long hypanthial nectar tubes, bright coloration and conspicuous fragrance suggest that they, like their North American relatives, are hawkmoth pollinated (see Gregory, 1963; Raguso et al., 2007), and indeed occasional visits by native *Hyles annei* hawkmoths (Lepidoptera: Sphingidae) have been seen during nocturnal observations at our study populations in coastal Chile (C.A. Villagra, unpublished data). At night, these flowers are also visited by non-native *B. orientalis* cockroaches, and by native tettigoniid grasshoppers (Orthoptera), both of which approach and visit flowers by walking between plants. These two insects feed opportunistically on pollen and nectar from newly opened flowers. Thus, the floral phenotype and visitor spectrum of *O. acaulis* presents an opportunity to study multimodal floral signals that attract both obligate and facultative floral visitors, whose impact on plant reproductive success could range from beneficial to harmful.

In this paper we concentrate on *O. acaulis* floral cues in relation to the flower-visiting behavior of *B. orientalis* cockroaches in the central Pacific coast of Chile, where this interaction takes place during poorly lit conditions from twilight to dusk. We experimentally manipulated the presentation of visual (flower corolla) and olfactory (floral fragrance) stimuli in order to generate treatments that physically decouple these floral signals and to test whether visual and olfactory floral stimuli, both separately and combined, are necessary and sufficient for different aspects of floral visitation by roaches.

We discuss our results in the context of the evolution of multimodal signaling and the establishment and conservation of plant-insect interactions from pollination to florivory.

## 1 Materials and Methods

### 1.1 Biology of *Oenothera acaulis*

*O. acaulis* belongs to the plant family Onagraceae, a group of plants that has served as a model system for the study of plant reproductive ecology and evolution for over half a century (Raven, 1969). Like its close relatives from the *Lauvaxia* section (Raimann, 1893; Spach, 1835), *O. acaulis* has self-compatible, night-blooming flowers with long, nectar-filled hypanthial tubes and large pollen grains, which are attached to sticky viscin threads (see Raven, 1969). Flowers of *O. acaulis* show remarkable population variation in floral dimensions, including anther-stigma distances (herkogamy) suggesting that outcross pollen is required for fertilization in some sites (C.A. Villagra, unpublished data), and that this species has a mixed mating system. In Chile, *O. acaulis* occurs in coastal areas between IV and X Regions of Chile, and also in the sub Andean belt in the high Andes between 2200–2700 m (Arroyo et al., 1982).

### 1.2 Study sites

The manipulative experiments were carried out in a large coastal scrub population located at BioParque Piquén, Los Molles (32°14'16"S, 71°31'16"W), Petorca Province, V Region of Chile, between 13 Nov–18 Dec, 2009. Characterization of the spontaneous behavior of *B. orientalis* visiting *O. acaulis* flowers was based on observations made at the above field site and in a second coastal population located near Cachagua (32°34'30"S, 71°26'51"W), some 50km south of Los Molles.

### 1.3 Floral scent and spectral reflectance

To analyze the fragrance of *O. acaulis* flowers, we performed scent collections using the dynamic headspace method, which allows scent collection from living plant tissues (Raguso and Pellmyr, 1998). For these, we enclosed a living flower within a nylon resin oven bag (10 × 20 cm<sup>2</sup>, Reynolds, Inc., Richmond, VA), from which air was drawn using a battery-operated vacuum pump (Spectrex, Inc., Redwood City, CA) at a flow rate of c. 200 ml/min, in order to trap volatile-laden air within a glass Pasteur pipette packed with 10 mg of absorbent beads (Super Q polymer, Alltech Associates, Waukegan, IL). After 1 h of headspace collection, we extracted trapped volatile compounds with 300 µL of redistilled high-grade hexane (Merck, Inc., Lichrosolv

for Linear Chromatography) and stored the eluted sample in 2 ml amber vials, at –20°C. We analyzed the chemical composition of flower fragrance collected in the field by combining gas chromatography (GC; Perkin Elmer Clarus 500) with a mass detection spectrometer (MS; Perkin Elmer Clarus 560D) at the Depto. de Química, Universidad de Tarapacá, Arica, Chile, equipped with an elite-5 capillary column (30 m × 0.20 mm) and using Helium as a carrier gas. We identified unknowns by comparing the retention times with authentic standards of 3-methylbutyraldoxime and 2-methylbutyraldoxime (syn and anti forms, courtesy of Roman Kaiser, Givaudan, Switzerland). Furthermore, we compared mass spectra of GC peaks with the NIST 2005 database (mass spectral library GC-EM-NIST2005) for tentative identifications when the correlation index was greater than 98% (using Turbomass Perkin Elmer software). We quantified the compounds by integrating the area below each peak of the total ion chromatogram, and compared this area with the area obtained from known concentrations of a standard compound (3-methylbutanol, Sigma Aldrich, Inc.) interpolating the amount of nanomoles obtained from the calibration curves based on this external standard. This compound was chosen as the standard due to its structural similarity to the major compounds detected in the scent of *O. acaulis* flowers. Using these methods, we evaluated whether the freshly picked flower (NC treatment, below) and the freshly picked flower concealed within the bag (olfactory only treatment, “O” below) differed in the composition of the fragrance released from the treatment, as well as in the rates of emission. In addition, we tested whether the green mesh bags themselves were a source of detectable olfactory cues by also collecting scent from this material. Pilot data demonstrated that plucked flowers did not differ in scent composition or crude emission rate whether they were enclosed within mesh bags (O treatment) or placed above them (NC treatment) (Online Appendix 1).

We measured the spectral reflectance for *O. acaulis* in the field using a USB—2000+ Ocean Optic portable Spectrometer and Spectrasuite software. We used paper that best approximates these reflectance properties for the purposes of behavioral assays (Fedex-Kinkos, Inc., “grey fleck” resumé paper; see Goyret and Raguso, 2006).

### 1.4 Flower-visiting behavior of *B. orientalis* on *Oenothera acaulis*

We recorded the visitation behavior of *B. orientalis* to unmanipulated, intact flowers of *O. acaulis* with a

hand-held video camera using a red-light headlamp or with the infrared-detecting option on the camera (Sony, Inc.). Video recordings were made during 2008 at the Cachagua population ( $n = 24$ ) and during 2009 at the Los Molles population ( $n=12$ ) over nine different days, resulting in 11 hours of filmed cockroach behavior. We grouped insect responses into five classes of behaviors: (1) feeding on floral resources (i.e. pollen or nectar); (2) exploring (contacting the petals with its palpi); (3) interacting (with other cockroaches, typically antennal or leg contact); (4) grooming (eating pollen from its body) and (5) feeding on floral tissues (i.e., petals or reproductive parts (which was not observed during this study)). Details on how specific behaviors were assigned to these five general classes are provided in the online Appendix 2. For scoring and analyzing cockroach flower-visiting behavior, we used JWatcher 10.1 software (Blumstein and Evans, 2006), which allowed us to obtain the relative frequency and proportion of time spent on each behavioral event defined. The natural flower-visiting behavior was depicted as the frequency (events/minute) for each of the defined activities. Due to the fact that these behavioral data were not normally distributed, we first ran a two-way ANOVA, manually ranking the data before the analysis, searching differences of the total distribution of behaviors within sites, in order to test for the potential effects of study sites (Cachagua and Los Molles) on roach responses. There was no significant effect of study site (frequency;  $F_{3,310}=1.47$ ,  $P=0.213$ ; proportion of time;  $F_{9,310}=2$ ,  $P=0.096$ ). We thus performed Kruskal-Wallis and post hoc Tukey tests on the combined data on SPSS 17.

### 1.5 Manipulative decoupling of visual and olfactory floral cues

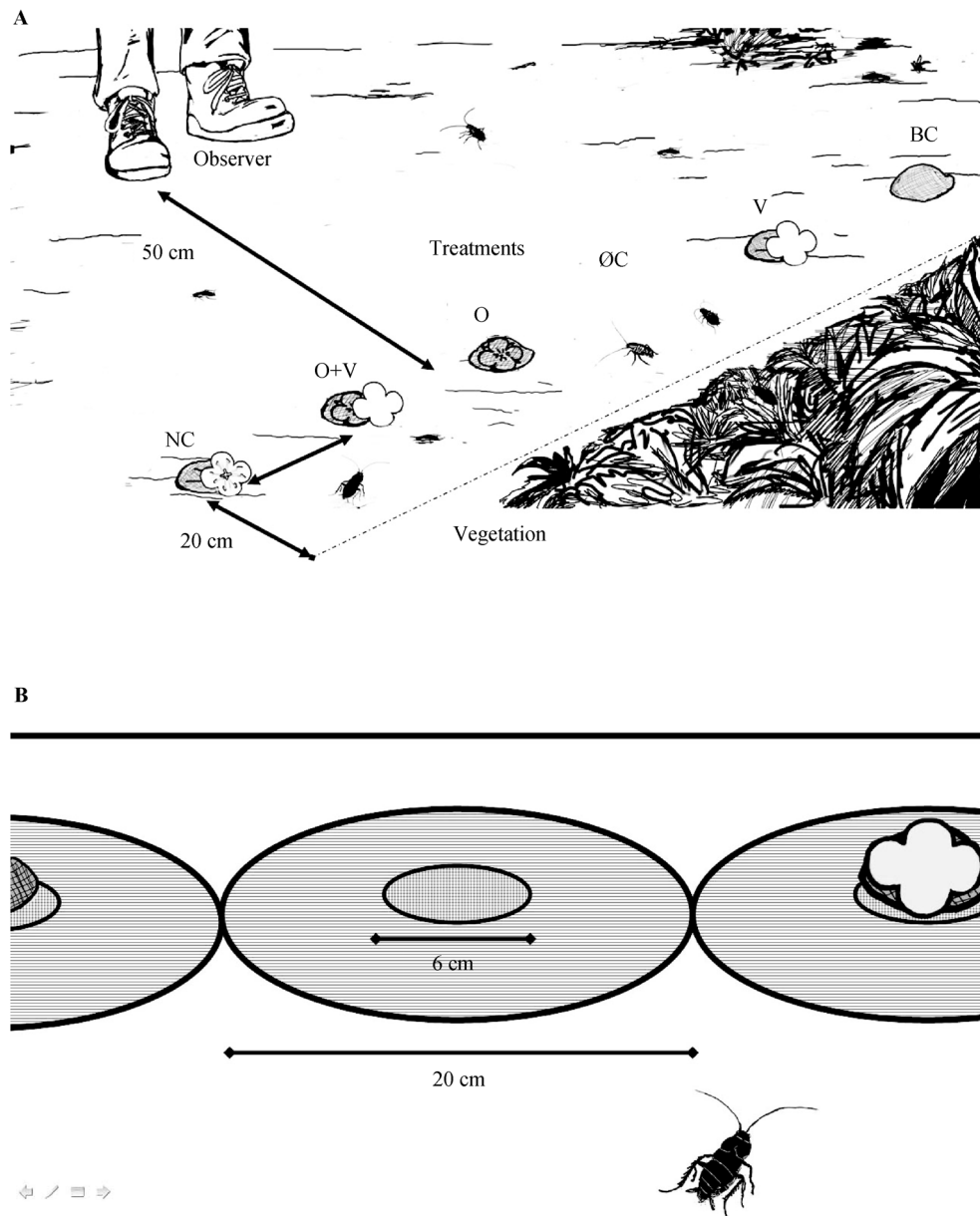
Based on previous manipulative experimental studies showing that hawkmoths synergistically use floral odor and visual contrast to find and feed from *Oenothera* and similar flowers (Raguso and Willis, 2002; Raguso and Willis, 2005; Goyret et al., 2007), and here we used a similar experimental approach to test whether the cockroaches use similar rules to find such flowers at night. This approach involves the experimental decoupling of visual and olfactory floral cues in the field, in order to explore the relative importance of visual and olfactory stimuli in cockroach visitation. Roaches were presented with different combinations of the two floral cues. Freshly-picked *O. acaulis* flowers placed inside green mesh nylon bags (10 threads/cm) were used to represent the olfactory cue in the absence of visual cues. Artificial corollas made from white paper were used to present the

visual cue in the absence of olfactory cues. Flowers were cut below the ovary (in order to avoid nectar loss) about 15 min before experiments began. To avoid possible differences in fragrance quantities potentially related to flower size (see Raguso, 2008b), we restricted our choice of flowers to those with a corolla diameter of  $6.0 \pm 0.5$  cm (mean  $\pm$  SEM). The paper corollas measured 6 cm in diameter.

We designed three experimental treatments and three controls treatments to experimentally manipulate floral stimuli. (1) "Normal" floral stimuli (olfactory and visual) were reconstituted (see Raguso and Willis, 2005) by placing the paper corolla model over the mesh-bagged freshly-picked flower (olfactory + visual = O+V). (2) An olfactory-only treatment was represented by the green mesh bag containing a hidden freshly-picked flower inside (olfactory = O). (3) The visual-only treatment was represented by placing a paper corolla model over an empty green mesh bag, without a freshly-picked flower inside (visual = V). (4) A null treatment consisted of an "empty" space ( $\emptyset$ C = controlling for the presence of active roaches on bare ground). (5) A freshly-picked flower was placed over the same mesh-bag used in the other treatments as a positive control for natural floral stimuli (natural control = NC). (6) An empty green mesh bag was used to control for any possible effect of the bagging material employed (bag control = BC).

The roaches were simultaneously exposed to the complete suite of treatments as shown in Fig. 1A. Experiments occurred on open ground between shrubs, at a minimum distance of 20 cm from vegetation to avoid the possible effects of stimuli from other plant species. Prior field observations of the natural flower-visiting behavior of these roaches showed that they typically circulated in cleared patches surrounded by taller vegetation, comprised of *Echinopsis chiloensis* and *E. litoralis* (Cactaceae), *Haplopappus foliosus* and *H. chrysantemifolius*. shrubs (Asteraceae) and *Puya venusta* and *P. chilensis* (Bromeliaceae) succulents among others. We selected similar locations for placement of our treatment arrays. Finally, before commencing observations, we removed all other *O. acaulis* flowers (including old and faded ones) from the experimental area.

The experimental and controls treatments were placed in random order 20 cm apart (Fig. 1A), so as to represent typical distances between *O. acaulis* plants in natural populations (D. Dominguez and C.A. Villagra, unpublished data). In order to avoid landmark learning by the roaches (see Durier and Ribault, 2000), the experimental array was moved to a different location each



**Fig. 1** Diagrams of experimental procedures

**A.** Schematic presentation of the experimental array and placement of human observers. The array was presented near foraging patches on open ground. Letters: O + V = olfactory & visual; O = olfactory only; V = visual only, ØC = empty ground or null control, NC = natural flower control (full complement of stimuli), BC = bag control. **B.** Dimensions and rationale for the defined “total” and “stimulus” areas of observation. Note that the actual order of treatment presentation was randomized in different replicates of this experiment.

night. For the quantification of roach visits to the different treatments, we defined two zones surrounding each experimental and control treatments (Fig. 1B). The “total” area (a 20 cm diameter) encompassed the portion of the experimental arena representing half the distance between each experimental stimulus and its (20 cm distant) flanking neighbors (Fig. 1A). The “stimulus” area (a 6 cm diameter) recreated the mean diameter of an *O. acaulis* flower (6 cm) surrounding the experimental and control treatments. For treatments with real

or artificial flowers, this essentially describes visitation to the flower. We considered these two areas as biologically meaningful for the study of cockroach flower-foraging as, in contrast with others flower visitors (that approach flowers in flight), our study organisms walk between flowering plants. This behavior is quite different from the rapid, in-flight approaches and visits scored in comparable studies (e.g., the stereotypical approach flights of hawkmoths; Raguso and Willis, 2005; Goyret et al., 2007), in that the roaches appear to spend more

time assessing the foraging options on foot. Thus, based on our preliminary observations, chose to evaluate sensory aspects of roach foraging behavior at these two distances from the experimental stimuli, which represent the floral stimuli and their immediate vicinity.

**Observation procedures** Three persons observed one experimental array for the 2 h duration of each experimental replicate. Observations were made at least 1 m from the experimental array (Fig. 1A), and observers could approach up to 50 cm only if they were not able to see the roach clearly, while stepping carefully to minimize vibrations (to which cockroaches are highly sensitive; Shaw, 1994). Each observation began at 21:30 h and ended at 23:30 h. We selected this time interval because during our preliminary observations we found that roach activity peaked at roughly 22:30 h. (data not shown). The light conditions during this time varied from twilight at the beginning of the experiments, to starlight or full moon light conditions at most of the time. The most frequent condition was starlight and overcast light, during this time we were unable to measure this due to technical limitation imposed by poor light conditions. Thus, the observation period bracketed an hour before and an hour after the roaches' activity peak. Observers were equipped with red-light headlamps, in order to minimize visual disturbance, as *B. orientalis* does not have red photoreceptors (Mazokhin-Porshnyakov and Cherkasov, 1985). Each observer scored the time when a cockroach approached any of the six treatments either at the "total" or the "stimulus" zone. We did not mark any of the cockroaches or make any other marks in the field to avoid additional artificial situations besides the treatments themselves. Because we did not mark the individual cockroaches, there is always a chance that some of the observed insect visits represented revisita-

tion. In total, we performed 23 replicates of the experiment. No insect visitors besides roaches were observed during the course of this experiment.

**Dependant variables** Since cockroaches moved among different treatments during the experiment, we tried to eliminate the influence of previous visitation on subsequent choice and evaluate only the first approach. We determined the frequency of initial cockroach approaches to each treatment during each trial, and **mean** times (min) spent by all visiting cockroaches in each area. The mean time was calculated as the averaged time spent by all cockroaches on a given treatment per replicate (following Roy and Raguso, 1997). Taken separately, not all the treatments were normally distributed, but we found normal distribution for the entire data set when all treatments were grouped (Kolmogorov-Smirnoff Normality test), thus we used untransformed data in our statistical tests. The data were analyzed using ANCOVA with the number of conspecific roaches in the experimental array as a random factor. This was done because we observed a large amount of variation in the number of cockroaches among experimental replicates, suggesting that we could potentially observe differences in behavior due to social interactions. In addition we tested all other potentially relevant variables (such as temperature, humidity, light condition, cohort, etc.) collected during our assays as random factors, but did not find any significance associated with these factors, neither individually nor in combinations (data not shown). The alpha value was adjusted to 0.015 to account for multiple comparisons inherent to the full model for 3 dependent variables (Tables 1, 2). For post hoc tests we performed the least squares differences test. All the analysis described above were performed with SPSS 17 statistic software.

**Table 1 Total area analysis results (20 cm diameter around experimental treatments)**

Total Area ANCOVA	Dependant Variable	df	F	P	Partial Eta Squared
Intercept	Frequency	1	28.638	*0.000	0.652
	Mean Time	1	39.223	*0.000	0.707
Treatment	Frequency	5	1.997	0.088	0.109
	Mean Time	5	5.173	*0.000	0.237
Treatment * N° Cockroaches	Frequency	75	4.353	*0.000	0.886
	Mean Time	75	3.387	*0.000	0.858

Significance at alpha = 0.015 is indicated with an asterisk. The intercept corresponds to the model, and partial eta squared is a standardized measure of effect size for each variable on the ANCOVA model.

**Table 2 Stimulus area analysis results ( 6 cm diameter around experimental treatments)**

Stimulus Area ANCOVA	Dependant Variable	df	F	P	Partial Eta Squared
Intercept	Frequency	1	22.342	*0.001	0.665
	Mean Time	1	9.653	*0.007	0.386
Treatment	Frequency	5	5.911	*0.000	0.334
	Mean Time	5	2.349	0.048	0.124
Treatment * N° Cockroaches	Frequency	55	8.229	*0.000	0.873
	Mean Time	55	1.126	0.320	0.484

Significance at alpha = 0.015 is indicated with an asterisk; marginally significant values are denoted with †. The intercept correspond to the model, corresponds to the model, and partial eta squared is a standardized measure of effect size for each variable on the ANCOVA model.

## 2 Results

### 2.1 Flower phenotype

Floral fragrance in *O. acaulis* comprises a few major nitrogenous compounds derived from amino acid metabolism (Fig. 2A). Moreover, flowers were found to reflect over a wide range of human visible wavelengths, and less intensely in the UV range (Fig. 2B).

### 2.2 Flower visiting behavior of *B. orientalis*

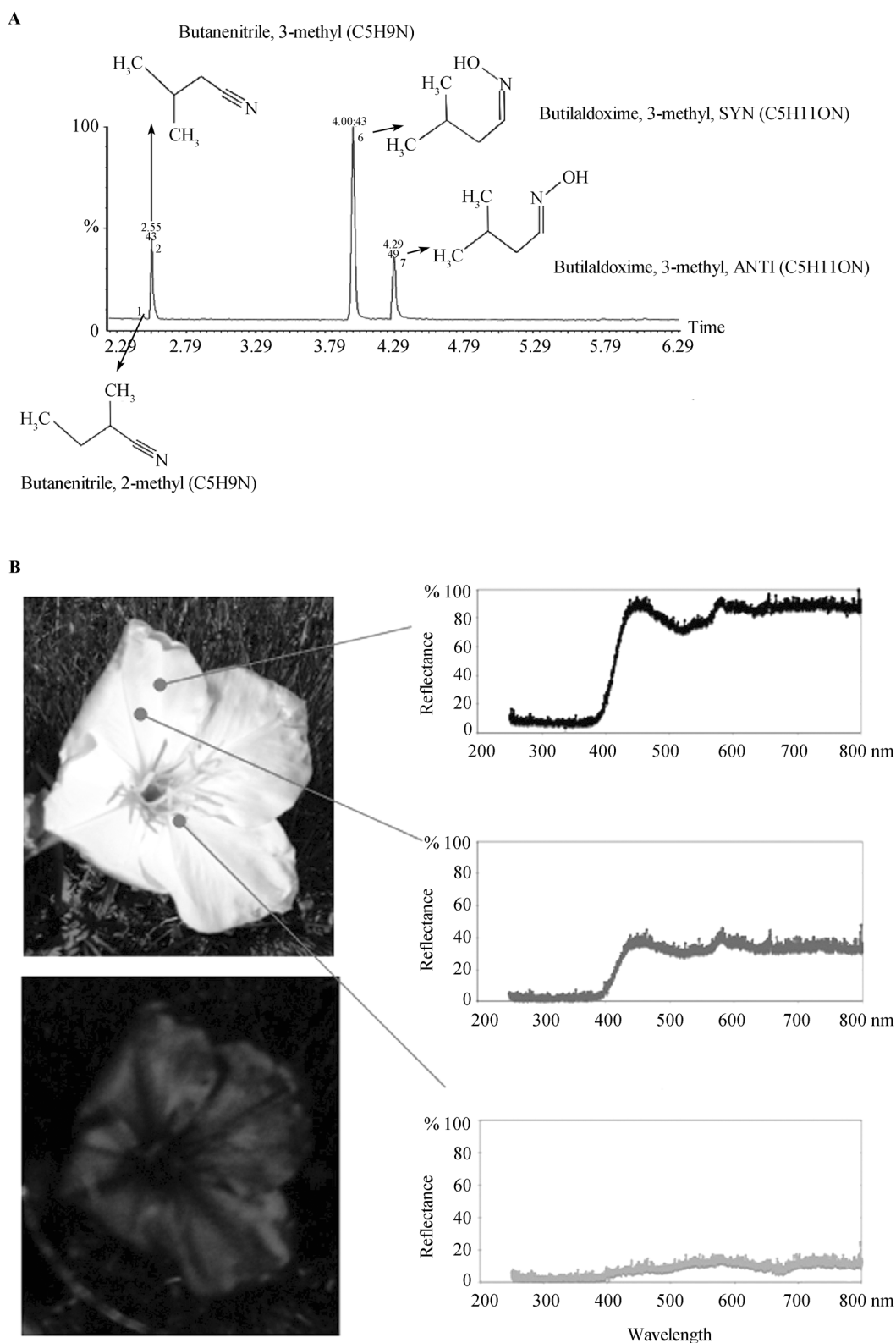
We found statistically significant differences between different behavioral events both for the frequency (Kruskal-Wallis One Way ANOVA on Ranks,  $H_{4,34} = 84.219$ ,  $P \leq 0.001$ ) and proportion of time (Kruskal-Wallis One Way ANOVA on Ranks,  $H_{4,34} = 96.252$ ,  $P \leq 0.001$ ) spent for each behavioral event. Post hoc all pairwise multiple comparison procedure (Tukey Test) showed that the primary behaviors performed by roaches corresponded to “feeding on floral resources” (nectar and pollen) and “exploring” (Fig. 3). We observed two distinctive phases during *B. orientalis* floral visits: the approach phase, during which roaches oriented their bodies towards the flowers and performed intense antennal movements (not quantified), and subsequent access of newly opened flowers, during which the behaviors described in Fig. 3 occurred.

### 2.3 Manipulative decoupling of visual and olfactory floral cues

The various subsets of visual and olfactory cues differed significantly in the frequency of cockroach visitation at both spatial scales measured in this study (Tables 1, 2). Within a 20 cm diameter of each stimulus (total area), treatment alone was not associated with a significant term (ANCOVA;  $F_{5,164}=1.997$ ;  $P=0.088$ ). However, when we included the number of cockroaches as a covariate random factor in the model, we found significant differences among treatments in frequency of visits

(ANCOVA;  $F_{5,164}=4.353$ ,  $P<0.001$ ), and this combined model explained almost 90% of the variance in our data set (Table 1). Based on the distribution of visitation frequencies in Fig. 4A, there is tendency of decrement in strength of responses by the insects towards different treatments; from natural flower control (NC), the reconstituted olfactory and visual stimuli (O+V) and the olfactory-only treatment (O), to which most roach visits occurred. The treatments that presented less attractiveness to cockroaches included the visual-only treatment (V), the empty treatment (∅C) and the empty mesh bag control (BC). These observed patterns were consistent when scored within a 6 cm diameter (stimulus area) of the presented stimuli (Fig. 4B). At both scales, the frequency of visits to O+V did not differ significantly from those to O (LSD; Fig. 4A, mean difference = 0.00,  $P = 1.00$ ; Fig. 4B, mean difference = 0.04,  $P = 0.77$ ).

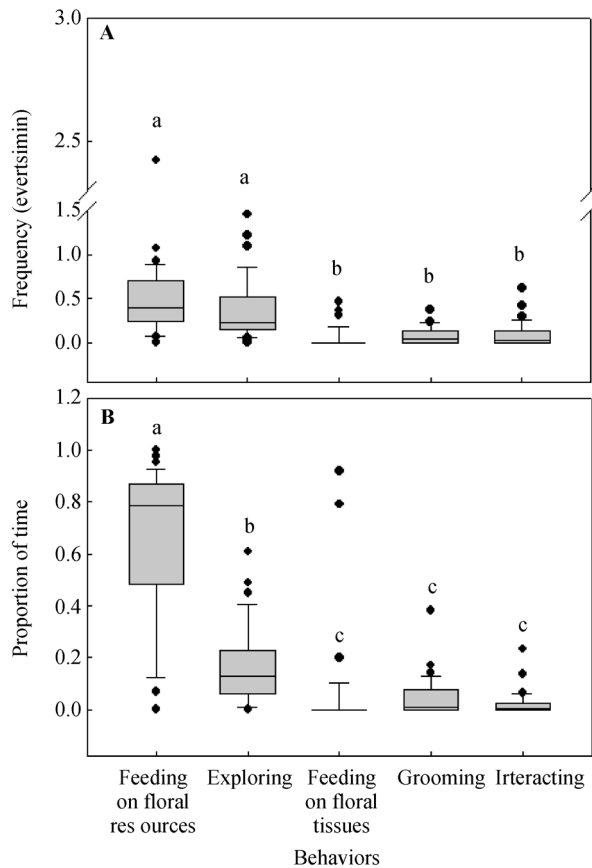
At the larger spatial scale (total area), the mean time spent by visiting roaches at each treatment was a significant source of variance (ANCOVA;  $F_{5,183}=3.387$ ,  $P<0.001$ ) when the combined model (treatment and number of cockroaches present) was analyzed (Table 1). This result was not observed at the smaller spatial scale (stimulus area), neither for the complete model nor for treatment alone (ANCOVA;  $F_{5, 183}=0.320$ ,  $P=0.048$ ; Table 2). The mean time spent by roaches at total area showed a tendency for longer residence time at each treatment that presented both (NC and O+V) of the multimodal floral signal (Fig. 4A). Interestingly, the olfactory treatment alone did not differ statistically from O+V treatment, nor from V, BC and ∅C. However there were no significant differences among experimental treatments at the stimulus area, whereas only the NC treatment differed significantly from all other treatments (Fig. 4B).



**Fig. 2 Flower fragrance and reflectance**

**A.** Floral volatiles collected from *O. acaulis* flowers. Total ion chromatogram (GC-MS) shows peak abundance (y-axis) vs. time (min, x-axis). The identified volatiles are common to other night-blooming species of *Oenothera* and are derived from amino acid metabolism. **B.** Visual characteristics of *O. acaulis* flowers. The upper left panel shows a flower photographed with a digital camera, lower left panel shows a photo of the same flower taken with a digital camera and an ultraviolet filter (Baäder “2 ®”). The right panel shows reflectance (% of white standard [y-axis] vs. wavelength, in nanometers (nm) [x-axis]) measured from different points over the flower surface (see arrows).





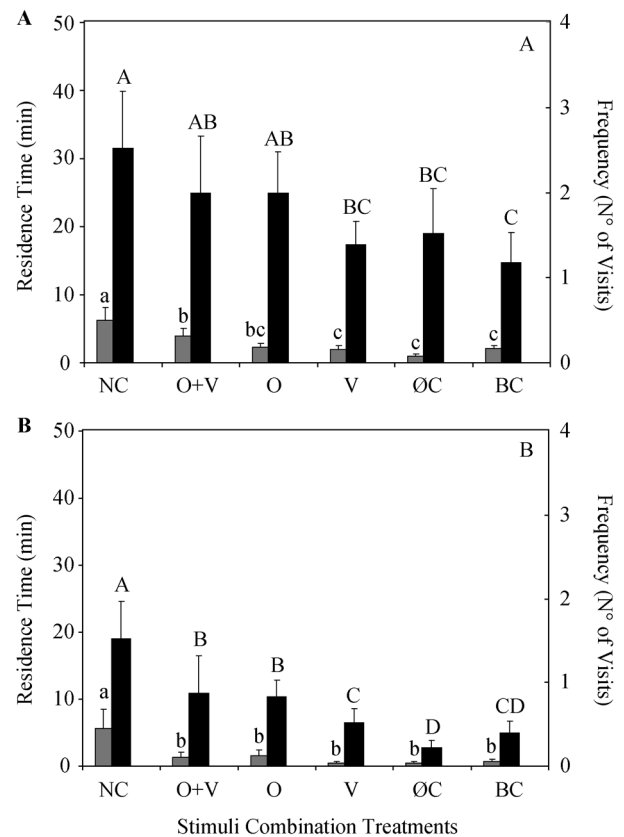
**Fig. 3** Box plots of the five general categories of intact *O. acaulis* flower visiting behavior exhibited by roaches

Panel A shows the frequency of performed behaviors (per minute) on flowers, whereas (B) shows the proportion of time budget spent engaged in each behavioral category. Different letters represent significant differences resulting from Tukey pairwise comparison tests. These behaviors were scored from video-taped field visits.

### 3 Discussion

#### 3.1 Is the attraction of roaches to flowers multimodal?

At the total area (20 cm diameter around the experimental stimuli), intended to simulate recruitment of *B. orientalis* roaches to the vicinity of flowering plants, visitation frequency was primarily odor-guided (Fig. 4A). Observations at the stimulus area (6 cm diameter) were intended to simulate actual floral visits by the roaches, as depicted in Fig. 3 and observed at the natural control (NC; Fig. 4B). It is clear from Fig. 4B that olfactory cues were necessary but insufficient to account for the levels of all response variables measured at the intact flower. In contrast, visual display in the absence of odor was no more attractive than the open ground or the empty mesh bag control at either spatial scale, with the exception of frequency at the 6 cm diameter scale,



**Fig. 4** Two response variables analyzed at different spatial scales

upper panel = total area, 20 cm diameter; lower panel = stimulus area, 6 cm diameter. Right X-axis show the residence time of the insects in the different experimental treatments. Left X-axis corresponds to the frequency of visits to the treatments (Represented with letters in the Y-axis). Grey bars indicate mean + standard error for mean duration (grey) of visits and frequency (black). Different letters represent significant differences among treatments obtained from LSD post hoc test ( $P < 0.05$ ) calculated after significant differences obtained in ANCOVA (see Table 1 and 2) for each variable.

where visual stimuli alone (V) elicited more approaches than the open ground (ØC; Figs. 4A and B). No subset of traits elicited visitation frequencies or durations comparable to the natural control, suggesting that artificial flowers either did not adequately reconstitute the stimuli presented by living petals, or that additional floral stimuli are required to elicit actual visits and the behaviors outlined in the Online Appendix 2. These stimuli may include olfactory, mechanosensory, gustatory and / or visual aspects of the floral nectar, pollen and petals sought by the roaches and then the capacity to feed from the flower during floral visits (Fig. 3).

In experiments on pollinator behavior, it is not unusual for floral reconstruction with artificial materials to fall short in comparison to actual unmanipulated flowers at the closest spatial scale (rev. by Raguso, 2006). These

shortcomings often reflect subtle aspects of the floral phenotype that are difficult to reconstruct in artificial flowers (e.g., texture), requiring the use of additional sensory channels, such as mechanoreception (Goyret and Raguso, 2006; Whitney et al., 2009), contact chemoreception (Gibernau et al., 1998) or gustation (taste) (Afik et al., 2006). In some cases, flowers remain turgid for several days after losing their odor, which researchers can exploit experimentally by adding scent extracts to such flowers (e.g. Angioy et al., 2004). Unfortunately, the flowers of *O. acaulis* lose their turgor and turn pink 24 hours after opening (C.A. Villagra, personal observation), so we could not use second day flowers (and thus present more texturally accurate scentless flowers).

Despite our expectations that both visual and olfactory signals would attract *B. orientalis* roaches to *O. acaulis* flowers, attraction was primarily mediated by floral scent under our experimental conditions. Our expectations were based primarily on laboratory-based studies of roach sensory biology performed under highly controlled conditions, including electrophysiological studies showing that roaches have remarkable vision under dim light conditions (Heimonen et al., 2006; Kyösti, 2008). In the case of *B. orientalis*, Mazokhin-Porshnyakov and Cherkasov (1985) identified UV and long- wavelength green photoreceptors. The existence of these receptors together with the presence of modest UV reflection on the petals of *O. acaulis* (Fig. 1B), suggested that *B. orientalis* could use chromatic contrast during flower searching, as has been demonstrated for other nocturnal insects under poor light conditions (Kelber and Roth, 2006). A study with shade response behavior (Okada and Toh, 1998) placed the limits of cockroach chromatic vision at 0.001 lux (starlight), with optimal visual capabilities at 10 lux (twilight), with illuminance measured with a lamp at 2 m distance.

Major nitrogenous compounds from *O. acaulis* flowers suggest that the composition of the scent of this night blooming plant is similar to that produced by other members of *Oenothera* section *Lauvaxia* (Raguso et al., 2007) (Fig. 2A). Also, the flower petals' reflectance (Fig. 2B) covered a broad range of visible wavelengths with less UV reflectance in comparison to the patterns found in North American *Oenothera* species (e.g., *O. neomexicana*, Raguso and Willis, 2002), as well as other night blooming flowers (e.g., *Datura wrightii*; Solanaceae: Raguso and Willis, 2005). Under our experimental conditions, floral scent was necessary and sufficient for

roach recruitment, whereas visual cues were not necessary and definitely were not sufficient for attraction at the spatial scales studied here. Thus, as the results stand, we found no clear evidence for the efficacy backup hypothesis. In contrast, either visual display or odor are sufficient to attract the nocturnal hawkmoth *Manduca sexta* to flowers such as those of *O. acaulis*, but their combination synergizes proboscis extension and feeding (Raguso and Willis, 2002; Goyret et al., 2007), and floral CO<sub>2</sub> functions as a redundant odor in this system (Goyret et al., 2008). The reliance of *B. orientalis* on olfactory stimuli was comparable (but in the converse sense) to the strong innate preferences shown by diurnal *Macroglossum stellatarum* hawkmoths (Balkenius et al., 2006) and *Vanessa indica* butterflies (Ōmura and Honda, 2005) for blue and yellow flowers, respectively, regardless of the presence of odor. However, under different light conditions and in the absence of their preferred color cue, both of these insects will visit flower models with less attractive colors or odors (Ōmura and Honda, 2005; Balkenius et al., 2006).

In addition to keying into floral cues, roaches actively walked on open ground, were found in smaller numbers in open and bag controls (Fig. 4A), and were highly responsive to the presence of other roaches (discussed below). Nevertheless, a more exhaustive test of the efficacy backup hypothesis will require experimental manipulations of environmental quality (e.g., light intensity and temperature), potentially in a laboratory environment (see Hebets and Papaj, 2005). On many nights coastal central Chile is foggy or cloudy, and we did not explicitly test whether the presence and amount of moonlight contributed to variance in our data set at the relatively pristine site of Los Molles, or at the highly human-impacted suburban setting in Cachagua. Future work should directly address this possibility either in lab or field tests.

### 3.2 How do roaches behave on *O. acaulis* flowers?

The primary behaviors shown by *B. orientalis* roaches at intact *O. acaulis* flowers, in terms of frequency and duration, were feeding from floral resources and exploration of the floral surface (Fig. 3). Video analysis showed that floral exploration was accompanied by almost constant antennation, suggesting that the differences between authentic corolla surfaces and paper surrogate flowers would have been apparent to the roaches regardless of visual similarities. During the experiments, we observed that *B. orientalis* performed antennation while visiting all of the six experimental and controls treatments (Fig. 2A). However, this kind of

behavior was more intensive in the scented treatments. Antennation by cockroaches has traditionally been attributed to odor detection (e.g., by Lent and Hyung-Wook, 2003). The occurrence of antennation also in visual-only treatments suggests that visual stimulation may modulate the expression of a behavior related to active olfactory-guided foraging (e.g., in lobsters; Koehl et al., 2001), just as Kunze and Gumbert (2001) suggested that odor may increase the attention of *Bombus terrestris* bees to visual cues. This possibility suggests a more indirect role for visual cues in cockroach foraging.

Among the remaining behavioral categories we assessed, "feeding on floral tissues" was the least frequently observed, suggesting that *B. orientalis* does not function as a florivore in this system. However, our previous observations revealed that these roaches consume flower petals when flowers already have sustained a wound, and thus may function as conditional antagonists (C. A. Villagra, personal observation). In a complementary study, we assessed the single-visit effectiveness of these roaches, and found that bagged flowers visited once by a roach and then re-bagged did not differ significantly in seed set ( $n=22$ , mean  $\pm$  SEM =  $45.2 \pm 8.4$  seeds/capsule) from flowers receiving no visits ( $n=26$ , bagged controls, seed set =  $30.4 \pm 4.9$ ) nor from those open to unlimited visitation ( $n=23$ , unbagged controls, seed set =  $35.0 \pm 6.9$ ; ANOVA,  $F_{2,25}=1.26$ , MS=681.764,  $P=0.29$ ; C.A.Villagra, unpublished data), to the best of our knowledge besides of our own research there is no other study on this subject to compare these findings. However, it is possible that pollen stripping after numerous roach visits could indirectly affect plant reproductive success by reducing the amount of pollen available for self or cross pollination, especially in flowers whose anther-stigma distance prevents self-pollination in the bud. Considering these patterns, eavesdropping roaches are most accurately described as facultative larcenists in this system, as they do not appear to have strong negative or positive impacts on the floral reproduction of *O. acaulis*.

### 3.3 Attention to other roaches

Our observations suggest that foraging *Blatta orientalis* roaches are affected by the behavior of other roaches. When we included the number of conspecifics in the experimental arena as a random factor in our ANCOVA models, we observed a strong interaction with nearly all response variables measured (Tables 1, 2). Actual interactions between roaches accounted for relatively low proportions of total visit time (or frequency) in comparison to other activities (mainly for-

aging; Fig. 3). However, these short intraspecific interactions may have modified subsequent behavioral responses in ways that we were not able to quantify in this study. For example, the presence of a conspecific insect (or a semiochemical from it) could affect the visiting behavior, as is well established for other flower visiting insects such as bumble-bees (Kawaguchi et al., 2006). Future experiments should measure roach behavior at flowers that have or have not been visited previously by roaches.

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**Appendix 1 Main *O. acaulis* fragrance compounds found in the headspace volatile collection obtained after one hour of sampling the natural control (“NC”), and the olfactory treatment (“O”)**

Treatments	Main fragrance compounds	p-moles		Relative abundance
		<i>X</i>	SEM	%
NC <i>n</i> =5	Butane nitrile, 3-methyl (*)	0.041	0.028	11.929
	Butyraldoxime,3-methyl,SYN (*)	0.232	0.031	67.593
	Butyraldoxime,3-methyl, ANTI (*)	0.070	0.011	20.491
	Total	0.343	0.070	100
O <i>n</i> =5	Butane nitrile, 3-methyl (*)	0.019	0.013	3.645
	Butyraldoxime,3-methyl,SYN (*)	0.370	0.282	71.026
	Butyraldoxime,3-methyl, ANTI (*)	0.132	0.100	25.338
	Total	0.521	0.396	100

Amounts of molecules detected by GC-MS are expressed in p-moles together with their relative abundance.

**Appendix 2 Behavior performed by *B. orientalis* cockroach at *O. acaulis* flowers, arranged according to functional clusters and brief descriptions for each cluster**

Type of Behavior	Behaviors	Description
Feeding on Floral Resources	Anther- anther feeding	The cockroach feeds on pollen placed over the first named structure, while perched on the second named structure. The first 5 behaviors correspond to pollen feeding while the last corresponds to nectar feeding
	Anther- stigma feeding	
	Anther- petal feeding	
	Stigma- stigma feeding	
	Stigma- anther feeding	
Grooming	Nectar feeding	The cockroach eats the pollen trapped on its own body, while perched on different parts of the flower.
	Grooming on petal	
	Grooming on reproductive parts	
Exploring	Grooming outside the flower	The cockroach explores the floral surface inspecting with its palps while antennating.
	Exploring the flower	
Interacting	Inspecting nectar tube opening	The cockroach meets another cockroach resulting in an aggressive encounter, or not.
	Aggressive interactions	
Feeding on Floral Tissues	Non aggressive interactions	The cockroach feeds on floral tissues, either petals or reproductive parts (anthers or stigma)
	Feeding on petals	
	Feeding on reproductive parts	