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Extreme variation in floral characters and its consequences for pollinator attraction among populations of an Andean cactus

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- *Background and aims* A South American cactus species, *Echinopsis ancistrophora* (Cactaceae), with dramatic among-population variation in floral traits is presented.
- *Methods* Eleven populations of *E. ancistrophora* were studied in their habitats in northern Argentina, and comparisons were made of relevant floral traits such as depth, stigma position, nectar volume and sugar concentration, and anthesis time. Diurnal and nocturnal pollinator assemblages were evaluated for populations with different floral trait combinations.
- *Key Results* Remarkable geographical variations in floral traits were recorded among the 11 populations throughout the distribution range of *E. ancistrophora*, with flower lengths ranging from 4·5 to 24·1 cm. Other floral traits associated with pollinator attraction also varied in a population-specific manner, in concert with floral depth. Populations with the shortest flowers showed morning anthesis and those with the longest flowers opened at dusk, whereas those with flowers of intermediate length opened at unusual times (2300–0600 h). Nectar production varied non-linearly with floral length; it was absent to low (population means up to 15 μL) in short- to intermediate-length flowers, but was high (population means up to 170 μL) in the longest tubed flowers. Evidence from light-trapping of moths, pollen carriage on their bodies and moth scale deposition on stigmas suggests that sphingid pollination is prevalent only in the four populations with the longest flowers, in which floral morphological traits and nectar volumes match the classic expectations for the hawkmoth pollination syndrome. All other populations, with flowers 4·5–15 cm long, were pollinated exclusively by solitary bees.
- *Conclusions* The results suggest incipient differentiation at the population level and local adaptation to either bee or hawkmoth (potentially plus bee) pollination.

Key words: Pollination, floral biology, *Echinopsis ancistrophora*, cactus, Cactaceae, hawkmoth, bee.

INTRODUCTION

The degree to which flower–pollinator relationships are specialized (or not) has stimulated much research and discussion during the past decade (Waser *et al.*, 1996; Johnson and Steiner, 2000). One outcome of this debate has been a reconsideration of what it means to be specialized. Obligate mutualism (e.g. between yuccas and yucca moths or between senita cactus and senita moth) is a relatively rare form of specialization, largely limited to nursery pollination systems in which a subset of fertilized ovules are consumed by the progeny of the pollinator (Holland and Fleming, 1999). More commonly, plants are specialized to pollination by guilds or functional groups of pollinators, e.g. sphingid moths with certain proboscis dimensions (Moré *et al.*, 2006), or oil-collecting bees (Cosacov *et al.*, 2008). One problem is that floral specialization on certain visitor guilds or species is often visible only at second glance, as different types of filters (e.g. chemical filters as in Johnson *et al.*, 2006; morphological filters as in Moré *et al.*, 2007) can act on the (potential) visitor spectrum. Furthermore, only a subset of floral visitors may act as pollinators, and only a subset of pollinators may account for most pollen transfer (Bloch *et al.*, 2006). For pollination systems based on animals that forage for nectar or pollen rewards, specialization has traditionally been thought to evolve as an adaptation to the most effective pollinator (Stebbins, 1970). Alternatively, Aigner (2001) has suggested that specialization even to a relatively inefficient pollinator can occur if it results in a net fitness increase. For example, Bloch *et al.* (2006) have shown that high visitation frequency can compensate for low pollination efficiency (i.e. number of conspecific pollen grains deposited on a stigma per visit) by butterflies at *Dianthus* flowers, an example of what Herrera (1989) has termed the 'quantity component of pollination'.

To test hypotheses about floral adaptation, Aigner (2001) has advocated the study of model systems with a range of floral phenotypes. However, the study of adaptive potential in floral evolution has been hampered by the fact that traits under directional or stabilizing selection tend to lose their variance in natural populations (Fenster, 1991; Herrera, 1996). Ironically, natural, heritable variation in floral colour, scent or shape, considered the *sine qua non* for adaptive evolution via natural selection (Endler, 1986), often is found to have neutral or non-significant effects on plant reproductive fitness (e.g. Ackerman *et al.*, 1997; Jersakova *et al.*, 2006). Thus, it can be difficult to explore the potential for pollinator-mediated selection if there is not sufficient floral variation left

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upon which selection can act. There are several opportunities to observe incipient phenotypic sorting:

(1) the study of hybrid swarms, with their attendant arrays of recombinant phenotypes associated with different hybrid and backcross generations (Hodges and Arnold, 1994);
(2) the use of selective breeding (Schemske and Bradshaw, 1999; Galliot *et al.*, 2006) or transgenic approaches (Kessler and Baldwin, 2007; Kessler *et al.*, 2008) to modify specific traits of interest;
(3) the experimental alteration of floral phenotypes (Nilsson, 1988; Castellanos *et al.*, 2004); and
(4) the study of naturally varying populations in a widely distributed species complex.

These approaches share the common goal of measuring the extent to which variation in floral traits could be moulded by pollinator-mediated natural selection.

The South American cactus genus *Echinopsis* contains several highly variable taxa, including the *E. ancistrophora* species complex, in which different natural populations vary in floral shape, colour, scent, nectar and time of anthesis. Owing to its dramatic phenotypic variability, numerous names have been assigned to different populations of *E. ancistrophora* and its relatives, until Hunt (1999) circumscribed *E. ancistrophora* to encompass four subspecies, including taxa with white or colourful flowers and short (approx. 5 cm) to very long (>20 cm) flowers. Remarkably, almost all flower colour × length combinations can be found within this group, often departing dramatically from the conventional trait combinations outlined by the floral syndrome concept for cacti and Angiosperms in general (Porsch, 1938, 1939; Faegri and van der Pijl, 1971). Here we focus on the white-flowered populations, i.e. *E. ancistrophora sensu* Lowry (2002) and Hunt (2006), that include the full extent of floral morphological variation. Preliminary molecular phylogenetic analyses confirm the monophyly of *E. ancistrophora* (*sensu* Lowry, 2002 and Hunt, 2006), and indicate that further subdivision of these entities into separate taxa (e.g. subspecies) is not supportable (B. O. Schlumpberger, unpubl. res.). Thus, the plants studied here represent a cluster of closely related entities undergoing unusual phenotypic sorting, and present a rare opportunity to study floral trait combinations not often observed in nature.

Flowers of the populations included in this study are white (to slightly pink in some cases) and funnel-shaped with highly variable tube length, and thus have been assumed to bloom nocturnally and to be moth-pollinated (Lowry, 2002). However, as the shortest flowers do not resemble typical hawkmoth-pollinated cactus flowers (e.g. Raguso *et al.*, 2003), and diurnal anthesis has been suggested for some of these populations (Backeberg, 1959), we predicted that pollinator affinities would vary among populations, offering the chance to study intraspecific divergence and potential adaptation to different functional groups of pollinators.

We tested specific predictions concerning floral trait combinations and associated pollinator affinities among 11 populations of *E. ancistrophora* in northern Argentina, as follows.

If floral variation is not adaptive, we should fail to reject the null hypothesis of random association between floral traits and visitor assemblages.

Alternatively, if flowers of different populations are adapted to different pollinator groups, anthesis time and nectar production should vary in concert with floral length as follows:

(1) Populations with long-tubed flowers were predicted to be pollinated primarily by sphingid moths. Consequently, anthesis should be nocturnal, with copious nectar production (Haber and Frankie, 1989).
(2) Populations with short-tubed flowers are pollinated primarily by diurnal bees. Consequently, we expected morning anthesis and lower quantities of nectar, depending upon whether the bees seek nectar or pollen as primary rewards (Wilson *et al.*, 2004).

In contrast, we have no clear predictions based on pollination syndromes for two populations of *E. ancistrophora* with intermediate floral tube lengths and anthesis times. One possibility is for bimodal pollination by sphingid moths and bees seeking nectar and pollen, respectively (see Barthell and Knops, 1997; Miyake and Yahara, 1998). A second possibility is for pollination by a novel class of pollinator or functional group (see Fenster *et al.*, 2004), whose presence would not have been predicted by classical syndromes (e.g. hummingbirds on white cactus flowers of *Echinopsis atacamensis* ssp. *pasacana*; Schlumpberger and Badano, 2005). A third possibility is that unusual combinations of floral traits may be discriminated against by potential pollinators due to incompatibility of floral rewards or mechanical fit with visitors (e.g. Wilson *et al.*, 2004), which would perhaps explain why such combinations are not observed more commonly in nature. These populations were of specific interest, as they allowed us to evaluate the potential constraints associated with transitional stages between discrete syndromes (e.g. Baker, 1963) versus the possibility of adding an additional pollinator with no reduction in fitness (e.g. Aigner, 2001).

METHODS

Plants and animals, study periods and locations

Echinopsis ancistrophora grows in the northern Argentine provinces of Tucuman, Salta and Jujuy along the eastern Andean slopes or on pre-Andean hills above the Chaco forest zone. Field studies took place during the flowering period of *E. ancistrophora* from mid October to mid December in 2002 and 2003. Sixteen populations of *E. ancistrophora* were studied, covering the entire range of its distribution in the Argentine provinces Tucuman, Salta and Jujuy. However, only 11 populations for which sufficient sample sizes of floral trait data and flower visitors could be gathered are included in the present analysis, representing a mostly latitudinal transect, which also encompasses the small longitudinal extension of the species (Table 1). Plant identifications were determined by the first author. Voucher specimens are deposited in the herbarium of the Museo Botánico de Córdoba (CORD), Argentina. Bees were determined by Arturo Roig-Alsina, Museo Argentino de Ciencias Naturales ‘Bernardino Rivadavia’ (MACN), Buenos Aires, Argentina,

TABLE 1. *Studied populations of* E. ancistrophora *in Argentina*

Population	Province	Co-ordinates and elevation
Quebrada del Toro, Puente del Toro	Salta	24°52′S, 065°41′W; 1800 m
Quebrada del Toro, El Mollar	Salta	24°51′S, 065°42′W; 1800 m
Campo Quijano	Salta	24°54′S, 065°39′W; 1550 m
Cuesta del Cebilar	Salta	25°40′S, 065°29′W; 1800 m
La Caldera, Campo Alegre	Salta	24°34′S, 065°21′W; 1550 m
Quebrada de Escoipe, Escoipe	Salta	25°11′S, 065°46′W; 2250 m
Quebrada de Humahuaca, Volcán	Jujuy	23°55′S, 065°28′W; 2130 m
Abra Santa Laura	Jujuy	24°29′S, 065°18′W; 1800 m
Calderilla	Salta	24°40′S, 065°22′W; 1400 m
Termas de Reyes	Jujuy	24°10′S, 065°28′W; 1740 m
El Fuerte	Jujuy	24°15′S, 064°25′W; 1450 m

where voucher specimens were deposited as well. Sphingids were determined by the second and third authors, and all vouchers were deposited at the Laboratorio de Biología Floral, Instituto Multidisciplinario de Biología Vegetal (CONICET-UNCba), Córdoba, Argentina.

Floral traits

Flower characters were evaluated in the field. Additional observations on cultivated plants confirmed that floral traits are stable under differing environmental conditions (data not shown). Flower depth was measured with a ruler (to the nearest millimetre) during full anthesis. When flower depth was measured, we also recorded stigma position in these flowers, in order to control for potential correlation of flower length and stigma position, and four different positions were distinguished: (1) exserted, above upper anthers; (2) nested among or below upper anthers; (3) central in flower; and (4) nested among or below lowest anthers. We chose to distinguish four functional categories rather than measuring style length, because precise measuring of the styles would have required cutting the flowers open, and functional (relative) stigma position is more informative than absolute style length, especially in flowers with such grossly different lengths. We calculated the Spearman's rank correlation coefficient to test for correlation between flower length and the four distinguished stigma positions.

Nectar characters were evaluated in all populations for which sufficient numbers of flowers were obtained. Nectar quantity was measured towards the end of anthesis, i.e. in the afternoon for flowers with morning anthesis and in the morning for flowers with evening anthesis, with calibrated micropipettes (5, 10 or 20 µL). Subsequently, sugar concentration (% Brix = sucrose equivalents) was identified to the nearest 0·5 % with a temperature-controlled hand refractometer (Atago Co., Ltd, Tokyo, Japan), when nectar quantity was sufficient (>1 µL). In flowers with smaller volumes, the nectar of several flowers had to be pooled for refractometer measurement. Owing to the narrowness of the flower tubes the flowers were sampled destructively in order to extract nectar, but care was taken to collect only floral nectar and not fluids from wounded floral tissues. We tested for a correlation between nectar quantity and quality (i.e. sugar concentration) using Spearman's rank correlation coefficient.

Limited numbers of flowers were available for only a few days each year, and were needed for the other studies described in this paper and in Schlumpberger and Raguso (2008). Thus, in some cases nectar had to be taken from open, unmanipulated flowers exposed to possible visitors, at the end of their anthesis time. However, observing nectar production in bagged flowers of several populations (all flowers in Puente del Toro, one-third of the flowers in El Mollar, and some flowers in Escoipe and Volcán, respectively) suggested that using open flowers did not strongly bias the results, as 70–75 % of flowers in these populations were not visited (see below). Furthermore, the fact that the longest flowers were unbagged and still yielded by far the highest nectar volumes indicates that visitor frequency was low. In the worst case, the substantial nectar production in those flowers was underestimated. The low nectar volumes measured in the relatively long flowers from Volcán (see Fig. 1 below) were confirmed by both large sample size and the inclusion of bagged flowers. In order to test for reliability of the nectar data obtained in the field, we sampled nectar from some additional flowers of plants cultivated in the Munich Botanical Garden, Germany, descended from (*a*) the El Fuerte population, (*b*) the outskirts of our Campo Quijano population and (*c*) a population near Valle Grande, Jujuy, Argentina, with long flowers with evening anthesis, which was not part of our field study.

Anthesis times were observed in the field; additional data were obtained from cultivated plants by direct observation or by means of a surveillance camera. In the latter case, plants were illuminated with infrared diodes, and photographs were taken automatically by 'EyeCU2' surveillance software (JPC Software, Northport, AL, USA) and directly stored on a computer.

Flower visitor assemblage and behaviour

Nocturnal flower visitors. In order to evaluate the potential for hawkmoth pollination in *E. ancistrophora*, we combined two indirect methods to study hawkmoth visitation.

(1) We analysed more than 300 stigmas of unmanipulated, freshly wilted flowers for the presence of lepidopteran scales (Nilsson and Rabakonandrianina, 1988), surveyed from flowers of all lengths in all populations. Stigmas were examined either with a hand lens (×20) directly in the field, or in the laboratory with a microscope (×64–100) after preserving stigmas individually in 1·5-mL Eppendorf tubes with 70 % ethanol.
(2) In the four populations for which the previous method suggested hawkmoth visitation, we trapped sphingids using a vertical sheet light-trap, removed pollen from their bodies and determined visitors of *E. ancistrophora* flowers by their pollen loads. Light-traps were equipped with two 160-W MB bulbs, powered by a generator. Traps were installed on nights coinciding with *E. ancistrophora* flowering, and/or one or two nights thereafter. Sphingid moths were collected and handled as described by Nattero *et al.* (2003). We determined species and sex, and measured proboscis length and the location of pollen deposits on each moth's body. Reference pollen samples from flowers (also taken from co-blooming sphingophilous plants) were prepared in glycerine gelatine on microscope slides for comparison with pollen loads taken from moths' bodies (Kearns and Inouye, 1993).

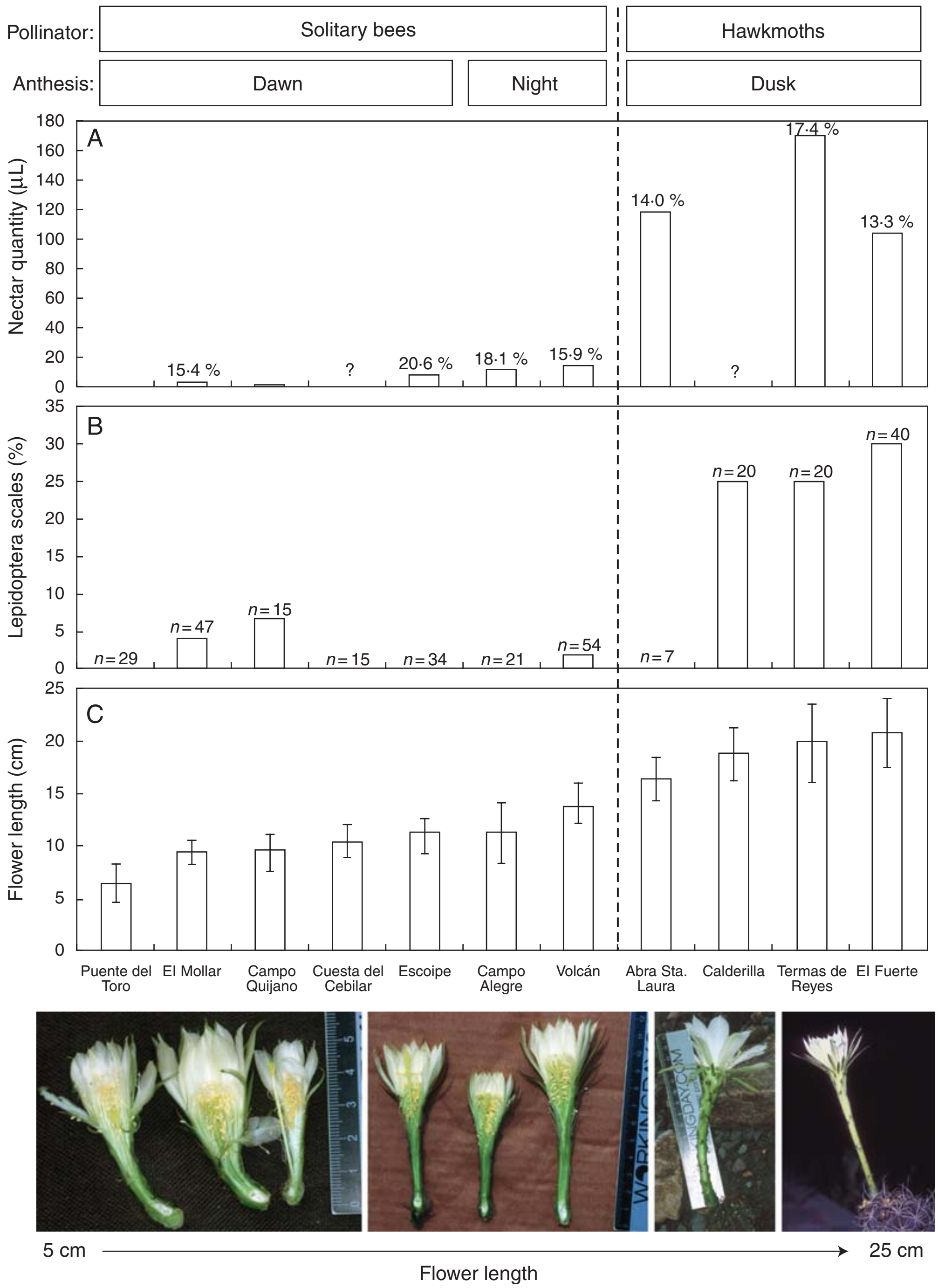

FIG. 1. (A) Mean nectar quantity per population and mean sugar concentration. $n \geq 10$, except Calderilla $n = 4$. (B) Percentage of stigmas with Lepidoptera scales and number of observed flowers per population. (C) Mean flower lengths in 11 *E. ancistrophora* populations. Error bars indicate maximum and minimum lengths. Dotted vertical line separates moth- from bee-pollinated populations.

Diurnal flower visitors. In the seven populations in which no indication of hawkmoth pollination was found, we studied the spectrum of diurnal flower visitors by two methods.

(1) Individual flowers were observed for several time intervals of 15 min each, counting the frequency of flower visits for all visitors (four populations); behavioural aspects such as stigma contact, nectar uptake, pollen collection and search for mating partners were recorded during 15-min observation periods on different individuals, with a total of 8–12 periods per population. Extremely short and synchronized flowering events prevented a more intense visitor evaluation.

(2) Flower visitors were collected and prepared for later determination from the seven populations for which flower size and anthesis time led us to expect pollination by diurnal

visitors, i.e. bees. Collections and observations were made during the time of maximum anthesis and visitor activity, i.e. from flower opening until flowers began to wilt in the afternoon (i.e. 0900–1400 h).

Due to the rarity and brevity of flowering events, the long-tubed populations were not watched extensively for bee visitation on the mornings following anthesis.

Sympatric moth-adapted flowers. In order to understand the role of moth-adapted *E. ancistrophora* flowers in their communities, we evaluated flower lengths of all sympatric night-blooming flowers likely to be pollinated by moths in both the El Fuerte and the Termas de Reyes populations.

RESULTS

Floral traits

Flower length and stigma position. In *E. ancistrophora*, flower length varied little within populations, but dramatically among populations, with the shortest flowers measuring 4·5 cm in the Puente del Toro population (mean 6·40 cm, s.d. $= 0{\cdot}89$, $n = 14$), and the longest flower tubes in El Fuerte measuring up to 24·1 cm (mean 20·80 cm, s.d. $= 2{\cdot}09$, $n = 10$; Fig. 1C). Observation of cultivated plants revealed that open flowers continue to grow after anthesis; haphazardly chosen individuals increased their total floral length between opening and wilting by 3–9 %. Stigma position was highly variable within all populations, with no correlation to flower length (Spearman's rank correlation coefficient $r = -0{\cdot}08$, $P = 0{\cdot}14$, flowers from all populations pooled). The continued elongation of flowers after anthesis also changed the relative position of the stigma over time, giving the appearance that the stigma retreated further into the floral tube. Flower measures obtained from cultivated plants originating from five study populations were found to be within the distribution of field-collected data (not shown).

Anthesis. Strong differences in the timing of anthesis were found among populations. Matinal flower opening occurred in the five populations with the shortest flowers, and vespertine flower opening occurred in the four populations with the longest flowers (Fig. 1, top). Short flowers usually opened between 0900 and 1030 h, and long flowers opened between 1930 and 2100 h, but low temperatures, cloud cover or precipitation could delay opening. In the two populations with intermediate flower lengths, anthesis time was intermediate as well, with first flowers opening at 2300 h in Campo Alegre and at 0130 h in Volcán. In populations with short or long flowers, the process of flower opening was synchronous, i.e. all opening flowers were in similar opening stages at a given time. In contrast, flowers in Volcán opened during an extended period from 0130 h to sunrise, with different opening stages found at all times. Anthesis times of cultivated plants did not differ from times observed in the field (data not shown).

Flowers usually remained open for less than 24 h in long-flowered populations, but longer blooming duration (up to 48 h) was observed in short-flowered populations, depending on environmental conditions: field observations suggest that hot and dry conditions cause shorter flowering, and delayed wilting was observed in bagged flowers. For the short-flowered plants from Puente del Toro, El Mollar, Cuesta del Cebilar, we observed that flowers close in the afternoon or evening and re-open the next morning. On other occasions, flowers in the same populations (e.g. El Mollar) bloomed for only 1 d, depending on the weather. The wilting of flowers (or closing before re-opening on the second day in shorter flowers) began before 1000 h in long flowers (e.g. Termas de Reyes) and no earlier than 1200 h in short to medium flowers (e.g. El Mollar, Volcán).

Nectar. Nectar production was low in flowers from the populations with short and intermediate flower lengths (i.e. those with diurnal or intermediate anthesis times), with highest nectar production in the Volcán population (mean 14·9 μL). No nectar was found in the shortest flowers from the Puente del Toro population, despite the fact that all flowers were bagged (Fig. 1A). Mean nectar production was more than ten-fold higher in the four populations with very long-tubed flowers and nocturnal anthesis than the average of the other populations (Fig. 1A). In the three populations for which sufficient sampling was performed, large nectar crops were present even in unbagged open flowers, with a maximum of 262 μL (mean 170 μL per flower) in Termas de Reyes (Fig. 1A).

Nectar sugar content ranged from 13·4 to 20·6 %, with no obvious relationship between sugar concentration and nectar quantity or flower length (Fig. 1A). There was a marginally significant, negative correlation between sugar concentration and nectar volume (Spearman's $r = -0{\cdot}377$, $P = 0{\cdot}056$, flowers from all populations pooled).

Results from cultivated plants mirror the patterns observed in the field: the diurnal flowers from plants from the Campo Quijano region have relative low nectar production (mean 16·3 μL, $n = 5$); flowers of descendants from El Fuerte produced on average 92·6 μL ($n = 5$), and one nocturnal flower from Valle Grande produced 99·7 μL. In three of the five flowers of cultivated plants from the El Fuerte population, we measured a concentration gradient from the top to the base of the nectar column. Sugar concentrations ranged from 12 % in the uppermost samples to 27·5 % in the lowest samples.

Nocturnal flower visitors: (a) stigma observations. In three of the four populations of *E. ancistrophora* with the longest flower tubes and evening anthesis (Calderilla, Termas de Reyes, El Fuerte), 25–30 % of all stigmas had lepidopteran scales adhering to their surface, indicating moth visitation (Fig. 1B). In the fourth of these populations (Abra Sta. Laura) not enough stigmas could be examined, due to low levels of flowering. Among the seven populations with short to intermediate flower tubes, moth scales were found on stigmas in only three populations. Within these populations, the frequency of individuals with moth scales was low (1·8, 4·3 and 6·7 % of observed flowers; Fig. 1B).

Nocturnal flower visitors: (b) light-trap results. In the three populations of *E. ancistrophora* with the highest frequency of moth scales on floral stigmas (Calderilla, Termas de Reyes, El Fuerte), we captured 476 sphingid moths belonging to 20 species from eight genera (Table 2), of which 19 individuals (three species from two genera) carried pollen of *E. ancistrophora* (Fig. 2). All but one of these moths

TABLE 2. *Spectrum and abundance of sphingid moths collected with light-traps in three of the long-flowered, moth-adapted populations of* E. ancistrophora, *with mean proboscis length and standard deviation*

Location	Hawkmoth species	*n*	Proboscis length (mm)	s.d.
El Fuerte	*Callionima grisescens*	3	16·54	0·9
	Eumorpha analis	5	42·84	1·78
	Erinnyis ello	1	35·01	0
	Erinnyis lassauxii	1	36·21	0
	Eumorpha neuburgeri	5	42·35	2·2
	Hyles euphorbiarum	3	26·78	1·87
	Manduca armatipes	12	45·21	4·39
	Manduca bergi	35	55·9	3·42
	Manduca diffissa	18	61·1	4·51
	Manduca sexta	3	75·09	25·51
	Manduca tucumana	6	77·57	2·51
	Sphinx maura	36	57·37	3·88
	Sphinx phalerata	5	48·45	2·83
	Xylophanes pluto	5	34·04	1·02
	Xylophanes tersa	2	31·42	2·96
	Total	140		
La Calderilla	*Hyles lineata*	1	35·37	0
	Manduca bergi	2	56·01	6·46
	Manduca diffissa	1	52·05	0
	Manduca tucumana	2	77·55	5·36
	Sphinx maura	2	61·47	1·16
	Total	8		
Termas de Reyes	*Callionima grisescens*	1	15·69	0
	Eumorpha analis	5	44·76	5·64
	Euryglottis aper	12	65·83	2·71
	Eumorpha neuburgeri	28	45·24	2·74
	Erinnyis oenotrus	1	36·45	0
	Manduca bergi	3	55·64	4·07
	Manduca diffissa	5	71·63	2·76
	Manduca stuarti	11	78·42	5·44
	Manduca tucumana	125	79·42	5·13
	Sphinx maura	45	60·8	4·46
	Sphinx phalerata	28	48·74	3·11
	Xylophanes pluto	4	33·78	1·27
	Xylophanes schreiteri	58	35·04	2·63
	Xylophanes tersa	2	31·63	0·11
	Total	328		
Total samples		476		

(94·7 %) belonged to the genus *Manduca* (*M. stuarti* $n = 4$, *M. tucumana* $n = 14$), and were caught in the Termas de Reyes population (Table 3). These two species had the greatest proboscis lengths of all captured sphingids, with means of 79·3 mm for *M. tucumana* and 78·6 mm for *M. stuarti*. The mean proboscis length for all captured sphingids was 57·9 mm, and the mean for the subset of all *Manduca* moths was 71·1 mm. Only one specimen of *Sphinx phalerata* with *E. ancistrophora* pollen was caught in the El Fuerte population, with a proboscis 53·3 mm long. In addition to pollen of *E. ancistrophora*, the sphingids carried pollen of various other putatively sphingophilous plants belonging to the families Apocynaceae, Asteraceae, Martyniaceae, Onagraceae, Rubiaceae, Solanaceae and Verbenaceae (Fig. 3).

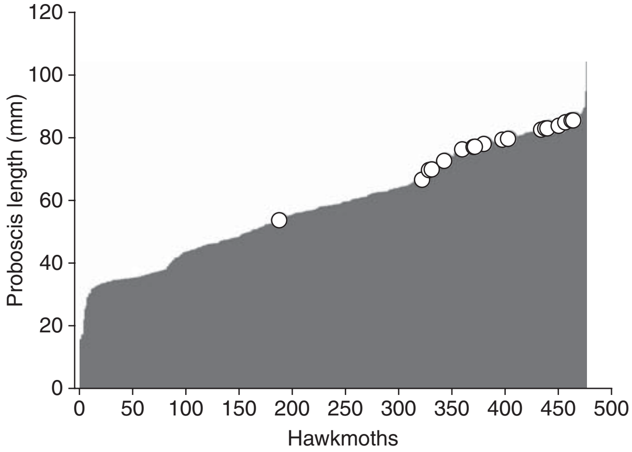

FIG. 2. All individual hawkmoths collected, sorted according to their extended proboscis lengths (mm). Specimens marked by circles are those that carried pollen of *E. ancistrophora*.

Notably, long flowers with nocturnal anthesis attracted dynastine scarab beetles of the genus *Cyclocephala* that fed on and destroyed flowers. These beetles were also attracted by our light-traps.

Diurnal flower visitors. Populations of *E. ancistrophora* with short- or intermediate-length flowers and early morning anthesis were visited primarily by solitary bees. In total, 21 bee species from 14 genera and five families were observed (Table 4). Most abundant were bees of the family Halictidae, with 48 % of all collected bees (e.g. *Dialictus* sp. and *Pseudagapostemon jensenii*), followed by Apidae (28 %, e.g. *Ceratina catamarcensis*, *C. morrensis* and *Diadasia* sp.), Andrenidae (11 %, e.g. *Anthrenoides* spp. and *Callonychium* sp.), Colletidae (8 %, i.e. *Brachyglossula communis*) and Megachilidae (5 %, i.e. *Megachile* sp.). Sixteen per cent of all collected bees were males. Direct observations of visitor behaviour revealed that female bees visited flowers exclusively for pollen collection, whereas male bees visited flowers in search of mates (Table 4). Bees were not observed to probe for floral nectar. Only two of the observed bee taxa, namely *Brachyglossula communis* and *Lithurgus* sp., are known as cactus specialists. The overall frequency of bee visits was 8·1 bees per flower per hour for females only, and 11·5 bees per flower per hour if both genders were considered. Roughly two-thirds (64 %) of all bee visits resulted in stigma contact. If only female bees were considered, the frequency of stigma contacts increased to three in four visits (76 %). Visits by ants, beetles and flies were infrequent, and largely destructive and ineffective, as the observed visits rarely resulted in stigma contact.

DISCUSSION

Among northern Argentine populations of white-flowered *Echinopsis ancistrophora*, also referred to as *E. ancistrophora* ssp. *ancistrophora* (e.g. Hunt, 1999; Schlumpberger and Raguso, 2008), we document extreme variation of floral characters between populations, with only moderate variation within populations. Our first two predictions, driven by pollination

TABLE 3. *All sphingid specimens carrying pollen of* E. ancistrophora *ssp.* ancistrophora*, out of a total of 476 examined individuals*

Population	Species	Proboscis length (cm)	Sex	Date	Time
El Fuerte	*S. phalerata*	53·28	f	24·11·2003	2150
Termas de Reyes	*M. stuarti*	66·23	m	22·10·2003	2235
Termas de Reyes	*M. stuarti*	76·75	m	22·10·2003	2237
Termas de Reyes	*M. stuarti*	85·22	m	22·10·2003	2334
Termas de Reyes	*M. stuarti*	85·34	m	22·10·2003	2334
Termas de Reyes	*M. tucumana*	69·29	m	22·10·2003	0000
Termas de Reyes	*M. tucumana*	69·53	f	9·11·2003	2054
Termas de Reyes	*M. tucumana*	72·29	f	9·11·2003	2204
Termas de Reyes	*M. tucumana*	75·88	m	22·10·2003	0015
Termas de Reyes	*M. tucumana*	76·64	m	9·11·2003	2305
Termas de Reyes	*M. tucumana*	77·59	m	9·11·2003	2235
Termas de Reyes	*M. tucumana*	78·94	m	9·11·2003	2307
Termas de Reyes	*M. tucumana*	79·42	m	22·10·2003	2147
Termas de Reyes	*M. tucumana*	81·52	m	22·10·2003	2248
Termas de Reyes	*M. tucumana*	82·24	f	9·11·2003	2047
Termas de Reyes	*M. tucumana*	82·62	f	25·11·2003	2134
Termas de Reyes	*M. tucumana*	82·76	m	9·11·2003	2242
Termas de Reyes	*M. tucumana*	83·48	m	9·11·2003	2239
Termas de Reyes	*M. tucumana*	84·68	m	9·1·2003	2258

S. = *Sphinx*, *M.* = *Manduca*; time is local time.

syndrome expectations based on floral morphology, were largely supported by our results. First, the four populations with the longest ($\geq$15 cm) flowers showed evening anthesis, presented copious amounts of nectar ($>$100 μL) and were pollinated by sphingid moths. Second, the five populations with short-tubed flowers ($\leq$12 cm) showed morning anthesis, presented meagre ($<$10 μL) nectar volumes and were pollinated by diurnal bees. For populations with intermediate floral morphology, we predicted either a generalized pollination strategy (i.e. moths and bees) or a novel pollinator, but observed neither. The two populations whose flowers ranged from 12 to 15 cm opened between midnight and dawn, presented only slightly more substantial (10–20 μL) nectar volumes, and, despite their length, and also were pollinated by diurnal bees. In some populations, *Cyclocephala* scarab beetles were abundant and destructive nocturnal floral visitors whose presence was not predicted by floral syndromes. Below we discuss these patterns and their implications for floral evolution in *Echinopsis* cacti.

Floral traits

In natural populations of *E. ancistrophora*, flower length was associated with floral traits of visual display, reward presentation and pollinator fit. These floral traits also were associated with the observed flower visitor assemblage in patterns consistent with the expectations of pollination syndromes. Thus, we reject the null hypothesis of random association between floral traits and visitor assemblages, as hawkmoths did not heavily visit or pollinate short-tubed *E. ancistrophora* populations. Previously, naïve *Manduca sexta* were shown to be attracted to *E. ancistrophora* flowers of all lengths in wind tunnel assays (Schlumpberger and Raguso, 2008); here, we found significant amounts of moth scales only in long flowers. In addition, we found low amounts of moth scales on a few short-tubed individuals from El Mollar and Campo Quijano ($n = 1$ flowers each, i.e. 1·7 % of all short-tubed flowers). The combination of evening flower closure (see above), low scale frequency on stigmas (Fig. 1B) and poor mechanical fit for moths with long proboscides (Fig. 2, Table 2) suggests that hawkmoths are unsuitable or at best uncommon and inefficient pollinators of short-tubed *E. ancistrophora*. Instead, the presence of scales may indicate that diurnal butterflies are occasional visitors to these flowers, which are closed during the active periods for most hawkmoths in this region (Moré, 2008).

One interesting finding was the apparent misinterpretation of functional floral depth among some of the *Echinopsis* populations studied. We were surprised to observe diurnal bee pollination in all examined populations with mean flower lengths $\leq$15 cm, despite their white corollas and relatively long, narrow floral tubes. Most non-cactus hawkmoth-pollinated plants growing in sympatry with *E. ancistrophora* populations had significantly shorter floral tubes or spurs (Fig. 3), several of them even shorter than the shortest bee-adapted *E. ancistrophora* flowers known (Fig. 1C). However, additional *E. ancistrophora* plants studied in cultivation, belonging to several other populations, confirm that the taxon is split into two groups: flowers shorter than 15 cm had predominantly diurnal anthesis and low nectar production (bee-pollinated group) and flowers longer than 15 cm had predominantly nocturnal anthesis and high nectar production (moth-pollinated group; data not shown). Similar patterns have emerged in an ongoing study on floral evolution in three other lineages of the genus *Echinopsis* (B. O. Schlumpberger, unpubl. res.). The paucity of nectar in most *E. ancistrophora* populations with flowers shorter than 15 cm (Fig. 1A), combined with the observed foraging behaviour of bee visitors, indicates that these flowers primarily offer pollen as a reward. Their functional depth probably does not include the floral tube, which may simply serve as a pedestal raising the corolla above the ground (see Cocucci, 1991), or could indicate a phylogenetic constraint reflecting the tubular floral architecture of this lineage's ancestor.

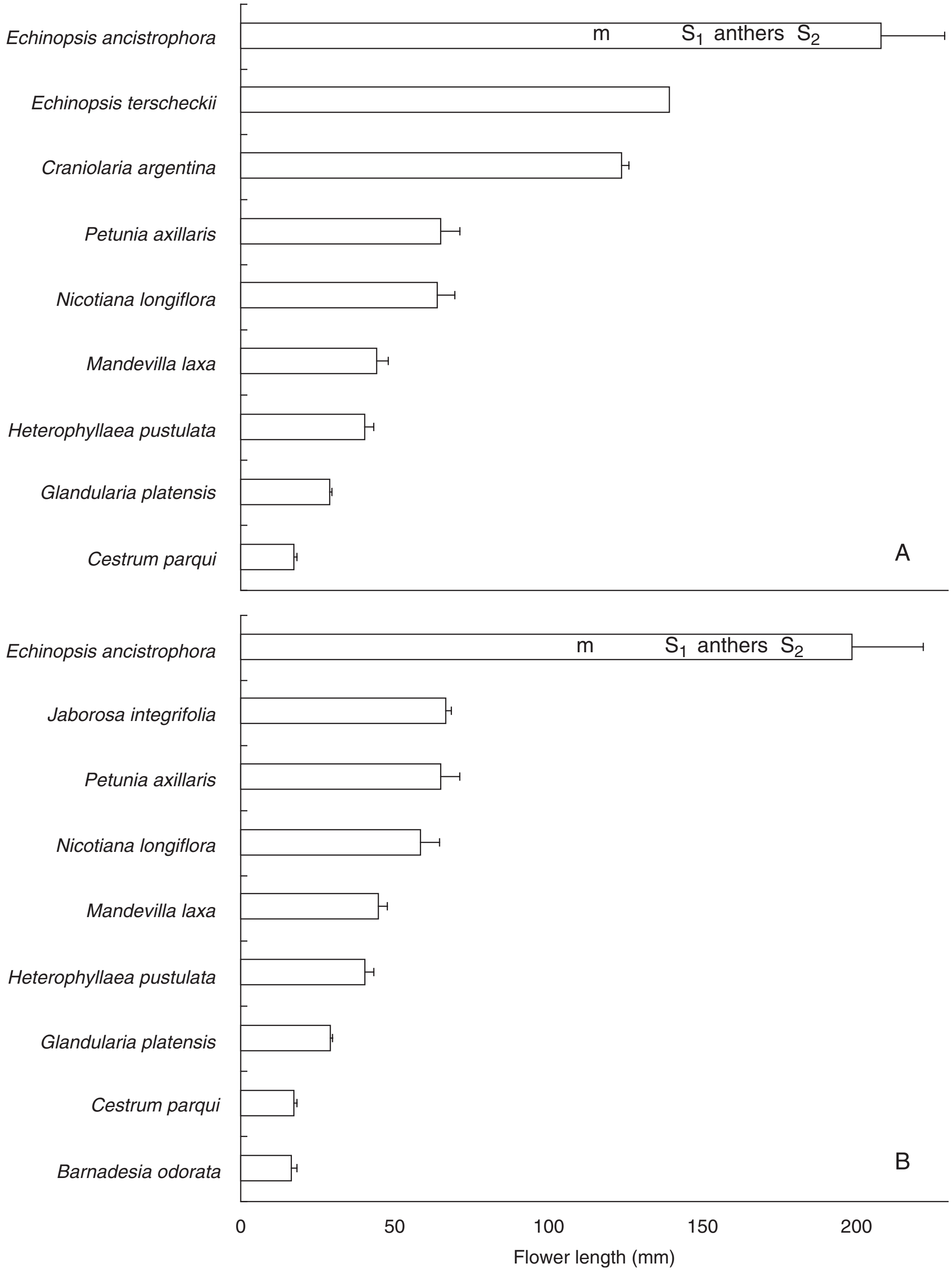

FIG. 3. Mean flower lengths (+ s.d.) of sympatric moth-pollinated species in two of the *E. ancistrophora* populations with moth-adapted flowers: (A) El Fuerte, and (B) Termas de Reyes. m = estimated maximum entering position of sphingids (i.e. their heads); s_1 = minimum stigma position; s_2 = maximum stigma position; 'anthers' = zone of densely packed anthers.

Another surprising finding was the variation in stigma position independent of pollination strategy, which we observed even within populations. For hawkmoth-pollinated flowers, both approach herkogamy (i.e. exserted stigma) and reverse herkogamy have been described (e.g. Raguso *et al.*, 2003). It is unclear whether approach herkogamy vs. shorter stigmas should be more effective at receiving outcrossed pollen in trumpet-shaped, hawkmoth-pollinated flowers (see Kulbaba and Worley, 2008). The flower morphology of *E. ancistrophora* and proboscis lengths of the visiting hawkmoths suggest that the latter need to land and crawl into the flowers in order to deplete the nectar. Within-population

TABLE 4. *Spectrum of bees visiting* E. ancistrophora *in six populations with short to medium flowers*

	Puente del Toro	El Mollar	Cuesta del Cebilar	Escoipe	Campo Alegre	Volcán
Anthrenoides sp.1 (Andrenidae)				f		
Anthrenoides sp.2 (Andrenidae)					f	
Augochloropsis sp. (Halictidae)	x	x			x	x
Bombus bellicosus (Apidae)	f		f			
Brachyglossula communis (Colletidae)			x	f/m		
Caenohalictus sp. (Halictidae)		x	x	f		
Callonychium sp. (Andrenidae)				m		
Ceratina catamarcensis (Apidae)	f					f
Ceratina morrensis (Apidae)	x				f	
Ceratina sp. (Apidae)		x				
Diadasia sp. (Apidae)						f/m
Dialictus sp. (Halictidae)	x	x	x	x	x	
unknown halictid 1 (Halictidae)				x		
unknown halictid 2 (Halictidae)		x				
Lithurgus sp. (Megachilidae)		x	f			
Megachile sp. (Megachilidae)		x		x		x
Melissoptila cf. *carinata* (Apidae)					f/m	
Psaenythia sp. (Andrenidae)		m				
Pseudagapostemon (Neagap.) *jensenii (*Halictidae)			x	x		
Pseudagapostemon (Pseudagap.) sp. (Halictidae)	x					
Svastrides zebra (Apidae)			f			

f = females and m = males of collected bees; x = observed but not collected bees.

variation in stigma position probably results from disruptive selection from different pollinators (bees vs. hawkmoths), as was suggested by Baker (1964) and Grant and Grant (1983). In our study plants, flower age does play a role as well, but does not explain the extensive observed variation.

We had expected a positive, linear correlation between floral depth and nectar volume among populations, given that all *E. ancistrophora* flowers are trumpet shaped and could offer nectar to different sized pollinators. Instead, the distribution of nectar standing crop volume among populations was a step function, with low (approx. 15 μL) to non-existent standing crops in populations with flowers <15 cm long, and copious mean volumes (≤170 μL) in the longest tubed flowers (Fig. 1). The observed differences in nectar quantity are not the result of enlarged nectaries in longer flowers, as nectar volume increased abruptly, while flower length increased linearly and intermediate nectar volumes were not observed (see Fig. 1). Therefore, these differences appear to represent adaptations to pollen-collecting bees vs. nectar-drinking moths in the absence of bimodally pollinated intermediate-length flowers. In nectar of long *E. ancistrophora* flowers, we occasionally measured an increasing sugar concentration from the top to the bottom of the floral tube. Such nectar gradients were described recently by Martins and Johnson (2007) from the floral spurs of sphingophilous orchids in Kenya. These authors suggested that nectar gradients might compel hawkmoths to probe more deeply into the flowers, enhancing physical contact with reproductive organs. Additional studies are needed to determine whether nectar concentration gradients in *Echinopsis* flowers result from active or passive secretion patterns within the nectar tube.

Interestingly, previous studies revealed that floral scent in this species complex does not covary with morphological characters in patterns predicted by floral syndromes. For reasons discussed elsewhere (Schlumpberger and Raguso, 2008), populations with hawkmoth-pollinated flowers (e.g. Termas de Reyes) did not have the strongest or most attractive floral scent, although the odours of long- and short-tubed flowers alike were sufficient to attract naïve *Manduca sexta* hawkmoths in wind tunnel bioassays. Further field experiments with bees and detrimental floral visitors (see below) will be needed to explore alternative explanations for the negative correlation between odour emission rates and floral tube length in this species complex.

Pulsed mass flowering

Flowering in natural populations of *E. ancistrophora* occurred in a few short, synchronized waves during the year, but our observations were limited by the need to monitor 11 remote populations during several field trips. Additional observations made on cultivated *E. ancistrophora* plants grown under controlled conditions confirm pulsed flowering in only one to three events per year, each lasting 1–4 d (data not shown). The flowers usually last less than 24 h, possibly due to their size (e.g. high water investment), combined with water loss through evapotranspiration. We have observed similar flowering phenology in other *Echinopsis* taxa, as well as in more distantly related species in the genus *Peniocereus* (Raguso *et al.*, 2003). From the standpoint of plant–pollinator food webs, *Echinopsis* flowers provide brief meals for their bee and hawkmoth pollinators, whose foraging demands are satisfied by other, more consistently available floral resources during the days to weeks of their adult lives (see Alarcón *et al.*, 2008).

Pollinator assemblages and evolutionary shifts

In *E. ancistrophora* we found that pollination by hawkmoths occurred only in populations whose floral morphology fits the sphingophilous pollination syndrome, with abundant nectar, maximum depth and anthesis at dusk. In contrast, bees pollinate not only populations with short flowers and purely diurnal anthesis, but also those with flowers of intermediate length and intermediate opening time, which appeared (superficially) to be adapted to sphingids and may represent transitional states.

Our evaluation of the spectrum of hawkmoth-adapted flowers growing in sympatry with two long-tubed populations of *E. ancistrophora* showed that only *Craniolaria argentina* has flowers of similar functional length (Fig. 3). At first glance, long-tubed *E. ancistrophora* flowers might be considered the most specialized moth flowers in these communities, with nectar being entirely accessible only to moths with the longest proboscides. Even moths with proboscis lengths up to 100 mm (*Manduca* spp., Fig. 2) must enter the upper, funnel-shaped part of the flowers in order to access the nectar resources fully (see Haber and Frankie, 1989). However, our evidence for moth pollination in the four longest-tubed populations of *E. ancistrophora* stems from the presence of moth scales on the stigmas of wilted flowers (Fig. 1) and the presence of pollen on light-trapped moths in long-tubed populations (Tables 2 and 3). Limited observations in the Termas de Reyes population revealed halictid bees gathering pollen in the wilting flowers. It is likely that pollen-seeking bees provide additional pollination to long-flowered, moth-pollinated *E. ancistrophora* by gathering pollen in the morning, as was recently found in three other moth-adapted *Echinopsis* species (B. O. Schlumpberger, unpubl. res.). Thus, the most conservative conclusion at present is that the four longest-tubed populations of *E. ancistrophora* have bimodal pollination strategies, with nectar-seeking moths and pollen-collecting bees as nocturnal and diurnal pollinators, respectively. Such a reproductive assurance strategy would be especially beneficial in the genus *Echinopsis*, most species of which are obligate outcrossers (Berger, 1929). Per-visit effectiveness experiments combined with measurements of gene flow are needed to assess how plants serviced by hawkmoths as well as bees might differ in fruit set and outcrossing rates.

Bimodal pollination involving bees has been described in other apparently hawkmoth-adapted species (Barthell and Knops, 1997; Miyake and Yahara, 1998). Examples for mixed pollination in cacti with predominantly nocturnal anthesis by both diurnal and nocturnal visitors were found in *Echinopsis atacamensis* ssp. *pasacana* (Schlumpberger and Badano, 2005) and a few other columnar cacti (Sahley, 1996; Dar *et al.*, 2006; Rivera-Marchant & Ackerman, 2006). Such adaptation to sets of different pollinators is thought to have especially evolved near the northern and southern distributional limits of columnar cacti, as a response to low reliability of specialized pollinators, i.e. nectarivorous bats (Fleming *et al.*, 2001). Aigner (2001) has argued that the attraction of additional pollinator taxa should be favoured if the required phenotypic trade-offs do not reduce the plant's net reproductive fitness. If long-tubed flowers evolved from a short-tubed, bee-pollinated ancestry in *E. ancistrophora*, changes associated with effective hawkmoth pollination (e.g. extended nectar tube and supplemented nectar volumes) would be unlikely to affect pollen foraging bees. In this case, we would have increased apparent floral specialization in respect to nectar accessibility, coupled with a decrease in the specificity of the potential pollinator spectrum. Similarly, Armbruster (2006) distinguished between evolutionary and ecological specialization. Although the floral phenotype of plants may evolve towards specialization to a subset of available pollinators or pollinator groups, their net ecological specialization may actually decrease (Aigner, 2001). If, instead, the direction of evolution in this lineage is from a long-tubed, nectar-rich ancestry to shorter-tubed, nectarless flowers, then some populations will have specialized in bee pollination through the loss of traits that attract sphingids and translate their visits into effective pollen transfer. Phylogenetic analysis (in progress) will be used to determine which of these scenarios most accurately depicts the order of evolution in *E. ancistrophora*.

The assemblages of flower-visiting bees differed among populations with short-tubed flowers, which may simply reflect limited sampling time spread across many ephemerally blooming populations. However, with few, short, synchronized flowering events these flowers are best considered as transients within the established flower–pollinator networks in their communities (e.g. Medan *et al.*, 2006), and flower visitor assemblages may simply vary among flowering events within and among years. Interestingly, specialized cactus bees, which are abundant in these regions and visit *Echinopsis* and other cactus genera (Roig-Alsina and Schlumpberger, 2008) were only occasionally found in some of the studied populations (Table 4). We assume that the extremely short, pulsed flowering of *E. ancistrophora* together with relatively low cactus diversity at some of the sites cannot support viable populations of these specialists. Despite these observations, generalized bee pollination appears to be an effective strategy for most populations of *E. ancistrophora*, given the relatively high (mean 76 %) observed stigma contact frequency for female bees, and that specialized bees accounted for a low percentage of all observed visits. Pollination exclusively by generalized bees was observed in cacti from Córdoba, Argentina (Schlumpberger, 2002). In contrast, studies of cacti in southern Brazil revealed that specialized cactus bees (e.g. *Arhysosage*, Andrenidae) touched the stigmas in more than 90 % of their visits, whereas generalized bees (e.g. *Dialictus*, Halictidae) only contacted the stigmas in 6–29 % of their visits (Schlindwein and Wittmann, 1995). For North American bee-pollinated cacti, most studies documented specialized cactus bees as well, usually outperforming co-visiting polylectic bees (Simpson and Neff, 1987; Johnson, 1992; McIntosh, 2005).

Populations with intermediate flowers

Studies on the pollination of intermediate flowers are rare and offer unique opportunities to study floral syndrome evolution. One possible interpretation for the occurrence of intermediate populations in our study is that they are of hybrid origin, and offer too little nectar and open too late at night (see Gregory, 1964; Raguso *et al.*, 2003) to enlist hawkmoths as pollinators. Another possibility is that they represent a transition from a long-tubed ancestry, in which nectar reduction mitigates physiological or ecological costs in the absence of selection for hawkmoth pollination. In Volcán, flower opening times were spread over more than 5 h, indicating a less precise timing than in populations with shorter or longer flowers respectively. Anthesis time varied more strongly than did flower length and nectar quantity at Volcán, which may indicate that it is a more plastic trait. In the case of *E. ancistrophora*, the two intermediate populations were not intermediate in nectar volume, which resembled the low values of short-tubed, bee-adapted populations. It is unclear whether poor nectar rewards or the shifted anthesis time later at night was most responsible for the lack of observed moth scales. These flowers were long enough (10–15 cm) to have ensured scale deposition on stigmas if they had been visited by the moths observed in our study (see Fig. 2, Table 3).

Alternatives to pollinator-centrism: the impact of flower predation by beetles

Nectar production in the quantities measured for the four hawkmoth-pollinated populations has obvious physiological costs in a desert environment (Carroll *et al.*, 2001 and references herein). Our observations suggest that the ecological costs of making flowers apparent to florivores may be just as high, with nocturnal *Cyclocephala* beetles visiting long-tubed flowers of *E. ancistrophora*. Flowers adapted to these beetles as pollinators often are structurally reinforced so as to withstand their destructive behaviour (Young, 1988). However, the nocturnal flowers of *E. ancistrophora* have no such defences, and were damaged by the beetles (B. O. Schlumpberger, pers. observ.). Our previous study revealed an inverse relationship between floral length and scent emission rates among *E. ancistrophora* populations, against our expectations that hawkmoth pollination should require the strongest floral scents (Schlumpberger and Raguso, 2008). Perhaps *Cyclocephala* beetles have selected for reduced scent in populations with hawkmoth pollination. Manipulative field experiments will be needed to measure the magnitude and consequences of beetle responses to augmented floral scent.

Conclusions

Our results suggest incipient specialization in *E. ancistrophora*, with population-level differences in apparent flower–pollinator adaptation, either to bees or hawkmoths (potentially plus bees). However, the presence of destructive beetles in populations with nocturnal anthesis underlines the potential for enemies to influence the evolution of floral phenotype. The observed intraspecific variation of floral traits and flower visitors among isolated populations, including intermediate, potentially transitional stages, makes this plant group an ideal model for studying plant–pollinator interactions and their evolutionary transitions.

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