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**SINDROME DE POLINIZACION GENERALIZADO: ¿ESTAN LOS RASGOS FLORALES
ADAPTADOS A DISTINTOS POLINIZADORES?**

Tesis

Entregada a la Universidad de Chile

En cumplimiento parcial de los requisitos para optar al grado de

Magíster en Ciencias Biológicas

Facultad de Ciencias

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INFORME DE APROBACIÓN

Se informa a la Escuela de Postgrado de la Facultad de Ciencias que la Tesis de Magíster presentada por la candidata:

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*"La Biología no solo es la ciencia de la vida,
sino la conciencia de la vida"*

Rego

A mi compañero de alegrías, penas, logros y sacrificios...

Sin tu apoyo y comprensión no lo hubiera logrado

Biografía



Desde muy pequeña, fue mi papá y fueron los scouts quienes me enseñaron el respeto, la admiración y el interés por la naturaleza. Nací (1985) y estudié biología (2003-2010) en uno de los países más ricos del mundo en diversidad biológica: Bolivia. Durante el pregrado, mi interés se enfocó en la ecología, lo cual me llevó a desempeñarme como auxiliar de investigación en el Centro de Biodiversidad y Genética de la Universidad Mayor de San Simón (2005-2010) y como voluntaria del Programa de Conservación de Murciélagos de Bolivia (2006). Particularmente, mi atención se enfocó en las interacciones planta-animal y es así que no pensé dos veces en postularme para realizar una pasantía de investigación en interacciones planta-animal en el Laboratorio de Química Ecológica de la Universidad de Chile. Allí conocí a la Dra. Alejandra Troncoso y al Prof. Hermann Niemeyer, quienes compartieron conmigo sus conocimientos y me guiaron con mucha paciencia por el camino de la ecología y la química. Como parte del proyecto de doctorado de Alejandra Troncoso estudié la interacción de parasitismo entre el muérdago *T. verticillatus* y sus hospederos en el Santuario de la Naturaleza Yerba Loca. Esta fue una experiencia enriquecedora y gratificante que me motivó a continuar mi formación académica persiguiendo estudios de magíster en la Universidad de Chile. Actualmente, mi interés principal es el estudio del rol de los compuestos químicos en las interacciones ecológicas y continuaré desarrollando este interés durante mis estudios de doctorado en Ecología y Biología Evolutiva de la Universidad de Chile.

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RESUMEN

Las flores que son polinizadas tanto durante el día como en la noche, a menudo ofrecen recompensas (néctar) y exhiben mecanismos de atracción (esencias florales) para atraer distintos tipos de polinizadores. En este trabajo, exploramos los patrones de emisión de esencias florales y producción de néctar en las flores del cactus *Echinopsis chiloensis ssp. chiloensis*, en relación a los patrones de actividad de sus polinizadores diurnos y nocturnos. Medimos la frecuencia de visitantes florales, la producción de néctar y la concentración de azúcar en él, desarrollamos experimentos de exclusión de polinización y colectamos y analizamos las esencias florales. Los principales polinizadores diurnos fueron abejas, mientras que durante la noche el principal polinizador fue el esfíngido *Manduca sexta*. Las esencias florales diurnas estuvieron dominadas por varios compuestos que atraen una amplia gama de polinizadores, mientras que las esencias nocturnas fueron menos diversas y dominadas por el (*E*)-nerolidol, un compuesto que provoca respuestas antenales en esfíngidos. El volumen de néctar y la concentración de azúcar en la noche fueron similares a los valores reportados en flores polinizadas por esfíngidos. El volumen de néctar del día fue mayor que el reportado en flores polinizadas por abejas, pero similar al reportado en flores con numerosos polinizadores. La concentración de azúcar del día fue similar al reportado en otras flores polinizadas por abejas. Aunque las flores del cactus parecen estar morfológicamente adaptadas a la polinización por esfíngidos, también permiten la polinización por abejas. Las esencias y el néctar fueron consistentes con los requerimientos y las habilidades de percepción de los polinizadores diurnos y nocturnos. Ambos grupos de polinizadores contribuyen de manera similar al éxito reproductivo de cactus y tienen un efecto aditivo sobre el conjunto de frutos y semillas por fruto. Estos resultados son discutidos en términos del ajuste entre rasgos florales, habilidad de percepción y requerimientos de cada grupo de polinizadores.

Palabras clave: Esfíngidos, abejas, esencias florales, néctar, contribución de polinizadores

ABSTRACT

Flowers that are pollinated both during the day and at night often offer distinct rewards (nectar) and display distinct attraction mechanisms (floral scents) for different pollinators. In this paper, we explored the patterns of emission of flower scents and production of nectar in the flowers of cactus *Echinopsis chiloensis* ssp. *chiloensis*, in relation to the patterns of activity of its diurnal and nocturnal pollinators. We measured frequency of flower visitors, nectar production and sugar concentration, performed pollination exclusion experiments and analyzed floral scents. Bees were the main pollinators at daytime and the hawkmoth *Manduca sexta* at nighttime. Diurnal scents were dominated by several compounds which can attract a wider 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 in nectar at night were similar to those reported for hawkmoth-pollinated flowers. Daytime nectar volume was higher than common values reported in bee-pollinated flowers, but similar to those reported in flowers pollinated by several different pollinators. Daytime sugar concentration was similar to that reported in bee-pollinated flowers. Although the flowers of cactus seem morphologically adapted to hawkmoth pollination, also allows bee pollination; moreover, floral scents and nectar rewards were consistent with requirements and perception abilities of diurnal and nocturnal pollinators. Both groups of pollinators contribute equally at reproductive success and have an additive effect on fruit set and seeds per fruit set. These results are discussed in terms of the linkage between floral traits, perception abilities and requirements of each group of pollinators.

Key words: Hawkmoth, Bees, Floral Scents, Nectar, Pollination Contribution

GENERALIZED POLLINATION SYSTEM: ARE FLORAL TRAITS ADAPTED TO DIFFERENT POLLINATORS?

Introduction

A pollination syndrome comprises the entire set of floral traits involved in the attraction of a particular group of pollinators (Proctor *et al.* 1996). Flowers with generalized pollination syndrome are those that attract more than one type of pollinator (e.g.: Young 2002, Schlumpberger & Badano 2005, Muchhala *et al.* 2008, Ortega-Baes *et al.* 2010, Schmid *et al.* 2010, Yokota & Yahara 2012) and are much more common than was thought until a few years ago (e.g., 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 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.: Schemske & Bradshaw 1999, Briscoe & Chittka 2001, Chittka & Raine 2006, Kim *et al.* 2011). For example, hawkmoths may be mainly attracted by olfactory cues (Yamamoto *et al.* 1969, Mechaber *et al.* 2002) and may require large amounts of nectar with low sugar concentration (Opler 1983, Baker & Baker 1983, Kaczorowski *et al.* 2005) and bees may be mainly attracted by visual cues (Milet-Pinheiro *et al.* 2012) and may require lesser amounts of nectar with high sugar concentration (e.g., bees: Opler 1983, Baker & Baker 1983). In this sense, flowers that are pollinated both day and night may produce nectar (Amorin *et al.* 2013) and flower scents (Dotterl *et al.* 2012) with different characteristics during the day and night, in relation to its day and night pollinators, respectively.

Moreover, for a generalized pollination syndrome to be considered as optimal, different groups of pollinators should contribute effectively to the reproductive success

of the plant (Flemming *et al.* 2001); these contribution depend in general on both the visitation rate and the efficiency of pollen transfer (e.g.: Thomson 2000, Aigner 2004). Different pollinators may differ in their frequency of visits and pollination efficiency (Waser *et al.* 1996). Hawkmoths are generally considered very efficient 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 & Liltved 1997), and a single visit can successfully fertilize a given flower (Willmot & Búrquez 1996). In contrast, bees are considered inefficient pollinators since, unlike hawkmoths that 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 & Adam 2010). In cacti from temperate areas both hawkmoths and bees are effective pollinators, but the most efficient group varies depending on the species of cactus (Ortega-Baes *et al.* 2010, Alonso-Pedano & Ortega-Baes 2012).

Several species of cacti from temperate regions exhibit generalized pollination syndromes with anthesis lasting from 24 to 48 hours; their main nocturnal visitors are moths while during the day their main visitors are bees (de Viana *et al.* 2001, Schlumpberger & Badano 2005, Schlumpberger *et al.* 2009, Ortega-Baes *et al.* 2010, Alonso-Pedano & 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.* 2013).

In this thesis, we explore the emission pattern 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 & 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 can stay open for 22 h on average. Indeed, Walter (2010) describe that the main nocturnal visitors and the major contributors to

fruit set and seeds per fruit set in this cactus are hawkmoths, and also during the day the main visitors are bees. Because of different perception abilities and requirements of their floral pollinators, we hypothesize that composition of flower scents, nectar volumes and sugar concentration in the nectar in *E. chiloensis ssp. chiloensis* differ between day and night, presenting characteristics related to 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 but, due to the morphological traits of the flowers, the higher efficiency of hawkmoths and the previous evidence in *E. chiloensis ssp. chiloensis* (Walter 2010), the contribution of nocturnal pollinators should be greater.

Methods and Materials

Study Area and Study Species- This study was conducted from November 2012 until 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 temperature was 19±0.5°C with an average relative humidity of 54±3% (data from the agro-meteorological station at RNRC). The vegetation of the area is dominated by evergreen plants of the sclerophyllous forest and matorral vegetational region (Gajardo, 1993). *Echinopsis chiloensis ssp. chiloensis* is endemic to central Chile from La Serena (29°02'S) to Talca (36°33'S) (Luebert & Pliscoff 2006) and exhibits different pollination syndromes along its distribution. In its northern distribution (National Reserve Las Chinchillas: 31°30'S, 71°06'W), it exhibits a diurnal pollination syndrome where flowers open between 06:00 and 07:00 h, remain open a maximum of 8 hours and their main visitors are bees (Ossa & 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 19:00 and 21:00 h, remain open between 17 and 42 h 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.

Floral Visitors- Anthesis in the study area began between 18:00 and 20:00 h; flowers remained open for 20 to 22 h on average, similar to the population in Rancagua (Walter 2010). 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 were selected to conduct direct observations of floral visitors for periods of 30 minutes 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 or Eppendorf tubes and they were later identified by specialists (see Acknowledgements). During the night period the flowers were filmed with infrared light for periods of 90 minutes every 3 hours and identified by the same specialists. During each visit, the behavior of visitors was observed to determine which species were potentially pollinators. 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 & Pellmyr 1998). Six flowers that opened around the same time (18:30-20:30) of different cactus individuals at different days were selected and individually bagged with tulle bags before their opening and after each collection to avoid the alteration of floral scents by visiting insects. The collection system consisted of an inert oven bag (Melitta, Minden, Germany), a silicone hose and teflon tape used for sealing the system. Synthetic 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) and a manual flow-meter and flow regulator. Floral scents were collected in Porapak Q

columns during the peak visiting activity hours within each period of anthesis (see Results), each collection lasting one hour.

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 column injection RTX-5MS (length 30 mm, diameter 0.25 and 0.25 micron film thickness). Helium was used as carrier gas with a flow rate of 1.3 ml/min. Temperature of injection port and detector were set at 250°C. The oven temperature was programmed at 35°C for 5 minutes and then increased by 5°C every minute until 200°C was reached; the final temperature was kept for 10 min. Volatile compounds were identified according to their mass spectra and their retention indices using the 2011 NIST 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 peak visiting activity hours within each period of anthesis (see Results), as a function of the time after opening; in this case, in addition to collections during the periods previously considered (opening, night and day), 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 (18:30-20:30) of 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 measurement was performed using 40 different flowers (10 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 measurement was made in the remaining 10 flowers repeatedly every 6 hours, simulating nectar consumption by floral visitors, therefore obtaining the nectar volume produced between hours (repeated nectar measurement).

All nectar samples were collected and measured using 100 μ l Hamilton syringes. Sugar concentration in the nectar (% of sucrose) was measured with a manual refractometer. For this, 40 μ l 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 μ l, a measured amount of distilled water was added to produce a final volume of 50 μ l; final concentrations were determined by applying the dilution factor to the measured concentration.

Contribution of Pollinators to Reproductive Success- One hundred flowers were selected and assigned 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, 1188 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 rate of visitors and relative abundance of volatile compounds were calculated because the totals showed substantial variations. The 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 & Keough 2002). To compare the assembly of visitors and flower scent composition between time periods (opening, night and day), Bray-Curtis (1957) similarity indices were calculated and similarity analysis of permutations (ANOSIM;

Clarke & Gorley 2006) subsequently performed with 10,000 random permutations; the Bonferroni correction factor was applied to reduce type I error (Quinn & Keough 2002). To identify the species and the compounds responsible for the variability in visitation rate and scents between time periods, similarities of percentages (SIMPER) analyses were performed. To compare the variation of visitors and scents between time periods, multivariate non-metric multidimensional scaling (NMDS) method was conducted 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 experimental exclusions treatments through the Chi-Square test of association for frequency tables (Dytham 2011) using online tools website: <http://faculty.vassar.edu/lowry/VassarStats.html> (Lowry, 2008). Parametric one-way ANOVA was used to compare nectar volume between periods and the number of seeds per fruit between exclusion types. Because these 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 & Cox 1964). A posteriori pairwise comparisons were performed through Tukey tests. Non-parametric one-way Kruskal-Wallis was used to compare nectar sugar concentration between periods; this analyzes was followed by non-parametric Dunn tests for unbalanced designs. Analyses were performed in STATISTICA 8 (StatSof Inc. 2006) and the graphics made using SIGMA PLOT 11.0 (Systat Software Inc. 2008). For easier interpretation of the results, the nectar untransformed is shown.

Results

Floral Visitors- Twenty-four insect species belonging to five orders were observed visiting flowers; sixteen of these species belonging to three different orders were potential pollinators (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 potential pollinators were the bees *Apis mellifera*, *Leioproctus semicyaneus* and *Ruizantheda mutabilis* (Table 1). The peak visiting activity hours were: 18:30-20:30 h during opening, 00:30-02:30 h during the night and 11:30-13:30 h during the day; and corresponded with the zero, sixth and 18th hour of anthesis, respectively, which allowed us to control the age of each sampled flower.

The assemblage of potential pollinators varied significantly between time periods (one-way ANOSIM: $R=0.37$ $P<0.001$). The pattern of visits during the day was significantly different between opening and night periods (one-way ANOSIM: opening vs. day $R=0.59$ $P<0.001$; night vs. day $R=0.58$ $P<0.001$) but the pattern of visits during the opening was non significantly different from the night period (one-way ANOSIM: opening vs. night $R=0.009$ $P=0.344$). According to the SIMPER analysis, the main species responsible for differences between time periods were: bees *Megachile distungüeda*, *L. semicyaneus*, *Manuelia gayi*, *Diadasia chilensis*, *R. mutabilis* and *A. mellifera*, the most present with high frequency during the day period. The resulting graph for the NMDS analysis (Fig. 1a) provides an adequate representation of the clear differences between the day and the other two periods (opening and night) and the similarity (non-significant differences) between opening and night periods.

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 (Table 2). 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: $R=0.55$ $P<0.001$). 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 from those of the night period (one-way ANOSIM: $R=0.46$ $P=0.044$) and from those of the day period ($R=0.34$ $P=0.052$). According to the 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 analysis (Fig. 1b) shows clear differences between day and night and similarity between opening and day.

Table 1 Potential pollinators, mean visitation rates (VR), standard error (SE) and percentage of visits (%) to the cactus *Echinopsis chiloensis* ssp. *chiloensis* during three different time periods (opening, night, day).

Order/species (family)	Opening		Night		Day	
	V. R. (SE)	%	V. R. (SE)	%	V. R. (SE)	%
COLEOPTERA						
<i>Bilyaxia concinna</i> (Buprestidae)					0.31 (0.21)	0.60
<i>Epiclines gayi</i> (Cleridae)					0.15 (0.10)	0.30
<i>Amecocerus</i> sp. (Melyridae)			0.11 (0.09)	33.33	3.54 (1.25)	6.94
<i>Arctodium vulpinum</i> (Scarabaeidae)					0.46 (0.22)	0.90
Sub-totals	0.00	0.00	0.11	33.33	4.46	8.75
HYMENOPTERA						
<i>Diadasia chilensis</i> (Apidae)					4.85 (1.56)	9.50
<i>Apis mellifera</i> (Apidae)	0.67 (0.67)	100.00			11.54 (4.36)	22.62
<i>Manuelia gayi</i> (Apidae)					3.31 (1.32)	6.49
<i>Leoproctus semicyaneus</i> (Colletidae)					10.08 (4.82)	19.76
<i>Coenohalictus delator</i> (Halictidae)					1.69 (0.70)	3.32
<i>Pseudagapostemon pissisi</i> (Halictidae)					0.85 (0.61)	1.66
<i>Lasiglossum herbstiellus</i> (Halictidae)					1.62 (0.77)	3.17
<i>Ruizantheda mutabilis</i> (Halictidae)					8.77 (3.40)	17.19
<i>Ruizantheda próxima</i> (Halictidae)					1.85 (0.88)	3.62
<i>Trichothurgus dubius</i> (Megachilidae)					1.00 (0.34)	1.96
<i>Megachile distinguenda</i> (Megachilidae)					1.00 (0.53)	1.96
Sub-totals	0.67	100.00	0.00	0.00	47.87	91.25
LEPIDOPTERA						
<i>Manduca sexta</i> (Sphingidae)			0.22 (0.18)	66.67		
Sub-totals	0.00	0	0.22	66.67	0.00	0.00
Grand-totals	0.67	100.00	0.33	100.00	52.33	100.00

Table 2 Frequency (f), 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 three different time periods (opening, night, day).

Compound class/name	Retention Index	Opening		Night		Day	
		f	area % (SE)	f	area % (SE)	f	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	1006	5	0.30 (0.91)	2	0.08 (0.29)	4	1.10 (2.57)
<i>m</i> -Cymene	1024	5	0.15 (0.96)	2	0.05 (0.16)	4	1.03 (1.37)
Limonene	1027	6	0.94 (2.09)	4	0.47 (1.15)	5	3.74 (0.85)
(<i>Z</i>)- β -Ocimene	1039	5	1.06 (4.05)	5	4.31 (2.17)	5	0.93 (2.43)
(<i>E</i>)-Ocimene	1050	5	18.49 (11.60)	5	38.73 (11.17)	6	5.65 (7.75)
Linalool	1099	1	0.01 (0.00)	4	2.51 (1.09)		
<i>Neo-allo</i> -ocimene	1131	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	1281	3	0.17 (0.91)	3	0.04 (0.05)	3	0.72 (0.12)
α -Cubebene	1346	1	0.05 (0.00)				
β -Farnesene	1462	2	1.52 (0.61)	5	0.26 (0.10)	1	1.08 (1.00)
Germacrene D	1489	2	0.28 (6.50)	2	0.94 (3.04)	2	0.06 (0.18)
α -Farnesene	1513	4	15.70 (3.28)	6	4.85 (2.12)	3	7.57 (2.35)
δ -Cadinene	1531	1	0.13 (1.00)	2	0.23 (0.50)	1	0.01 (1.00)
(<i>E</i>)-Nerolidol	1570	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	1098	1	15.00 (0.00)			6	47.21 (12.57)
Methyl salicylate	1198	1	0.43 (0.00)			1	8.15 (0.00)
Benzyl benzoate	1769					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	1097					1	0.08 (0.00)
Dodecane	1199					1	0.03 (0.00)
Tetradecane	1400	1	0.005 (0.00)	1	0.04 (0.00)	1	0.69 (0.00)
Hexadecane	1599					1	2.23 (0.00)
Sub-totals			0.42 (0.08)		0.14 (0.02)		3.21 (0.35)
D) Aldehyde							
Decanal	1206	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

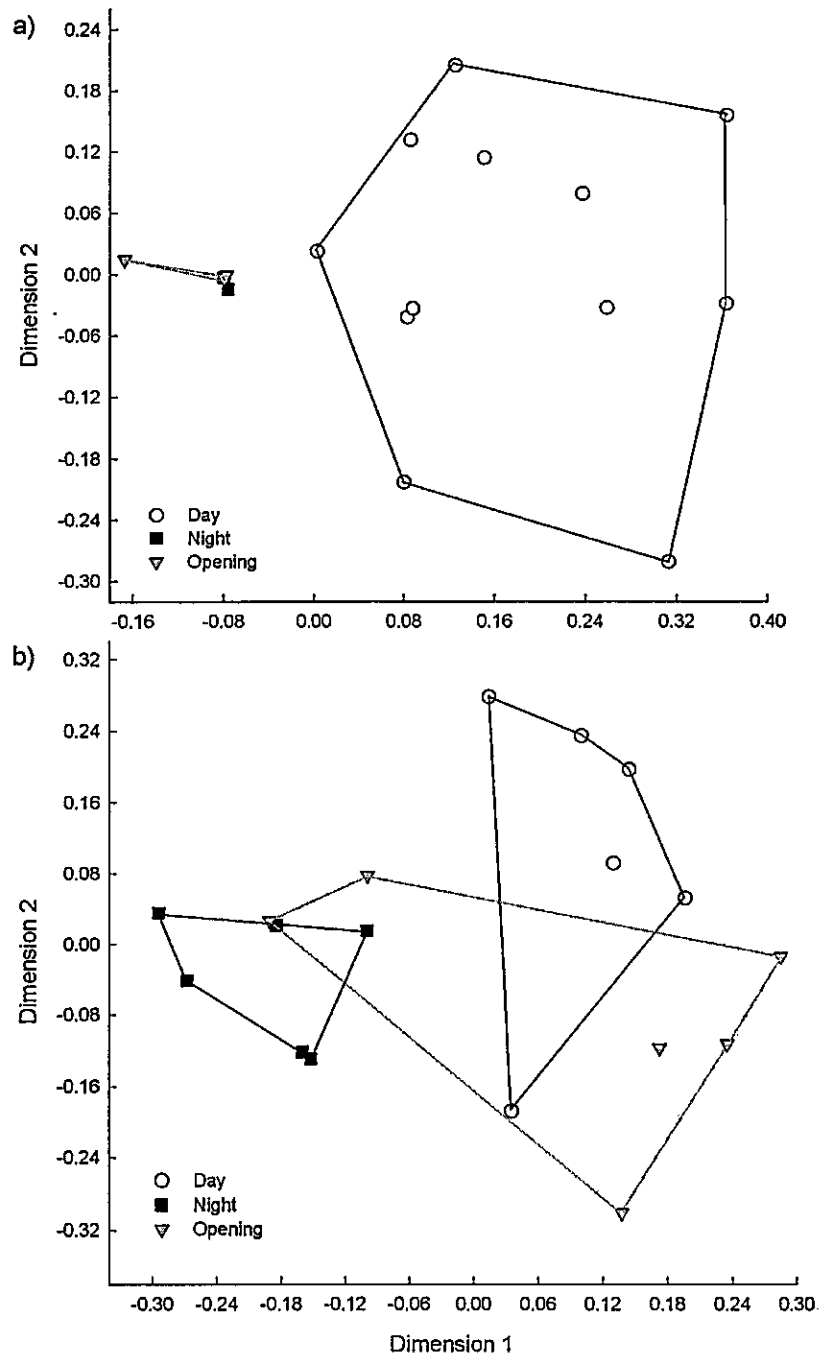


Fig. 1 Non-metric multidimensional scaling (NMDS) based on Bray-Curtis similarity matrix of: a) assemblages of potential pollinators (2D stress value: 0.126) and b) floral scents (2D stress value: 0.298), in flowers of the cactus *Echinopsis chiloensis* ssp. *chiloensis* at three times during anthesis (opening, nighttime, daytime). Each point in space represents the pattern of visits in a flower (13 per period) or the pattern of scents in a flower (6 per period). If the patterns are very similar between one and another flower, the points shown overlapped.

Nectar Production- Nectar is available at the opening of flowers and is 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 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 were significantly higher than that produced 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 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 showed a negative correlation 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). Flowers without exclusion produced a significantly higher proportion of fruit compared to flowers with nocturnal and diurnal exclusion (Chi-square: $X^2=5.56$, $P=0.038$ and $X^2=18$, $P<0.001$, respectively). The proportion of fruits produced from flowers with nocturnal exclusion was significantly higher than in flowers with diurnal exclusion (Chi-square: $X^2=4.35$, $P=0.037$). Number of seeds varied significantly along 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: $X_2=11.07$, $P<0.001$ and $X_2=9.96$ $P=0.001$, respectively); however, there was no difference in the number of germinated seeds between diurnal and nocturnal exclusions (Chi-square: $X_2=0.13$, $P=0.72$) (Table 3). The product of fruit set x seed set was similar for the two exclusion treatments and they were smaller than for the no exclusion treatment (Table 3).

Table 3 Fruit set, seed set (per fruit), fruit set x seed set and germinated seeds from flowers of the cactus *Echinopsis chiloensis* ssp. *chiloensis* under different pollinator exclusion experiments. SE= standard errors. Different letters within columns indicate significant differences at $P=0.05$.

<u>Exclusion</u>	<u>Fruits set</u> (Proportion)	<u>Seeds set</u> Nº (EE)	<u>Fruit set x seeds set</u> Nº	<u>Germinated seeds</u> Nº ± EE (%)
Total	0	0	0	0
Without	0.80 a	2850.2 (393.8) a	2280	17.3 ± 0.2 (96) a
Night	0.48 b	764.8 (221.1) b	367	16.2 ± 0.3 (89.9) b
Day	0.20 c	1599 (376.8) b	320	16.3 ± 0.4 (90.7) b



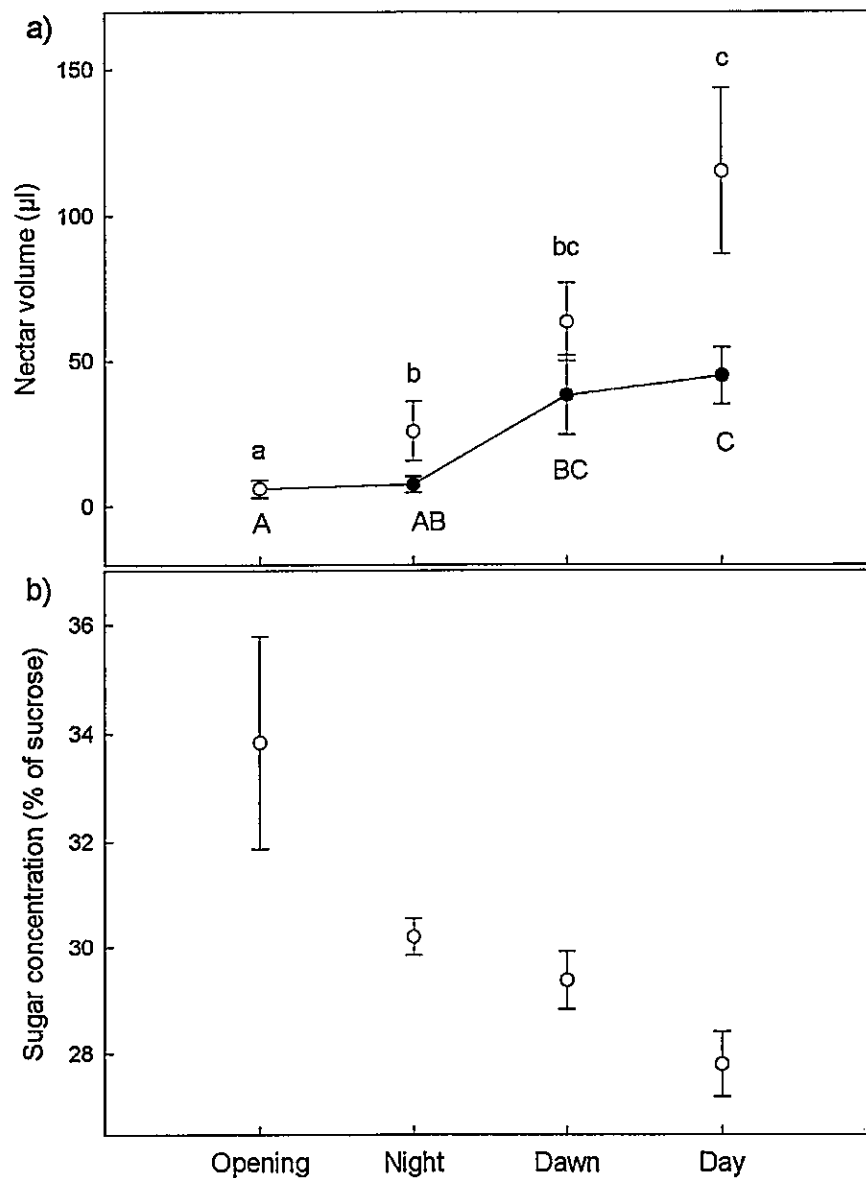


Fig. 2 a) Nectar volume (μl , mean ± 1 SE) and b) sugar concentration ($^{\circ}\text{Bx}$, mean ± 1 SE) in flowers of the cactus *Echinopsis chiloensis* ssp. *chiloensis* measured at four different periods using two different methodologies (○= accumulated nectar measurements; ●= repeated nectar measurements). Different letters (a, b, c and A, B, C) indicate significant differences between periods.

Discussion

The bee *A. mellifera* was the only species present during the opening period and was the most frequent one during the day period but it was absent at night. Other frequent visitors during the day were the bees *L. semicyaneus*, *R. mutabilis*, *D. chilensis* and *M. gayi*. Although *A. mellifera* has been described as a frequent visitor to several cactus species (Bustamante *et al.* 2010, Ortega-Baes *et al.* 2010, Larrea-Alcázar & López 2011, Alonso-Pedano & Ortega-Baes 2012), it is considered a generalist pollen collector (Michener 2000) as well as an inefficient (Keys *et al.* 1995) and detrimental pollinator (Schlumpberger & Badano 2005). In contrast, the bees *L. semicyaneus* and *R. mutabilis* belong to oligolectic families (Collins & Thomas 1991), some species of the genus *Diadasia* have been described as pollen collector specialized in cacti (Sipes & Tepedino 2005) and *M. gayi* has been reported as a pollinator of several plant species in the area where the present studies were performed (Smith-Ramírez & Yáñez 2010). Therefore, these four latter bee species are likely to be the main daytime pollinators of *E. chilensis ssp. chilensis*. The lower number of seeds in nocturnal exclusions may be caused by a lower quantity or quality of pollen deposited by *A. mellifera*, and also by a delayed pollination by bees in general (several hours after opening flowers) thus reducing pollen tube growth and may limit fertilization of some ovules (Bustamante *et al.* 2010).

The only potential pollinator species during the night were the beetle *Amecocerus sp.*, and the hawkmoth *Manduca sexta*. *Amecocerus 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 & Yáñez 2010). Nevertheless, the main nocturnal pollinator of *E. chilensis ssp. chilensis* probably is *M. sexta* since this hawkmoth has been described as frequent visitor of several cacti (Raguso *et al.* 2003, Schlumperger *et al.* 2009, Ortega-Baes *et al.* 2010) and was observed previously by chance inside one of the flowers of *E. chilensis ssp. chilensis* (Walter 2010). However, the hawkmoths are

generally considered to be locally and temporally scarce (Dar *et al.* 2006, Raguso *et al.* 2003, Schlumpberger and Badano 2005) and the lower number of fruits in diurnal exclusions may be caused by a lower frequency of visits by nocturnal pollinators, although the number of seeds encountered in diurnal exclusions indicates that this pollinators are very efficient.

Although compositional variation of flower scents along anthesis could simply be a consequence of circadian changes in light and temperature (Kolosova *et al.* 2001), the present variations were consistent with the differential requirements of daytime as well nighttime pollinators, as has been reported in other systems (Kaczorowski *et al.* 2005, Dotterl *et al.* 2012). Diurnal scents of *E. chiloensis ssp. chiloensis* were composed by a larger 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 bees and butterflies pollinated (Kaiser & Tollsten 1995). Moreover, (*E*)-nerolidol, α -farnesene and (*E*)-ocimene are common compounds in bee and moth-pollinated flowers (Kaiser & 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 & Tollsten 1993, Kaiser & Tollsten 1995, Kolosova *et al.* 2001, Pettersson *et al.* 2004, Dobson 2006, Schlumperger & 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 & Tollsten 1995, Raguso & Willis 2003, Dobson 2006). Nocturnal scents of *E. chiloensis ssp. chiloensis* were almost completely

dominated by terpenes, the most abundant compounds being the sesquiterpene (*E*)-nerolidol and the monoterpene (*E*)-ocimene, both commonly, but not exclusively, found in flowers pollinated by hawkmoths (Knudsen & Tollsten 1993, Jurgens *et al.* 2003, Raguso *et al.* 2003, Dobson 2006, Schlumpberger & Raguso 2008). Although we did not find aromatic esters and 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 & 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 & Fritz 1983, Macior 1986, Amorin *et al.* 2013). 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 & 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 & Sazima 1995; 129 μ l, de Viana *et al.* 2001; 170 μ l, Schlumpberger *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 % w/w, observed values; 21.2 – 77%, Pyke & Waser 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 & Waser 1981; 15 – 30%, Baker & Baker 1983, Kaczorowsky *et al.* 2005; 24 – 28%, Fleming *et al.* 1996; 25 – 29%, Raguso *et al.* 2003; 20 – 30%, Torres *et al.* 2013. In some

cactus mainly pollinated during the night, nighttime nectar is accumulated in absence of nocturnal pollinators, and increases the chance of visits by diurnal pollinators (Bustamante *et al.* 2010). In *E. chilensis ssp. chilensis* nectar is produced continuously both during day and night, the greatest production and volume being observed during the day and not at night as is common in moth pollinated cacti (Fleming *et al.* 1996, de Viana *et al.* 2001, Schlumpberger & 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 & Ortega-Baes 2012), the pollination system of *E. chilensis ssp. chilensis* is complementary, because the different groups of pollinators showed an additive effect on fruit set and also on seed set per fruit (Fleming *et al.* 2001). 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 ones are very efficient and diurnal ones 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 x seed set (Wolff *et al.* 2003), a bumblebee and a hummingbird affecting also the product of fruit set x seed set (Waser & Price 1990), and a bumblebee and a hummingbird affecting plant fitness through color choice (Waser & Price 1981). Moreover, the joint effect of diurnal and nocturnal pollinators on the product of fruit set and seed was over 3 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 effectiveness (Greenleaf & Kremen 2006, Brittain *et al.* 2013).

Flowers *E. chilensis* ssp. *chilensis* show external traits apparently adapted 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 is not the case. A possible explanation for this apparent contradiction is that in past ecological contexts the 'most effective pollinators' were those to which flowers seem to be morphological adapted, although presently a generalized pattern of visitors are observed for these same flowers (Ollerton 1996). In such cases, current main pollinators would not affect 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. chilensis* ssp. *chilensis* are hawkmoths and bees, and bees that seem to be attracted by a wide range of morphological floral traits, among which are included the particular morphological floral traits that attract hawkmoths. The floral tube of *E. chilensis* ssp. *chilensis* flowers was 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 or 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. chilensis* ssp. *chilensis* flowers also facilitate pollen removal by hawkmoths, which hover just at the stamens level when feeding on nectar (Grant & 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 & 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. These conditions may change over short spatial and temporal scales (Rathcke 1992, Torres & Galetto 1998, Lake & Hughes 1999, Biernaskie

& Cartar 2004, Longo & Fischer 2006, Macukanovic-Jocic *et al.* 2004, Valtuena *et al.* 2007, Ono *et al.* 2008) therefore allowing flowers of *E. chiloensis* ssp. *chiloensis* to produce nectar and floral scents with characteristics which match the requirements and perception abilities of diurnal and nocturnal pollinators without interference from each other.

Conclusions

In conclusion, flowers of *E. chiloensis* ssp. *chiloensis* show external traits apparently adapted to hawkmoth pollination but also allowing bee pollination; moreover, the floral scents and nectar rewards of *E. chiloensis* ssp. *chiloensis* have characteristics consistent with the requirements and perception abilities of diurnal (bees) and nocturnal pollinators (hawkmoths). Our results show that the pollination system of *E. chiloensis* ssp. *chiloensis* is complementary, 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 contribute more than diurnal ones to the reproductive success of a population of *E. chiloensis* ssp. *chiloensis*, and those of Ossa & 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 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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