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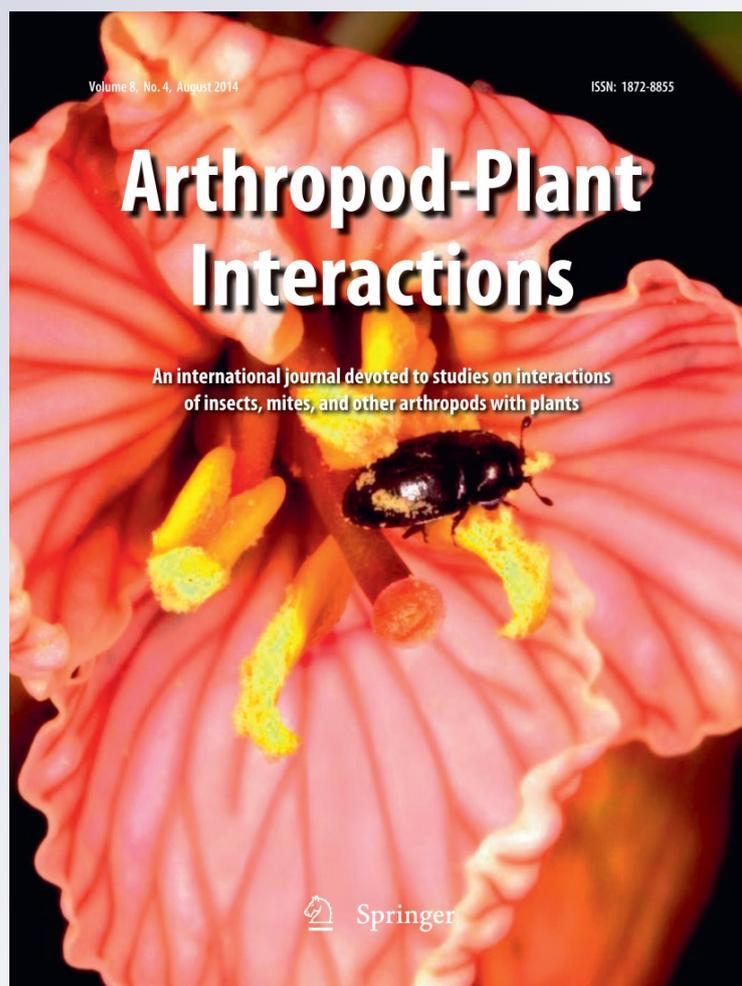
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Generalized pollination system: Are floral traits adapted to different pollinators?

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Abstract Flowers that are pollinated both during the day and at night could exhibit two different groups of pollinators and produce two different sets of attractants and rewards. We explored the patterns of emission of flower scents and production of nectar in the cactus *Echinopsis chiloensis* ssp. *chiloensis*, in relation to the patterns of activity of its diurnal and nocturnal pollinators. We measured frequency of flower visitors, analyzed floral scents, measured nectar production and sugar concentration, and performed pollination exclusion experiments. Bees were the main visitors at daytime and hawkmoths at nighttime. Diurnal scents were dominated by several compounds that can attract a wide range of pollinators, whereas nocturnal scents were less diverse and were dominated by (*E*)-nerolidol, a compound eliciting antennal responses in hawkmoths. Nectar volume and sugar concentration at night were similar to those recorded in hawkmoth-pollinated flowers. Daytime nectar volume was higher than those commonly found in bee-pollinated flowers, but similar to those found in flowers pollinated by several pollinators. Daytime sugar concentration was similar to those recorded

in bee-pollinated flowers. Flowers of *E. chiloensis* ssp. *chiloensis* seem morphologically adapted to hawkmoth pollination, but diurnal and nocturnal pollinators contribute to similar extents to reproductive success. Additionally, diurnal and nocturnal pollinators showed a synergic effect on the product of fruit set and seed set. The results are discussed in terms of the linkage between floral traits and perception abilities and requirements of pollinators.

Keywords Hawkmoth · Bees · Floral scents · Nectar · Pollination contribution

Introduction

A pollination syndrome comprises the entire set of floral traits involved in the attraction of a particular pollinator (Proctor et al. 1996). Flowers with generalized pollination syndrome are those that attract more than one type of pollinator (e.g., Young 2002; Schlumpberger and Badano 2005; Muchhala et al. 2008; Ortega-Baes et al. 2010; Schmid et al. 2010; Yokota and Yahara 2012) and are much more common than was thought until a few years ago (Fleming et al. 2001). This syndrome may be an optimal strategy in highly variable environments, where the abundance and distribution of pollinators fluctuate temporally and spatially, because it prevents plant reproductive success to be negatively affected by the absence of particular pollinators (Waser et al. 1996; Fleming et al. 2001).

Different types of pollinators may have different perception abilities and requirements (e.g., Dell'Olivo and Kuhlemeier 2013; Schemske and Bradshaw 1999; Briscoe and Chittka 2001; Chittka and Raine 2006; Kim et al. 2011). For example, hawkmoths are mainly attracted by olfactory cues (Yamamoto et al. 1969; Mechaber et al.

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2002) and require large amounts of nectar with low sugar concentration as reward (Opler 1983; Baker and Baker 1983; Kaczorowski et al. 2005), and bees are mainly attracted by visual cues (Milet-Pinheiro et al. 2012) and need lesser amounts of nectar with high sugar concentration (Opler 1983; Baker and Baker 1983). In this sense, flowers that are pollinated both day and night may produce flower scents (Dotterl et al. 2012) and nectar (Amorin et al. 2012) with different characteristics during the day and night, associated to their day and night pollinators, respectively.

Moreover, for a generalized pollination syndrome to be considered as optimal, different groups of pollinators should contribute to the reproductive success of the plant (Fleming et al. 2001); these contributions depend in general on both the visitation rate and the effectiveness in pollen transfer (e.g., Thomson 2000; Aigner 2004). Different pollinators may differ in their frequency of visits and pollination effectiveness (Waser et al. 1996). Hawkmoths are generally considered very effective pollinators: Due to their large body size and their foraging behavior, they are capable of carrying large amounts of pollen along great distances (Johnson 1995; Johnson and Liltved 1997), and a single visit can successfully fertilize a given flower (Willmot and Búrquez 1996). In contrast, bees are considered ineffective pollinators since, unlike hawkmoths which are nectar collectors, most bees are pollen collectors and the pollen mass transported, being mixed with nectar and resin, is not all available for pollination (Williams and Adam 2010).

Several species of cacti from temperate regions exhibit generalized pollination syndromes; their main nocturnal visitors are moths while their main diurnal visitors are bees (de Viana et al. 2001; Schlumpberger and Badano 2005; Schlumpberger et al. 2009; Ortega-Baes et al. 2010; Alonso-Pedano and Ortega-Baes 2012). Since the identity of the main pollinators changes drastically within a short period of time, these flowers are ideal models to test hypotheses of character adaptation to different pollinators (e.g., Dotterl et al. 2012; Amorin et al. 2012).

In this paper, we explore the patterns of emission of flower scents and nectar production in *Echinopsis chiloensis* ssp. *chiloensis*, in relation to the patterns of activity of its diurnal and nocturnal pollinators. Flowers of the columnar cactus *E. chiloensis* ssp. *chiloensis* (Colla) H. Friedrich and G. D. Rowley exhibit traits that conform to a hawkmoth pollination syndrome (Dobson 2006), i.e., they are large, cone shaped, white colored, without nectar guides, open at dusk and stay open for periods that vary from ca. 8 to 22 h depending on latitude. Indeed, Walter (2010) described that the main nocturnal visitors and the major contributors to fruit set and seeds per fruit set in this cactus are hawkmoths, and also that during the day the

main visitors are bees. Because of the different perception abilities and requirements of their floral pollinators, we hypothesize that composition of flower scents, nectar volumes, and sugar concentration in the nectar of *E. chiloensis* ssp. *chiloensis* differs between day and night, presenting characteristics related to the perception abilities and requirements of each group of pollinators. Furthermore, we hypothesize that both groups of pollinators contribute to the reproductive success of the cactus.

Methods and materials

Study area and study species

This study was conducted from November 2012 to February 2013 at the Reserva Nacional Río Clarillo (RNRC), located 45 km southeast of Santiago (33°41'–33°51'S, 70°24'–70°29'W) between 860 y 3057 m.a.s.l. The climate corresponds to a mediterranean regime with rainy winters and dry summers (Niemeyer et al. 2002). During the study period, the mean day temperature was 19 ± 0.5 °C and mean relative humidity was 54 ± 3 % (data from the agrometeorological station at RNRC; mean of data taken at 09:00, 14:00, and 18:00, during the 4 months that the study lasted). The vegetation of the area is dominated by evergreen plants of the sclerophyllous forest and matorral vegetational region (Gajardo 1993). *E. chiloensis* ssp. *chiloensis* is endemic to central Chile from La Serena (29°02'S) to Talca (36°33'S) (Luebert and Plissock 2006) and exhibits different pollination syndromes along its distribution. In its northern distribution (Coquimbo: 31°30'S, 71°06'W), it exhibits a diurnal pollination syndrome where flowers open between 06:00 and 07:00 h, remain open an average of 8.5 h and their main visitors are bees (Ossa and Medel 2011). In contrast, in the southern range (Rancagua: 34°10'S, 70°43'W), it exhibits a generalized pollination syndrome where flowers open between 18:00 and 20:00 h, remain open for 20–22 h on average and their main diurnal visitors are bees, and their main nocturnal visitors are moths (Walter 2010). These previous studies have determined the reproductive system of this cactus as self-incompatible and totally dependent on cross-pollination.

Individuals of *E. chiloensis* ssp. *chiloensis* were chosen from among the population growing along the main river at the RNRC, within an area of approximately 25 ha. Only one flower per individual was used. Anthesis in the study area began mostly between 18:00 and 21:00 h, the mean time of opening being ca. 19:30; flowers remained open for 20–22 h on average. During summer, dusk begins at around 21:00; in this scenario, potential diurnal pollinators have the chance to visit recently opened flowers up to 3 h before dusk.

Floral visitors

Three periods of observations during floral anthesis were defined: opening (18:00–21:00), night (21:00–06:00) and day (06:00–18:00). Thirteen different flowers that opened around the same time in different days (between 18:30 and 20:00) were selected to conduct observations of floral visitors. In daytime (opening and day periods), direct observations were made for periods of 30 min each hour, beginning with the opening of the flowers and ending with their closing during the following day. The visiting insects were collected using an insect net, placed in Eppendorf tubes and later identified by specialists (see Acknowledgments). In nighttime, the flowers were filmed with infrared light for periods of 90 min every 3 h, and insects observed during playback were identified by the same specialists. The mean visitation rate for each insect species was estimated as the number of individuals per hour per flower. The peak visiting activity hours during each of the observation periods were determined to establish the times to collect nectar and floral scents so that these traits may be related with visitors activity.

Collection of floral scents

Volatile scent collection was performed in fresh uncut flowers in the field using the dynamic headspace adsorption method (Raguso and Pellmyr 1998). Six flowers that opened around the same time (between 18:30 and 20:00) of different cactus individuals were selected; the flowers were at all times individually enclosed in tulle bags, except when they were submitted to scent collection. The collection system consisted of an inert oven bag (Melitta, Minden, Germany) which enclosed the flower, a silicone hose inlet, a silicone hose outlet, and Teflon tape used for sealing the system. Compressed synthetic air, manufactured by mixing pure oxygen (20 %) and nitrogen (80 %), was injected into the inlet and extracted at the outlet to collect the volatile compounds without getting contamination by compounds in the natural air around the plant. Airflow at the inlet and outlet was adjusted to 250 ml/min, the first using the regulator attached to a synthetic air tube and the second using an extraction pump (PAS-500), a manual flowmeter and flow regulator. Floral scents were collected in Porapak Q columns attached to the bag outlet during the periods of highest visitor activity (see Results) within each period of anthesis, each collection lasting 1 h.

In the laboratory, the Porapak Q columns were eluted with 1 ml of dichloromethane, and the extracts stored in 1.8-ml glass vials at -10°C . The analysis of the volatiles was performed on a gas chromatograph coupled to a mass spectrometer (Shimadzu QP2010 Ultra). Samples were analyzed in a capillary column (RTx-5MS: length 30 m,

diameter 0.25 mm, and film thickness 0.25 μm). Helium was used as carrier gas with a flow rate of 1.3 ml/min. Temperature of injection port and detector was set at 250°C . The oven temperature was programmed at 35°C for 5 min and then increased by 5°C every minute until 200°C was reached; this final temperature was maintained for 10 min. Volatile compounds were identified according to their mass spectra and their retention indices using the NIST 2011 standard reference database and The Pherobase (El-Sayed 2012).

Nectar production

In order to determine nectar production of *E. chiloensis* ssp. *chiloensis* along anthesis, nectar collection was performed during the periods of highest visitor activity (see “Results”), within each period of anthesis; additionally, a collection was performed at dawn (06:30–08:30) to determine whether the nectar was produced only during the night or also during the day. Fifty different flowers that opened around the same time (18:30–20:00) in different cactus individuals were selected and individually bagged with tulle bags before opening to avoid nectar consumption. Two types of nectar measurements were performed. The first type involved 40 different flowers (ten flowers for each time period, i.e., opening, night, dawn and day), thus obtaining the cumulative volume of nectar along anthesis when flowers are not visited (accumulated nectar measurement). The second type of measurement was made in the remaining ten flowers repeatedly every 6 h, therefore, obtaining the nectar volume produced between hours (repeated nectar measurement). All nectar samples were collected and measured using 100 μl Hamilton syringes. Sugar concentration in the nectar (% sucrose) was measured with a manual refractometer. For this, 40 μl of each nectar sample was used to cover completely the prism of the refractometer. When nectar volume of a single flower was less than 40 μl , a measured amount of distilled water was added to a final volume of 50 μl ; final concentrations were determined by applying the dilution factor to the measured concentration.

Contribution of pollinators to reproductive success

One hundred flowers belonging to different cactus individuals and opening at around the same time (between 18:30 and 20:00) were selected and assigned haphazardly to the following treatments: (1) total exclusion of pollinators: Flowers were excluded with tulle bags before opening until after closing ($N = 25$); (2) nocturnal exclusion of pollinators: Flowers were bagged from 20:00 until 06:00 the next morning ($N = 25$); (3) diurnal exclusion of pollinators: Flowers were bagged from opening until 20:00 and

from 06:00 until closing ($N = 25$); (4) without exclusion of pollinators: Flowers remained without bags ($N = 25$). The flowers were monitored until abortion or fruit production; the fruits produced were collected once they were mature (after dehiscence) to determine the number of seeds produced per fruit. Subsequently, 396 seeds were selected randomly per treatment (nocturnal exclusion, diurnal exclusion, without exclusion, 1,188 in total), for use in germination experiments under standard conditions: constant temperature of 23 °C and 14:10 light:darkness photoperiod for 30 days (e.g., Walter 2010). The seeds were separated in groups of 18 in Petri dishes with moist cotton and covered with aluminum paper.

Statistical analyses

Relative visitation rates and relative abundance of floral volatile compounds were calculated since raw values showed substantial inter-individual variations. All data were square root (datum + 1) transformed because the data matrix contained many zero values and to reduce the influence of particularly abundant species or volatile compounds (Quinn and Keough 2002). To compare the assembly of visitors and flower scent composition between time periods (opening, night and day), Bray and Curtis similarity indices were calculated and similarity analysis of permutations (ANOSIM; Clarke and Gorley 2006) subsequently performed with 10,000 random permutations; the Bonferroni correction factor was applied to reduce type I error (Quinn and Keough 2002). To identify the species and compounds responsible for the variability in visitation rate and scents between time periods, respectively, similarities of percentages (SIMPER) analyses were performed. To show the variation of visitors and scents between time periods, the nonmetric multidimensional scaling (NMDS) method was applied based on the matrix of Bray–Curtis similarities (e.g., Balao et al. 2011). Analyses were performed in the statistical program PAST (Hammer et al. 2001).

The number of fruits produced and the number of germinated seeds were compared between exclusion treatments through the chi-square test of association (Dytham 2011). Parametric one-way ANOVA was used to compare nectar volume between periods and the number of seeds per fruit between exclusion treatments. Because these variables did not follow a normal distribution, they were transformed with a Box–Cox linear function [$y^\lambda = (y^\lambda - 1) / \ln y^\lambda$, for ($\lambda \neq 0$)] (Box and Cox 1964). A posteriori pairwise comparisons were performed using Tukey's tests (Dytham 2011). Nonparametric one-way Kruskal–Wallis test was used to compare nectar sugar concentrations between periods; this analysis was followed by nonparametric Dunn test for unbalanced designs (Siegel and

Castellan 1988). A Spearman's rank order correlation was used to evaluate the relation between the volume produced and sugar concentration of nectar measured using the accumulated method (Siegel and Castellan 1988). Analyses were performed in STATISTICA 8 (StatSoft Inc. 2006), and the graphics made using SIGMA PLOT 11.0 (Systat Software Inc. 2008). For easier interpretation of the results, the nectar untransformed values are shown.

Results

Floral visitors

Twenty-four insect species belonging to five orders were observed visiting flowers (Table 1). Few visitors were observed during the opening and night periods; visits were particularly scarce during the night period (Table 1). In contrast, during the day, there were more visitor species and visits were more frequent, Hymenoptera being the most frequently observed group. The most common species was the fly *Dolichophaonia* sp., followed by the bees *Apis mellifera*, *Leioproctus semicyaneus*, and *Pseudagapostemon pissisi* (Table 1). The peak visiting activity occurred at approximately the zeroth, sixth, and 18th hour of anthesis and corresponded to 18:30–20:30, 00:30–02:30, and 11:30–13:30 h, respectively.

The assemblage of floral visitors varied significantly between time periods (one-way ANOSIM: $R = 0.37$, $P < 0.001$). All pairwise comparisons were significantly different between time periods (one-way ANOSIM: opening vs. night, $R = 0.06$, $P = 0.046$; opening vs. day, $R = 0.55$, $P < 0.001$; night vs. day, $R = 0.54$, $P < 0.001$). According to the SIMPER analysis, the main species responsible for differences between time periods were: the fly *Dolichophaonia* sp., very frequent during the opening and the day periods but absent at night; the bee *A. mellifera*, present during the opening period, very often during the day period but absent at night; and the bees *L. semicyaneus*, *P. pissisi*, and *D. chilensis*, present with high frequency only during the day period.

Floral scents

A total of 29 volatile compounds were identified in the flower scents (Table 2), 21 (74.2 %) of which occurred during the three time periods (opening, night and day). During the opening and night periods, scents were dominated by sesquiterpenes and monoterpenes; in contrast, during the day period, scents were dominated by aromatic esters (Table 2).

The composition of flower scents varied significantly between time periods (one-way ANOSIM: $R = 0.55$,

Table 1 Visitors, mean visitation rates (VR: visits/h), standard error (SE), and percentage of visits (%) to the cactus *E. chilensis* ssp. *chilensis* during three different time periods (opening, night, day)

Order/species (family)	Opening		Night		Day	
	VR (SE)	%	VR (SE)	%	VR (SE)	%
Coleoptera						
<i>Bilyaxia concinna</i> (Buprestidae)					0.31 (0.21)	0.59
<i>Epiclines gayi</i> (Cleridae)					0.15 (0.10)	0.30
<i>Amecocerus</i> sp. (Melyridae)			0.11 (0.11)	20.00	3.54 (1.25)	6.84
<i>Arctodium vulpinu</i> (Scarabaeidae)					0.46 (0.22)	0.89
Sub-totals			0.11 (0.11)	20.00	4.46 (1.57)	6.58
Diptera						
<i>Dolichophaonia</i> sp (Muscidae)	11.33 (1.45)	89.47			14.15 (5.36)	27.34
<i>Mitrodetus dentitarsis</i> (Mydidae)					0.23 (0.12)	0.45
<i>Trichophthalma murina</i> (Nemestrinidae)					0.15 (0.10)	0.30
<i>Mycteromyia conica</i> (Tabanidae)					0.38 (0.18)	0.74
<i>Seioptera importans</i> (Ulidiidae)					0.38 (0.18)	0.74
Sub-totals	11.33 (1.45)	89.47			15.31 (5.3)	22.56
Hemiptera						
<i>Leptoglossus chilensis</i> (Coreidae)	0.67 (0.33)	5.26	0.22 (0.15)	40.00	0.15 (0.10)	0.30
Sub-totals	0.67 (0.33)	5.26	0.22 (0.15)	40.00	0.15 (0.10)	0.22
Hymenoptera						
<i>Diadasia chilensis</i> (Apidae)					4.85 (1.56)	9.36
<i>Apis mellifera</i> (Apidae)	0.67 (0.67)	5.26			11.54 (4.36)	22.29
<i>Manuelia gayi</i> (Apidae)					3.31 (1.32)	6.39
<i>Leioproctus semicyaneus</i> (Colletidae)					10.08 (4.82)	19.47
<i>Coenohalictus dolator</i> (Halictidae)					1.69 (0.70)	3.27
<i>Pseudagapostemon pissisi</i> (Halictidae)					8.77 (3.40)	16.94
<i>Lasioglossum herbstiellu</i> (Halictidae)					1.85 (0.88)	3.57
<i>Ruizantheda mutabilis</i> (Halictidae)					1.62 (0.77)	3.12
<i>Ruizantheda proxima</i> (Halictidae)					0.85 (0.61)	1.63
<i>Trichothurgus dubius</i> (Megachilidae)					1.00 (0.53)	1.93
<i>Megachile distinguenda</i> (Megachilidae)					1.00 (0.34)	1.93
Unidentified Microhymenopteran					1.31 (0.66)	2.53
Sub-totals	0.67 (0.67)	5.26			47.85 (14.77)	70.64
Lepidoptera						
<i>Manduca sexta</i> (Sphingidae)			0.22 (0.18)	40.00		
Sub-totals			0.22 (0.18)	40.00		
Grand totals	12.67 (1.45)	100	0.56 (0.2)	100	48.92 (14.96)	100

$P < 0.001$). The scents of the day period were significantly different from those of the night period (one-way ANOSIM: $R = 0.80$, $P = 0.007$), while the differences between the scents emitted during the opening period were marginally significant with respect to the night period (one-way ANOSIM: $R = 0.46$, $P = 0.044$) and to the day period ($R = 0.34$, $P = 0.052$). According to SIMPER analysis, the main compounds responsible for differences between time periods were the terpenes (*E*)-nerolidol, (*E*)-ocimene and β -pinene and the aromatic ester methyl benzoate. The graph resulting from the NMDS method (Fig. 1)

shows the clear differences in scent composition between day and night and the similarity between opening and day periods.

Nectar production

Nectar was available at the opening of flowers and was produced continuously along anthesis (Fig. 2A). The accumulated nectar volume varied significantly along floral anthesis (ANOVA: $F = 17.370$, $P < 0.001$): Nectar accumulated during the whole anthesis period (collected during

Table 2 Frequency (*N*), average relative proportions (area %), and standard error (SE) of 29 compounds present in the floral scents of six flowers of *Echinopsis chiloensis* ssp. *chiloensis* during each of three different time periods (opening, night, day)

Compound class/name	Retention index	Opening		Night		Day	
		<i>N</i>	Area % (SE)	<i>N</i>	Area % (SE)	<i>N</i>	Area % (SE)
(a) Terpenes							
<i>Monoterpenes</i>							
α -Pinene	931	6	1.10 (2.25)	4	0.28 (0.57)	6	0.84 (0.88)
Camphene	947	3	6.04 (13.78)	3	1.17 (3.16)	2	2.03 (0.96)
β -Pinene	975	6	1.51(2.35)	5	0.32 (0.62)	6	3.01 (6.66)
β -Myrcene	990	1	0.01(1.00)	3	0.42 (0.13)		
3-Carene	1,006	5	0.30 (0.91)	2	0.08 (0.29)	4	1.10 (2.57)
<i>m</i> -Cymene	1,024	5	0.15 (0.96)	2	0.05 (0.16)	4	1.03 (1.37)
Limonene	1,027	6	0.94 (2.09)	4	0.47 (1.15)	5	3.74 (0.85)
(<i>Z</i>)- β -Ocimene	1,039	5	1.06 (4.05)	5	4.31 (2.17)	5	0.93 (2.43)
(<i>E</i>)-Ocimene	1,050	5	18.49 (11.60)	5	38.73 (11.17)	6	5.65 (7.75)
Linalool	1,099	1	0.01 (0.00)	4	2.51 (1.09)		
<i>Neo-allo-ocimene</i>	1,131	1	0.10 (0.00)	2	0.69 (0.38)	2	0.79 (4.46)
Sub-totals			29.71 (3.21)		49.04 (6.47)		19.13 (0.92)
<i>Sesquiterpenes</i>							
2,6,11-Trimethyldodecane	1,281	3	0.17 (0.91)	3	0.04 (0.05)	3	0.72 (0.12)
α -Cubebene	1,346	1	0.05 (0.00)				
β -Farnesene	1,462	2	1.52 (0.61)	5	0.26 (0.10)	1	1.08 (1.00)
Germacrene D	1,489	2	0.28 (6.50)	2	0.94 (3.04)	2	0.06 (0.18)
α -Farnesene	1,513	4	15.70 (3.28)	6	4.85 (2.12)	3	7.57 (2.35)
δ -Cadinene	1,531	1	0.13 (1.00)	2	0.23 (0.50)	1	0.01 (1.00)
(<i>E</i>)-Nerolidol	1,570	2	36.13 (9.15)	6	42.05 (6.85)	5	9.85 (2.00)
Sub-totals			53.97 (5.21)		48.36 (6.33)		19.29 (1.64)
(b) Benzenoids							
<i>Aliphatic benzenoid</i>							
<i>p</i> -Xylene	871	1	0.005 (1.00)	2	2.46 (3.60)	4	0.21 (0.04)
Sub-totals			0.005 (1.00)		2.46 (3.60)		0.21 (0.04)
<i>Aromatic esters</i>							
Methyl benzoate	1,098	1	15.00 (0.00)			6	47.21 (12.57)
Methyl salicylate	1,198	1	0.43 (0.00)			1	8.15 (0.00)
Benzyl benzoate	1,769					1	1.66 (0.00)
Sub-totals			15.43 (5.95)				57.02 (14.23)
(c) Alkanes							
3,4-Dimethylheptane	865	2	0.04 (0.45)			2	0.04 (0.17)
Nonane	896	4	0.38 (0.58)	1	0.10 (0.00)	2	0.14 (0.04)
Undecane	1,097					1	0.08 (0.00)
Dodecane	1,199					1	0.03 (0.00)
Tetradecane	1,400	1	0.005 (0.00)	1	0.04 (0.00)	1	0.69 (0.00)
Hexadecane	1,599					1	2.23 (0.00)
Sub-totals			0.42 (0.08)		0.14 (0.02)		3.21 (0.35)
(d) Aldehyde							
Decanal	1,206	1	0.46 (0.00)			1	1.15 (1.00)
Sub-totals			0.46 (0.00)				1.15 (1.00)
Grand totals			100		100		100

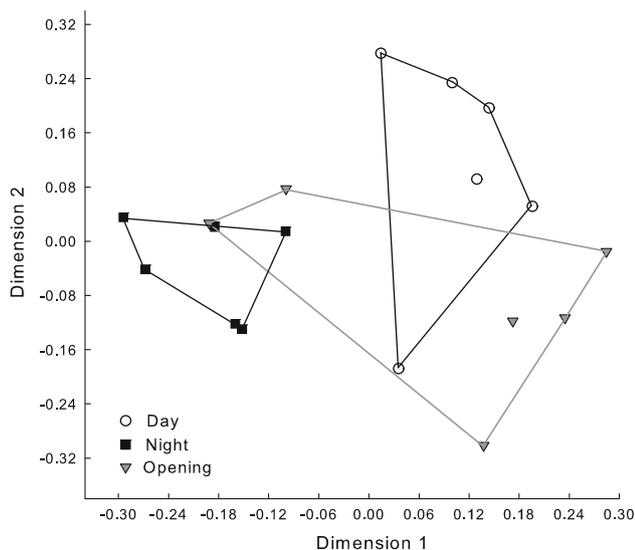


Fig. 1 Non-metric multidimensional scaling (NMDS) based on Bray–Curtis similarity matrices of floral scents, in flowers of the cactus *E. chiloensis* ssp. *chiloensis* at three times during anthesis (2D stress value 0.298). Each point in space is related to a singular pattern of scents of a flower during a particular collection event (maximum $N = 6$)

the day) was significantly higher than that accumulated from opening to night and at opening (Tukey: day vs. night, $q = 5.511$, $P = 0.002$; day vs. opening, $q = 9.502$, $P < 0.001$). On the other hand, nectar accumulated from opening to dawn and from opening to night was significantly higher than that accumulated at opening (Tukey: dawn vs. opening, $q = 7.460$, $P < 0.001$; night vs. opening, $q = 3.992$, $P = 0.037$). Nectar production determined through the repeated measurements method varied significantly along floral anthesis (ANOVA: $F = 8.336$, $P < 0.001$): A higher amount of nectar was produced during the day than during the night and opening periods (Tukey: day vs. night, $q = 5.412$, $P = 0.003$; day vs. opening, $q = 6.024$, $P < 0.001$) and during dawn the amount of nectar produced was higher than that produced before opening (Tukey: $q = 4.219$, $P < 0.025$).

Sugar concentration was significantly different among periods (Kruskal–Wallis: $H = 12.753$, $P = 0.005$), being higher at opening compared with the day period (Dunn's: $Q = 3.361$, $P < 0.05$). Other pairwise comparisons were not significantly different (Fig. 2B). Sugar concentration was only determined on samples obtained from the nectar accumulated measures experiment and was negatively correlated with nectar volume (Spearman: $r_s = -0.511$, $P = 0.001$).

Contribution of pollinators to reproductive success

Flowers that were excluded since their opening until their closing (total exclusion) did not produce fruit (Table 3).

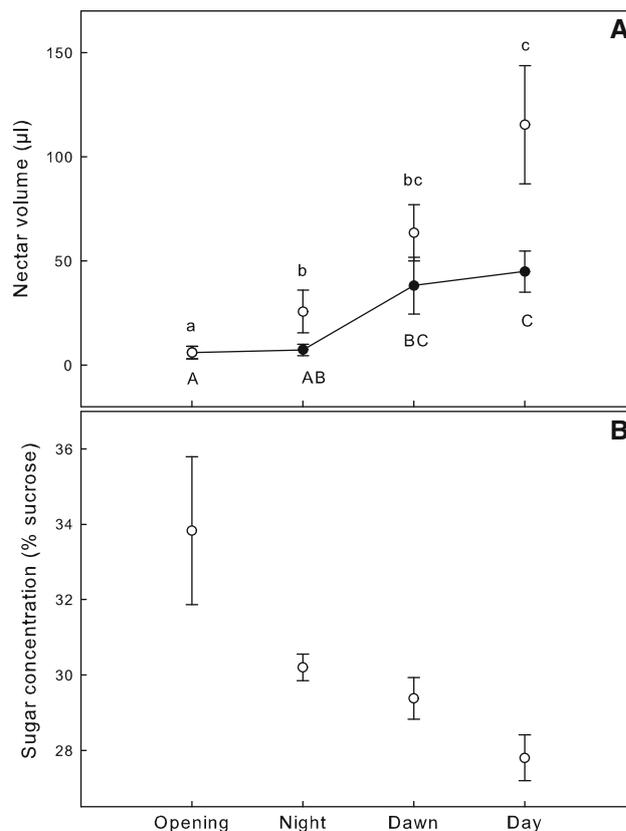


Fig. 2 **A** Nectar volume (μl , mean \pm 1 SE) and **B** sugar concentration (% sucrose, mean \pm 1 SE) in flowers of the cactus *E. chiloensis* ssp. *chiloensis* measured at three different periods within anthesis using two different methodologies (open circle accumulated nectar measurements; filled circle repeated nectar measurements). Different letters (a, b, c and A, B, C) indicate significant differences between periods at $P = 0.05$

Flowers without exclusion produced a significantly higher proportion of fruit compared to flowers with nocturnal and diurnal exclusions (chi square: $\chi^2 = 5.56$, $P = 0.038$ and $\chi^2 = 18$, $P < 0.001$, respectively). The proportion of fruit produced by flowers with nocturnal exclusion was significantly higher than by flowers with diurnal exclusion (chi square: $\chi^2 = 4.35$, $P = 0.037$). Number of seeds set per fruit varied significantly between floral exclusions (ANOVA: $F = 6.825$, $P = 0.003$): Flowers without exclusion produced a significant higher number of seeds per fruit in comparison with nocturnal and diurnal exclusions (Tukey: $q = 3.407$, $P = 0.045$ and $q = 4.737$, $P = 0.006$, respectively) (Table 3); however, there was no difference between diurnal and nocturnal exclusion treatments (Tukey: $q = 2.106$, $P = 0.309$). The number of germinated seeds from flowers without exclusion was significantly higher than those from the nocturnal and diurnal exclusions (chi square: $\chi^2 = 11.07$, $P < 0.001$ and $\chi^2 = 9.96$, $P = 0.001$, respectively); however, there was no difference in the number of germinated seeds between

Table 3 Fruit set, seed set (per fruit), Fruit set \times seed set, and germinated seeds from flowers of the cactus *E. chiloensis* ssp. *chiloensis* under different pollinator exclusion experiments

Exclusion treatment	Fruit set (Proportion)	Seed set N° (SE)	Fruit set \times seed set N°	Germinated seeds N° \pm SE (%)
Total exclusion	0	0	0	0
Without exclusion	0.80 a	2,850.2 (393.8) a	2,280	17.3 \pm 0.2 (96.0) a
Nocturnal exclusion	0.48 b	764.8 (221.1) b	367	16.2 \pm 0.3 (89.9) b
Diurnal exclusion	0.20 c	1,599.0 (376.8) b	320	16.3 \pm 0.4 (90.7) b

SE standard errors

Different letters within columns indicate significant differences at $P = 0.05$

diurnal and nocturnal exclusions (chi square: $\chi^2 = 0.13$, $P = 0.72$) (Table 3). The product of fruit set \times seed set was similar for the diurnal and nocturnal exclusion treatments, and they were smaller than that for the no exclusion treatment (Table 3).

Discussion

During flower opening and the daytime period, the fly *Dolichophaonia* sp. was the most frequent visitor; other frequent visitors during the daytime were the bees *A. mellifera*, *L. semicyaneus*, and *P. pissisi*. *Dolichophaonia* sp. did not make contact with the flower anthers, and no pollen was observed on its body. Moreover, some larvae of *Dolichophaonia* sp. were observed inside withered flowers; therefore, this fly is likely to visit flowers mainly in search of places to lay eggs and, if it has an effect on pollination, it might not be significant. On the other hand, all bee species observed belong to pollen feeding families (Grimaldi and Engel 2005; Williams and Adam 2010). Moreover, *A. mellifera* has been described as a frequent visitor to several cactus species (Bustamante et al. 2010; Ortega-Baes et al. 2010; Larrea-Alcázar and López 2011; Alonso-Pedano and Ortega-Baes 2012), some species of the genus *Diadasia* have been described as pollen collector specialists from cacti (Sipes and Tepedino 2005), and *Manuelia gayi* has been reported as a pollinator of several plant species in the area where the present studies were performed (Smith-Ramírez and Yáñez 2010). Therefore, bees are likely to be the main daytime pollinators of *E. chiloensis* ssp. *chiloensis*.

The only visitor species observed during the night were the hemipteran *Leptoglossus chilensis*, the beetle *Ameococcus* sp., and the hawkmoth *Manduca sexta*. The hemipteran is a generalist phytophagous insect (Levin 2000) that has not been reported as pollinator and that was not observed making contact with the flower anthers, most of the observations in the present study corresponding to mating events; hence, this insect most likely visits the cactus flowers as a herbivore or in search of a mate and therefore, if it has an effect on pollination, it might not be

significant. In contrast, *Ameococcus* sp. could be acting as a pollinator because many species of this genus feed on pollen and can transport it on their pilose bodies (Smith-Ramírez and Yáñez 2010). However, the main nocturnal pollinator of *E. chiloensis* ssp. *chiloensis* is likely to be *M. sexta* because hawkmoths are considered in general to be very effective pollinators due to their large body size, their foraging behavior, and their capability of carrying large amounts of pollen along great distances (Johnson 1995; Johnson and Liltved 1997); additionally, *M. sexta* has been described as a frequent visitor of cacti (Raguso et al. 2003; Schlumpberger et al. 2009; Ortega-Baes et al. 2010) and was observed previously by chance inside one of the flowers of *E. chiloensis* ssp. *chiloensis* (Walter 2010).

Although compositional variation of flower scents along anthesis could simply be a consequence of circadian changes in light and temperature (Kolossova et al. 2001), the present variations were consistent with the differential requirements of daytime as well as nighttime pollinators, as has been reported in other systems (Kaczorowski et al. 2005; Dotterl et al. 2012). Diurnal scents were composed by a large number of compounds; this is frequently the situation in flowers pollinated by different types of pollinators. Scents produced by bee-pollinated flowers are highly variable and may be dominated by benzenoids, terpenes, or fatty acid derivatives (Dobson 2006). Diurnal scents of *E. chiloensis* ssp. *chiloensis* were dominated by the aromatic esters methyl benzoate and methyl salicylate, and the terpenes (*E*)-nerolidol, α -farnesene and (*E*)-ocimene. All these compounds were found in the cactus *Pereskia aculeate*, which is mainly pollinated by bees and butterflies (Kaiser and Tollsten 1995). Moreover, (*E*)-nerolidol, α -farnesene, and (*E*)-ocimene are common compounds in bee and moth-pollinated flowers (Kaiser and Tollsten 1995; Raguso et al. 2003; Jurgens 2004; Grajales-Conesa et al. 2011), and methyl benzoate and methyl salicylate are commonly found both day and night in bee, moth, bat, and butterfly-pollinated flowers (Knudsen and Tollsten 1993; Kaiser and Tollsten 1995; Kolossova et al. 2001; Pettersson et al. 2004; Dobson 2006; Schlumpberger and Raguso 2008; Grajales-Conesa et al. 2011).

The less variable and less complex nocturnal scents of *E. chiloensis* ssp. *chiloensis* suggest they are possibly attracting a narrower range of pollinators. Scents of hawkmoth-pollinated flowers are generally dominated by terpenes, aromatic esters, and/or nitrogen compounds (Kaiser and Tollsten 1995; Raguso and Willis 2003; Dobson 2006). Nocturnal scents of *E. chiloensis* ssp. *chiloensis* were almost completely dominated by terpenes, the most abundant being the sesquiterpene (*E*)-nerolidol and the monoterpene (*E*)-ocimene, both commonly, but not exclusively, found in flowers pollinated by hawkmoths (Knudsen and Tollsten 1993; Jurgens et al. 2003; Raguso et al. 2003; Dobson 2006; Schlumberger and Raguso 2008). Although we did not find aromatic esters or nitrogen compounds in nocturnal scents of *E. chiloensis* ssp. *chiloensis*, the presence of (*E*)-nerolidol, an elicitor of antennal responses in several hawkmoth species (Raguso et al. 1996; Raguso and Light 1998) including *M. sexta* (Fraser et al. 2003), is of determining importance.

Although volume and concentration variation of nectar along anthesis could simply be a consequence of physical conditions of the plant and environmental conditions (Willmer 2011), the present variations were consistent with the differential requirements of daytime and nighttime pollinators, as has been reported in other systems (Morse and Fritz 1983; Macior 1986; Amarin et al. 2012). Diurnal nectar volumes in *E. chiloensis* ssp. *chiloensis* flowers were higher than those observed in other bee-pollinated flowers (ca. 115 μ l observed values; vs. 10 μ l, Opler 1983; 15 μ l, Perret et al. 2001; 54 μ l, Ossa and Medel 2011), but closer to the range recorded in other cactus flowers with generalized pollination syndrome (ca. 115 μ l, observed values; 110 μ l, Silva and Sazima 1995; 129 μ l, de Viana et al. 2001; 170 μ l; Schlumberger et al. 2009). Sugar concentration in *E. chiloensis* ssp. *chiloensis* during the day was within the wide range reported for other bee-pollinated species (ca. 28 % sucrose w/w, observed values; 21.2–77 %, Waser and Price 1981; 29 %, Perret et al. 2001). On the other hand, nectar volume and sugar concentration in flowers of *E. chiloensis* ssp. *chiloensis* at the night period suggest nectar as a reward trait related to moths' requirements. Values observed in this study were similar to those found for other cactus species pollinated by nocturnal moths, both in nectar volumes (ca. 26 μ l, observed values; 23–29 μ l, Ortega-Baes et al. 2010) as well as in sugar concentration (ca. 30 % w/w, observed values; 8.2–43.9 %, Pyke and Waser 1981; 15–30 %, Baker and Baker 1983; Kaczorowski et al. 2005; 24–28 %, Fleming et al. 1996; 25–29 %, Raguso et al. 2003; 20–30 %, Torres et al. 2013). In *E. chiloensis* ssp. *chiloensis*, nectar is produced continuously both during day and night, the greatest production and volume being observed during the day and not at night as are common in moth-pollinated

cacti (Fleming et al. 1996; de Viana et al. 2001; Schlumberger and Badano 2005; Ortega-Baes et al. 2010). These results suggest that although the nighttime nectar seems to be related to moths' requirements, the increased volume of nectar during the day seems to be of advantage to a large number of pollinators.

Similarly to other temperate cactus species (Fleming et al. 2001; Dar et al. 2006; Ortega-Baes et al. 2010; Alonso-Pedano and Ortega-Baes 2012), in the pollination system of *E. chiloensis* ssp. *chiloensis*, its different groups of pollinators showed an approximately additive effect on fruit set and also on seed set per fruit. On the other hand, the product of fruit set and seed set gave similar results under diurnal and nocturnal pollination, suggesting that both groups of pollinators contribute to similar extents to the reproductive success of the cactus, presumably because nocturnal pollinators are very effective and diurnal pollinators very frequent. This type of compensation in pollinator activity affecting plant fitness has been reported in different contexts, e.g., diurnal and nocturnal pollinators affecting the product of fruit set \times seed set (Wolff et al. 2003), a bumblebee and a hummingbird affecting also the product of fruit set \times seed set (Waser and Price 1990), and a bumblebee and a hummingbird affecting plant fitness through color choice (Waser and Price 1981). Moreover, the joint effect of diurnal and nocturnal pollinators on the product of fruit set and seed was over three times higher than the added effects of the groups measured individually (Table 3), suggesting a synergistic effect between the two groups of pollinators. Reports in the literature have shown that interspecific interactions can alter the behavior of pollinators leading to increased pollination efficiency (Greenleaf and Kremen 2006; Brittain et al. 2013).

Flowers of *E. chiloensis* ssp. *chiloensis* show external traits normally associated to hawkmoth pollination; hence, a higher contribution to reproductive success of nocturnal pollinators over diurnal pollinators may be expected (Sletvold et al. 2012). Table 3 shows that this was not the case. A possible explanation for this apparent contradiction is that flowers may be morphological adapted to their "most effective pollinators" in past ecological contexts which seem to differ to those presently prevailing (Ollerton 1996). In such cases, current main pollinators may not have affected the genetic integration of floral adaptations that evolved in the past (Stebbins 1970). The latter could occur if current main pollinators are attracted and are effective over a wide range of forms of floral traits including the narrower range of forms fitted to the former main pollinators (Aigner 2001). Apparently, current main pollinators of *E. chiloensis* ssp. *chiloensis* are hawkmoths and bees, and bees that seem to be attracted by a wide range of morphological floral traits, included among which are the particular morphological floral traits that attract

hawkmoths. The floral tube of *E. chiloensis* ssp. *chiloensis* flowers measured 13.25 ± 0.2 cm while the length of the proboscis of *M. sexta* ranges from 6 to 10 cm (Schlumpberger et al. 2009; Walter 2010); this forces the hawkmoth to penetrate into the flower to reach the nectar and in this way remove and deposit large amounts of pollen (Gibson 2001). On the other hand, the floral tube is sufficiently wide to allow bees to enter and reach the nectar. Exserted stamens of *E. chiloensis* ssp. *chiloensis* flowers also facilitate pollen removal by hawkmoths, which hover just at the stamens level when feeding on nectar (Grant and Grant 1983). This type of stamens can also attract bees collecting pollen (Miyake et al. 1998).

While morphological floral traits tend to be rather constant within a species (Worley and Barrett 2000; Herrera 2001), nectar production and composition and floral scents are physiological traits which depend on the physical conditions of the plant and environmental conditions, which may change over short spatial and temporal scales (Rathcke 1992; Torres and Galetto 1998; Lake and Hughes 1999; Biernaskie and Cartar 2004; Longo and Fischer 2006; Macukanovic-Jocic et al. 2004; Valtuena et al. 2007; Ono et al. 2008). In this way, flowers of *E. chiloensis* ssp. *chiloensis* may produce nectar and floral scents with characteristics which match the requirements and perception abilities of diurnal and nocturnal pollinators without interference from each other.

In conclusion, flowers of *E. chiloensis* ssp. *chiloensis* show external traits normally associated to hawkmoth pollination but also allowing bee pollination; moreover, the floral scents and nectar rewards of *E. chiloensis* ssp. *chiloensis* during the day and at night have characteristics consistent with the requirements and perception abilities of diurnal (bees) and nocturnal pollinators (hawkmoths), respectively. Furthermore, the pollination system of *E. chiloensis* ssp. *chiloensis* involves diurnal and nocturnal pollinators contributing to similar extents to its reproductive success. These results contrast with those of Walter (2010), who found that nocturnal pollinators contributed more than diurnal ones to the reproductive success of a population of *E. chiloensis* ssp. *chiloensis*, and those of Ossa and Medel (2011), who found populations of *E. chiloensis* ssp. *chiloensis* with strictly diurnal pollination syndrome. Additional studies exploring the variation in abundance and distribution of pollinators, their relative effectiveness and efficiency as pollinators, their requirements and perception abilities, and the relation of these factors with floral traits are needed to understand the geographical variation of this cactus–pollinator interaction.

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