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CELLULAR AND MOLECULAR BIOLOGY

Nectary structure is not related to pollination system in Trichocereeae cactus from Northwest Argentina

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Abstract: Floral nectaries are essential for plant reproduction but little is known about the relationship between these secretory structures and pollination system in cacti. To test phenotypic patterns in nectaries associated with pollination syndromes and/or with its pollinators, we selected from evolutionarily related genera *Cleistocactus*, *Denmoza*, and *Echinopsis*, a set of species with bird-pollinated flowers and floral traits that may fit with ornithophily or with sphingophily, and other set of sphingophilous species with moths as effective pollinator. Observations were made under light microscope and scanning and transmission electron microscopes. Nectaries are located at the base of the filaments welded to the tube, forming a chamber. The nectary consists of the epidermis with distinctive features in each genus, a secretory parenchyma which may be vascularized and a non-secretory vascularized parenchyma. Anatomical variants observed in nectaries of different species are not consistent with the floral pollination syndromes neither with groups of pollinators. The basic structure of the nectar chamber is relatively conserved, a fact that may be explained by phylogenetic conservatism among the genera investigated. Our results revalue the role of anatomical traits for the systematics of Cactaceae.

Key words: Bird-pollinated, Cactaceae, floral nectaries, pollination system, Trichocereeae.

INTRODUCTION

The great diversity of floral phenotypes present in angiosperms has been traditionally linked to the specialization onto different groups of pollinators based on the concept of pollination syndromes (Faegri & Van der Pijl 1979, Fenster et al. 2004). The variation of floral traits that defines floral phenotypes has been used to link cacti with certain groups of specialized pollinators, such as moths, non-specialized bees, birds, and bats (Grant & Grant 1979, Gibson & Nobel 1986, Pimienta-Barrios & del Castillo 2002). However, current evidence indicates that even though cacti may exhibit phenotypic specialization, most pollination systems are generalist (Fleming et al. 2001, De Viana et al. 2001, Schlumpberger & Badano 2005, Ortega-Baes et al. 2011, Alonso-Pedano & Ortega-Baes 2012, Eggli & Giorgetta 2015, Gorostiague & Ortega-Baes 2016, 2017), phenomenon that could be interpreted as only variations and not strictly functional adaptations. Their flowers can be visited by a broad spectrum of animals, including unexpected types given their floral traits (Waser et al. 1996). A significant proportion of species of the family exhibits phenotypic ornithophilous specialization, hence, the existence of specialized lineages has even been proposed by Anderson (2001) and Hunt (2006). However, recent research indicates that birds

can pollinate both cacti with specialized flowers and those adapted to pollination by other animal guilds (Fleming et al. 2001, Gorostiague & Ortega-Baes 2017).

The tribe Trichocereeae has diversified in the Southern Hemisphere, forming the second largest tribe of the subfamily Cactoideae (Gibson & Nobel 1986, Anderson 2001). Anderson (2001) proposed the classification followed widely today, which currently comprises 26 genera and about 400 species (Hunt 2006). However, phylogenetic relationships within the tribe are still controversial (Buxbaum 1953, Barthlott & Hunt 1993, Anderson 2001, Hunt 2006) and recent molecular data have indicated that important taxonomic rearrangements are needed (Nyffeler 2002, Arakaki et al. 2003, Arakaki 2008).

In northwestern Argentina, the tribe is represented by the genera *Cleistocactus*, *Denmoza*, *Echinopsis*, *Gymnocalycium*, *Harrisia*, *Oreocereus* and *Rebutia* (Hunt 2006). *Echinopsis* is one of the most diverse genera in terms of life forms and floral forms, including all the types of pollination described for the family. According to the phylogeny of Schlumpberger & Renner (2012), *Echinopsis s.l.* is a monophyletic group that includes closely related genera, such as *Cleistocactus* and *Denmoza* (Anderson 2001, Hunt 2006). The presence of bird-pollinated species stands out within this diverse lineage, including both flowers specialized to this guild of animals (Gorostiague & Ortega-Baes 2016) and flowers with morphological specialization aligned to moths (de Viana et al. 2001, Schlumpberger & Badano 2005, Gorostiague & Ortega-Baes 2017).

Floral nectaries are important structures involved in plant reproduction that produce and offer nectar, a sugar-rich solution implicated in plant-animal interactions (Pacini et al. 2003). These secretory structures vary in morphology, anatomy, topology and nectar secretion mode (Fahn 1952, 1954, 1979, 1988, Durkee 1983, Smets et al. 2000). It has been suggested that the variation recorded in floral nectaries is closely associated with their function (Fahn 1979, Giuliani et al. 2012) and is also related to the type of pollinator (Baker & Baker 1983, 1990, Pacini et al. 2003). For Cactaceae, three basic types of "nectariferous zones" were traditionally recognized, namely (1) furrow, (2) disc, and (3) chamber (Buxbaum 1953). In all cases studied to date, the secretion occurs along the basal portion of the hypanthium below the insertion of the innermost filaments surrounding the style, all of which fall within the hypanthial nectary type proposed by Bernardello (2007). Although cacti strongly depend on animals to disperse their pollen, and the ecological value of nectar as energy resource for pollinators is acknowledged, their nectaries have been scarcely addressed in the family (Stefano et al. 2001, Fuentes-Pérez et al. 2009, Almeida et al. 2012, 2013, Gutiérrez-Flores et al. 2017, Agüero et al. 2018, Camacho-Velázquez et al. 2019).

Seven species included in this study share pollination by birds (de Viana et al. 2001, Schlumpberger & Badano 2005, Ortega-Baes et al. 2011, Gorostiague & Ortega-Baes 2016, ichocere 2017, Gorostiague 2017). However, according to flower color, morphology and floral cycle, phenotypic specialization to birds can be recognized in *Cleistocactus* and *Denmoza* species, whereas *Echinopsis* species studied exhibit phenotypic specialization to moths (Anderson 2001, Hunt 2006).

Given the background, this study aimed to explore the relationships between floral nectary structure and floral phenotypic specialization in bird-pollinated flowers of Trichocereeae species from northwest Argentina. We studied the floral nectary morpho-anatomy in bird-pollinated species showing different floral phenotypes: specialization to bird pollination [*Cleistocactus baumannii* (Lem.) Lem., *C. hyalacanthus*

(K. Schum.) Grosellin, *C. smaragdiflorus* (F.A.C. Weber) Britton & Rose, and *Denmoza rhodacantha* (Salm-Dyck) Britton & Rose and supposed specialization to moths pollination [*Echinopsis atacamensis* (Phil.) Friedrich & G.D. Rowley, *E. leucantha* (Gillies ex Salm-Dyck) Walp. and *E. terscheckii* (Parm. ex Pfeiff.) Friedrich & G.D. Rowley]. In addition, we included three species with phenotypic specialization to pollination by moths, in which bird pollination was not recorded [*E. albispinosa* K. Schum., *E. ancistrophora* Speg., *E. schickendantzii* F.A.C. Weber] in order to enhance the understanding of plant-pollinator relationships in this tribe. This investigation was also conducted to fill gaps regarding to the knowledge of this secretory structure in this particular group of plants and intended to provide information that helps understand the evolution of floral nectary in relation to floral phenotypic specialization. Within this scope we expected to find differences in the morpho-anatomical characteristics of the nectaries among the studied species, associated with the different pollinating guilds.

MATERIALS AND METHODS

Examined material

Pre-anthesis buds and open flowers were collected from plants growing up in the wild and fixed in formalin–acetic acid–alcohol 70° (FAA) for anatomical and scanning electron microscopy (SEM) examination. The voucher specimens were deposited in the herbarium of the Institute of Botany of the Northeast (CTES), Argentina.

A list of species studied, with voucher specimens and information about floral cycle is presented in Table I.

Light microscopy

Permanent microscope slides of the floral nectary were prepared by processing the fixed material by dehydration through an ethanol series with a rinsing pre-impregnant of tertiary butyl alcohol (Gonzalez & Cristóbal 1997). Infiltration in paraffin Histoplast® (Biopack, Buenos Aires, Argentina) was performed according to Johansen (1940). The floral tubes were sectioned transversely and longitudinally (10–12 µm thickness) with a rotary microtome. The sections were stained with astra blue safranin (Luque et al. 1996), and then mounted with synthetic Canada balsam (Biopur, Buenos Aires, Argentina). Morphological and anatomical analyses were performed under a Leica MZ6 stereomicroscope and a Leica DM LB2 compound microscope (Leica, Wetzlar, Germany), respectively, both equipped with a digital camera.

Scanning electron microscopy (SEM)

The fixed material was dehydrated through a series of increasing ethanol concentrations. The material was then critical point-dried with solvent-substituted liquid carbon dioxide and coated with a thin layer of gold palladium. SEM micrographs were obtained with a JEOL 5800 LV scanning electron microscope (JEOL USA, Peabody, MA, USA) operating at 20 kV.

Transmission electron microscopy (TEM)

Floral nectary ultrastructure of *Echinopsis terscheckii* was studied in order to elucidate the probability of mixed secretion in the genus (through stomata and through epidermal trichomes). Floral nectary at pre-anthesis and anthesis were pre-fixed in 1% glutaraldehyde, 4% formaldehyde in phosphate buffer (pH 7.2) for 48 h and then post-fixed in OsO_4 at 2° C in the same buffer for 3 h. Then, they were dehydrated in ascending ethanol series and embedded in Spurr's resin (O'Brien & McCully 1981). Sections

Table I. Voucher information, comparison of floral characters and reproductive biology of the studied species of Trichocereeae.

Species	Voucher information	Floral nectary				Floral length (mm)	Floral diameter (mm)	Floral cycle	Floral phenotype	Pollinators/ visitors	Reference
		Type	Vascularization	Epidermis	Secretion mode						
Cleistocactus baumanii	Argentina. Salta, González, V.V. et al. 5 (CTES)	Semi-closed chamber	Xylem and phloem delimit the nectariferous parenchyma	Smooth	Stomata Holocrine	50.20 ± 9.53	7.19 ± 1.68	Diurnal, more than 48 h open	Ornithophilous	Hummingbirds	Gorostiague & Ortega Baes 2016
C. hyalacanthus	Argentina. Salta, González, V.V. et al. s.n. (CTES)	Semi-closed chamber	Xylem and phloem delimit the nectariferous parenchyma	Smooth	Stomata	c. 35-40	-	Diurnal	Ornithophilous	No available information	Hunt 2006
C. smaragdiflorus	Argentina. Salta, González, V.V. et al. 4 (CTES)	Semi-closed chamber	Xylem and phloem delimit the nectariferous parenchyma	Smooth	Stomata	44.71 ± 4.09	6.97 ± 1.89	Diurnal, more than 48 h open	Ornithophilous	Bees Hummingbirds	Gorostiague & Ortega Baes 2016
Denmoza rhodancantha	Argentina. Salta, González, V.V. et al. 17 (CTES)	Closed chamber	Xylem and phloem penetrate the nectariferous parenchyma	Papillose	Stomata	49.1 ± 7.9	9.9 ± 2.4	Diurnal, more than 48 h open	Ornithophilous	Bees Hummingbirds (*Oreotrochilus leucopleurus*)	Eggli & Giorgetta 2015, Gorostiague 2017
Echinopsis albispinosa	Argentina. Salta, González, V.V. et al. 9 (CTES)	Semi-closed chamber	Xylem and phloem delimit the nectariferous parenchyma	Unicellular trichomes	Stomata	128.6 ± 18.6	78.7 ± 13.6	nocturnal, extended to the following morning	Sphingophilous	Bees Moths	Gorostiague 2017
E. ancistrophora	Argentina. Salta, González, V.V. et al. 20 (CTES)	Semi-closed chamber	Xylem and phloem delimit the nectariferous parenchyma	Unicellular trichomes	Stomata	111.7 ± 17.4	80.0 ± 14.1	nocturnal, extended to the following morning	Sphingophilous	Bees Moths	Schlumpberger et al. 2009
E. atacamensis	Argentina. Salta, González, V.V. et al. 14 (CTES)	Semi-closed chamber	Xylem and phloem delimit the nectariferous parenchyma	Unicellular trichomes	Stomata	124 ± 2	96 ± 3	nocturnal, extended to the following morning	Sphingophilous	Bees Giant hummingbird (*Patagona gigas*) Moths Wasps	De Viana et al. 2001, Schlumpberger & Badano 2005
E. leucantha	Argentina. Salta, González, V.V. et al. s.n. (CTES)	Semi-closed chamber	Xylem and phloem delimit the nectariferous parenchyma	Unicellular trichomes	Trichomes	198.17 ± 13.20	113.62 ± 21.74	nocturnal, extended to the following morning	Sphingophilous	Bees Passerine birds (*Phrygilus gayi*)	Gorostiague & Ortega-Baes 2017
E. schickendantzii	Argentina. Salta, González, V.V. et al. 18 (CTES)	Semi-closed chamber	Xylem and phloem delimit the nectariferous parenchyma	Unicellular trichomes	Stomata	118.8 ± 2.3	105 - 140	nocturnal, extended to the following morning	Sphingophilous	Bees Moths	Alonso- Pedano & Ortega-Baes 2012
E. terscheckii	Argentina. Salta, González, V.V. et al. 10 (CTES)	Semi-closed chamber	Xylem and phloem delimit the nectariferous parenchyma	Unicellular trichomes	Stomata Trichomes	177.7 ± 16.0	163.8 ± 21.7	nocturnal, extended to the following morning	Sphingophilous	Bees Moths Beetles Hummingbirds Passerine birds	Ortega-Baes et al. 2011

1 µm thick were made on a Reichert-Jung ultramicrotome and stained with toluidine blue. Ultrathin sections (750-900 nm) were stained with uranyl acetate and lead citrate (Zarlavsky 2014). The sections were examined under a JEOL-JEM 1200 Ex II transmission electron microscopy (TEM) at 85 kV.

RESULTS

Floral morphology

The ten studied species have perfect, epigynous, solitary and sessile flowers, with a developed hypanthium, covered with bracts that have axillary hairs. The spiral perianth is composed of numerous lanceolate tepals of the same or different color as the tube, which are fused at the base forming the floral tube (Figure 1a-j). *Cleistocactus* and *Denmoza* flowers show phenotypic specialization to birds. These are tubular, with perianth limb slightly spread at the apex, actinomorphic or zygomorphic due to tube curvature. *Cleistocactus* flowers are usually borne laterally along the upper portions of the stems (Figure 1a-c), whereas in *Denmoza* the flowers appear at the top of the stem (Figure 1d). *Cleistocactus baumannii* has sigmoid flowers, with red pericarpel, floral tube and tepals (Figure 1a). *Cleistocactus hyalacanthus* exhibits slightly arched light red to orange flowers (Figure 1b). *Cleistocactus smaragdiflorus* has flowers with straight pink tube and emerald green tepals (Figure 1c). *Denmoza rhodacantha* red-magenta flowers vary in shape from straight to slightly or markedly sigmoid, depending on their location on the stem (Figure 1d). *Echinopsis albispinosa, E. ancistrophora, E. atacamensis, E. leucantha, E. schickendantzii and E. terschekii* flowers exhibit sphingophilous specialization; they are funnel-shaped with green floral tubes and white tepals (Figure 1e-j).

Nectar chamber morphology

In the analyzed species, the nectar chamber is located above the ovary, on the inner wall of the basal portion of the hypanthium associated with the base of the filaments, forming a hollow cylinder that surrounds the style (Figures 2a, e, h, 3a and 4f). In *C. baumannii*, an open vault-roof structure of non-secretory parenchyma is observed at the upper end of the chamber (Figure 2a). In *C. hyalacanthus* and *C. smaragdiflorus*, the primary staminal filaments remain fused at their bases when they begin to release from the tube (Figure 2e, h). In all cases, a semi-closed nectar chamber is formed. *Denmoza rhodacantha* presents a chamber with highly developed walls, consisting of multiple layers of nectariferous tissue (Figure 3a, b, g-i). In cross section, the chamber wall shows an undulating contour, following a pattern of peaks and valleys (Figure 3i). At the top of the chamber a dense set of curled trichomes form a plug that closes the chamber (Figure 3a-d), in some cases the bases of the trichomes are multicellular filiform prolongations originating in the upper portion of the nectariferous tissue, with papillose epidermis, similar to the nectariferous epidermis (Figure 3c-d). *Echinopsis albispinosa, E. ancistrophora*, *E. atacamensis, E. leucantha, E. schickendantzii* and *E. terscheckii* have semi-closed nectariferous chambers with a dense barrier of hundreds of staminal filaments surrounding the style and protecting the nectar chamber (Figure 4g). On the upper portion of the chamber wall there are vertical ribs parallel to the style axis, which are the base of the filaments fused to the tube (Figure 4f).

Nectar chamber anatomy

The nectaries of the analyzed species share structural similarities: the wall of the chamber is composed of a one-layered epidermis and underlying homogeneous

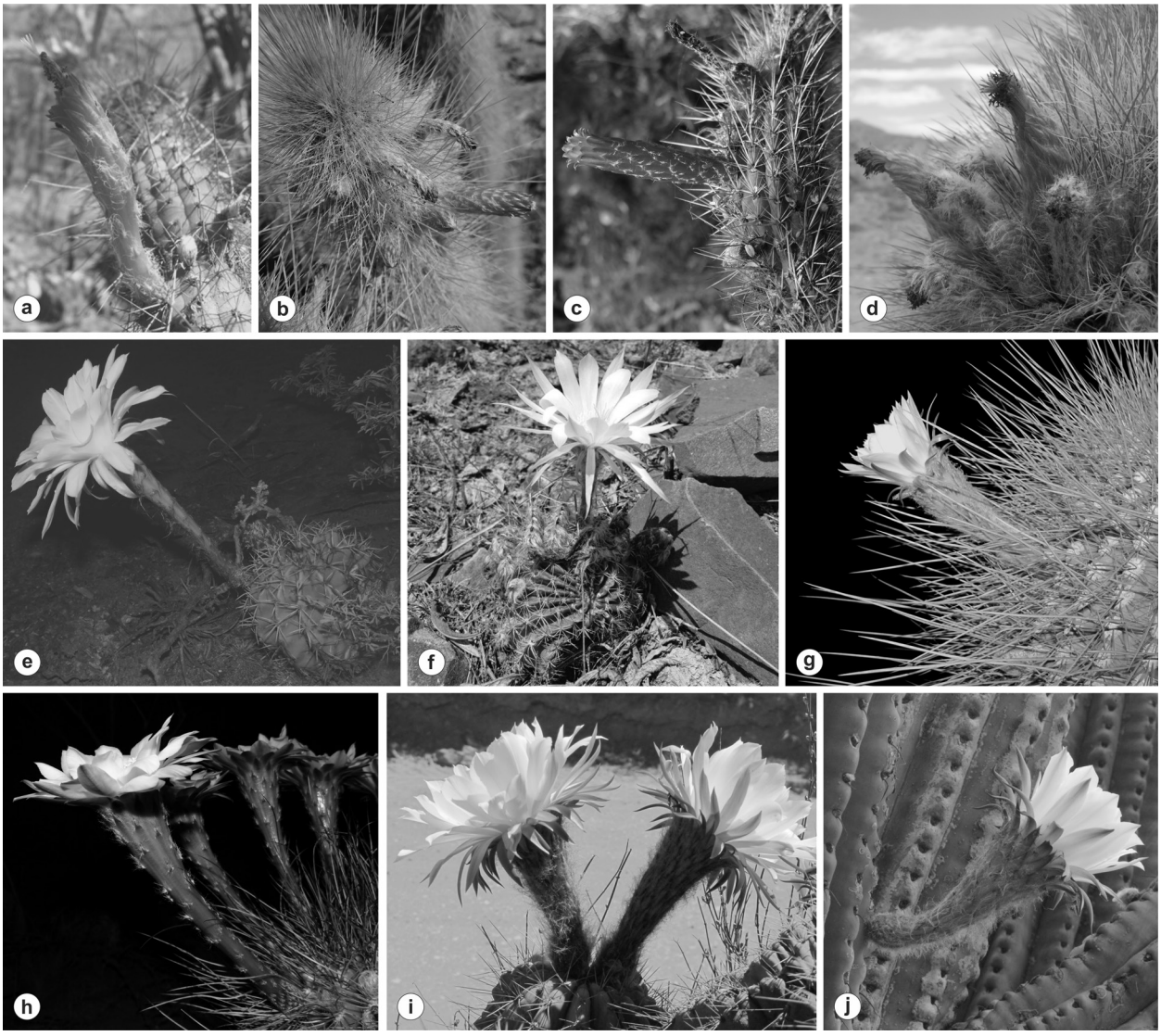

Figure 1. a-d Ornitophilous flowers of cactus. a *Cleistocactus baumannii,* detail of sigmoid tubular flower. b *C. hyalacanthus,* detail of upper portion of stem with buds, flowers in anthesis and fruits. c *C. smaragdiflorus*, detail of tubular flower, note green tepals. d *Denmoza rhodacantha,* detail of buds and sigmoid tubular flowers at the apex of the stem. e-j Sphingophilous flowers. e *Echinopsis albispinosa,* plant and infundibuliform flower. f *E. ancistrophora*, plant and flower still open early in the morning. g *E. atacamensis,* detail of infundibuliform flower. h *E. leucantha*, detail of a set of flowers. i *E. schickendantzii*, detail of two flowers. j *E. terscheckii*, detail of flower. Photographs: V. González a-c, P. Gorostiague d-j.

nectariferous parenchyma, delimited by non-secretory sub-nectariferous parenchyma, with associated vascular tissue. However, epidermal characteristics allowed us to differentiate the genera.

In the three species of *Cleistocactus*, the nectariferous epidermis is simple, with epidermal cells of thin cuticle; in cross section, those cells show quadrangular or rectangular contour, with a prominent nucleus and large vacuoles that occupy a large part of the cellular lumen (Figure 2b, f, i). In *Denmoza*, the epidermis shows short, single-celled trichomes, with conspicuous nuclei located at the base or centre

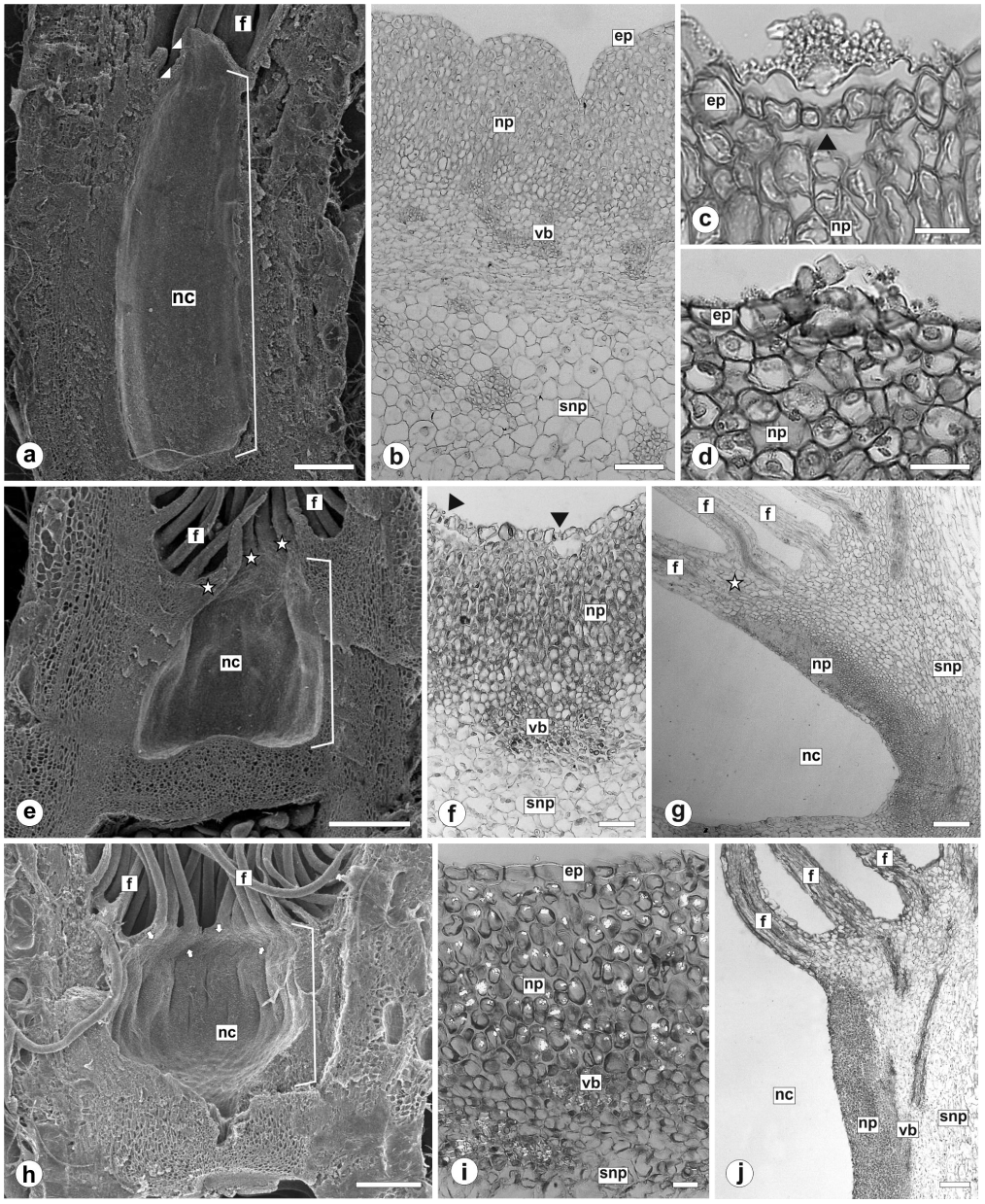

Figure 2. Floral nectary morph-anatomy in *Cleistocactus* species. a-d *C. baumannii* a Scanning Electron Microscope (SEM) of nectar chamber in longitudinal section, note at the upper portion the vault-roof structure (white arrowheads) formed by the fusion of the base of the internal stamens that begin to be released. b Cross section of floral nectary. c Detail of nectarostoma (black arrowhead) with remnants of crystallized secretion. d Detail of epidermis interrupted with traces of crystallized secretion. e-g *C. hyalacanthus.* e SEM of nectar chamber in longitudinal section, note the fused base of the filaments of the internal series (white stars) released in the upper portion of the chamber. f Cross section of floral nectary. g Longitudinal section of floral nectary. h-j *C. smaragdiflorus.* h SEM of nectar chamber in longitudinal section, white arrows indicate the bases of the fused filaments. i Cross section of floral nectary, with polarized light the starch grains show the figure of a Maltese cross. j Longitudinal section of nectar chamber. Abbreviations: (ep) epidermis; (f) filament; (nc) nectar chamber; (np) nectariferous parenchyma; (snp) sub-nectariferous parenchyma. Scale bars: a 2mm; b 100µm; c, i 20µm; d 30µm; e, h 1mm; f 50µm; g, k 200µm.

of the trichome, dense cytoplasm and large vacuoles (Figure 3h, j). In *Echinopsis* species, the epidermis exhibits unicellular trichomes, with a large nucleus of basal position and dense granular cytoplasm (Figure 4a-e, h-m). In *E. albispinosa* and *E. atacamensis*, trichomes are inclining toward the ostiolum in proximity to stomata, preventing nectar evaporation (Figure 4b, j). All the studied species have modified stomata (nectarostomata) of anomocytic type, which are involved in nectar exudation; they are located at the same level of the epidermis or slightly sunken, with conspicuous substomatal chambers. (Figures 2c, f, 3j and 4a, b, e, j). In *Cleistocactus* species, stomata are usually scarce, in *C. baumannii* a crystallized substance was observed on apparent ruptures of the epidermis (Figure 2d).

In the analyzed species, the secretory tissue is differentiated from the parenchyma of the base of the staminal filaments, which are adnate to the floral tube and show cohesion. In longitudinal section, the innermost wall of the floral tube is lined with nectariferous tissue, whereas the area comprising the roof of the ovary and the base of the style consists of non-secretory parenchyma (Figure 2g, j). The nectariferous parenchyma exhibits characteristics of tissues with high metabolic activity, probably related to nectar transformation and secretion processes. It is homogeneous, typically showing several layers of isodiametric cells; these cells are compact, smaller than the cells of the non-secretory parenchyma, thick-walled, with dense granular cytoplasm and prominent nucleus (Figures 2b, f, i, 3g-i and 4e, h, k-m). In *Cleistocactus* and *Echinopsis* species, cells have vacuoles containing calcium oxalate crystals in the form of druses and a few prismatic crystals. Additionally, the secretory parenchyma shows abundant cells with starch grains (Maltese cross in polarized light) (Figures 2i and 4i). In *D. rhodacantha*, only starch grains are observed in the nectariferous parenchyma close to the epidermis.

The subnectariferous parenchyma exhibits isodiametric cells that are larger than those of the secretory tissue, with large vacuoles and abundant starch grains, thin-walled, and with conspicuous intercellular spaces (Figures 2b, f, i, 3i and 4e, h, k, m).

The parenchyma underlying the secretory tissue contains periphlomatic vascular bundles in a ring arrangement, corresponding to the vascular bundles of the staminal filaments and other floral whorls that are fused together as part of the floral tube at the nectary level. These traces, which delimit the secretory tissue, are those that irrigate the nectary in *Cleistocactus* and *Echinopsis* species (Figures 2b, f, i, and 4h, i, k-m). However, in *D. rhodancatha*, the innervation of the nectar chamber is more complex; traces of xylem and phloem originated from the vascular ring of the non-secretory parenchyma penetrate and branch deeply into the nectariferous tissue (Figure 3g-i).

These results are summarized in Table I, which also includes floral phenotype and reproductive biology information of these species.

Floral nectary ultrastructure of *Echinopsis terscheckii*

At pre-anthesis, epidermal trichomes covering the wall of the nectariferous chamber, have a prominent nucleus in a basal position, and dense cytoplasm that shows characteristics of high metabolic activity, with abundant mitochondria and compound amyloplasts (Figure 5a, b). Towards the base, the cell wall of the trichome that comes into contact with the secretory tissue is widened (Figure 5a, c). Plasmodesmata are not observed in the connection between the epidermis and the nectariferous parenchyma (Figure 5d). The latter

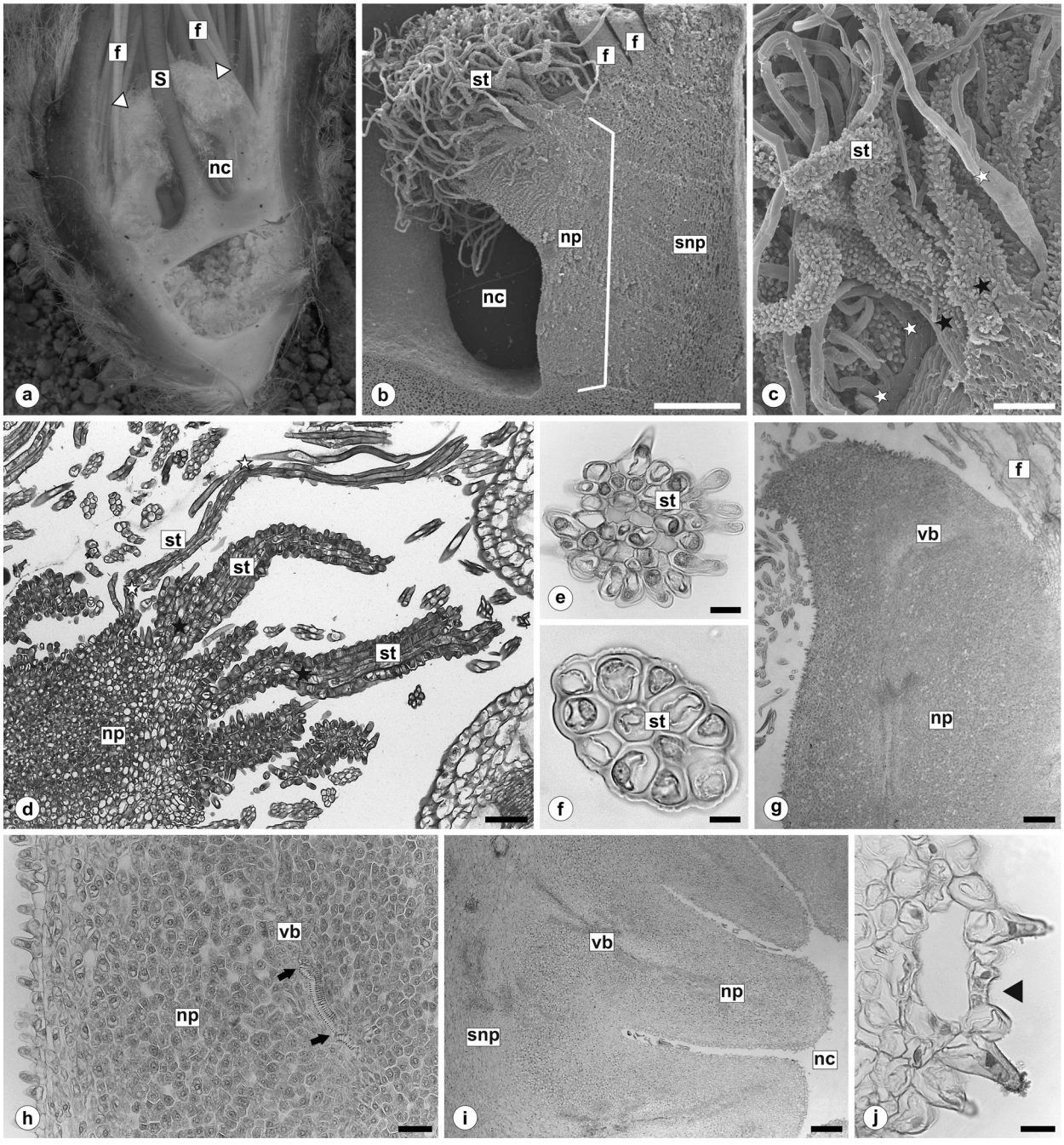

Figure 3. Floral nectary morph-anatomy of *Denmoza rhodacantha*. a-b Longitudinal sections. a Basal portion of flower, note the nectar chamber surrounding the base of the style, with a "plug" of curled trichomes (white arrowheads). b SEM of portion of nectar chamber. c Detail of staminodes born in the upper portion of the nectar chamber, note differences in the bases of the curled trichomes (black starts and white starts). d Light micrographs of non-vascularized staminodes, note the morphological differences in the bases (black starts and white start). e and f transversal section of staminodes. g Longitudinal section of floral nectary wall. h Detail of nectary wall showing traces of xylem and phloem penetrating and branching deeply into the nectariferous parenchyma (arrows). i Cross section of nectar chamber, observe the pattern of peaks and valleys of the wall. j Detail of nectarostoma (arrowhead), with conspicuous sub-stomatic chamber. Abbreviations: filaments (f), nectar chamber (nc), nectariferous parenchyma (np), sub-nectariferous parenchyma (snp), style (S), vascular bundles (vb). Scales: b 500 µm; c, e, g 200 µm; f 20 µm; d, h 50 µm.

shows cells with similar characteristics to those of the epidermal trichomes, such as rich and active cytoplasm, with abundant mitochondria, endoplasmic reticulum, dictyosomes, large amyloplasts with compound starch grains, and numerous small vacuoles (Figure 5e, f). Secretory cells are interconnected through primary pit-fields (Figure 5f).

At floral anthesis, the tissues of the nectary show certain ultrastructural differences regarding to the pre-anthesis stage. In the cytoplasm of epidermal trichomes, amyloplasts persist although they contain somewhat degraded starch grains (Figure 5g, h), in addition, abundant rough endoplasmic reticulum is observed (Figure 5h). At the tip of trichomes, were seen remnants of apparent secretion released by rupture of the cuticle (Figure 5i). No plasmodesmata were observed connecting the cytoplasm of epidermal cells, nor between them and the underlying secretory tissue (Figure 5j). The nectariferous parenchyma shows less dense cytoplasm than in pre-anthesis stage; mitochondria of conserved size and shape are observed, together with partially degraded mitochondria (Figure 5k). Abundant electrodense material located at intercellular spaces, suggests that sugary secretion accumulates in these spaces (Figure 5l).

DISCUSSION

The main findings of this work reveal that the diversity of floral phenotypic specialization in Trichocereeae Argentinian species has not relation with the floral nectary structure. The basic morpho-anatomy and location of the nectar chamber evidence phylogenetic conservatism. However, epidermis surface and patterns of vascularization of the secretory parenchyma are the most interesting differences between genera. The variants observed are not consistent with the pollinator system.

Floral morphology

The systematic value of certain patterns of flower internal organization, particularly of floral nectaries, has been poorly explored in Cactaceae (Buxbaum 1953, Tiagi 1955, Boke 1963, 1964, 1966, 1968, 1980, Pimienta-Barrios & del Castillo 2002, Strittmatter et al. 2002, Fuentes-Pérez et al. 2009, Almeida et al. 2010, 2013). For example, the structure of floral nectaries in Hylocereeae and Rhipsalideae is a distinctive tribal feature, and this character also has strong taxonomic implications at the generic level in the Rhipsalideae (Almeida et al. 2013).

With reference to the pollination process, bee-mediated pollination has been indicated as the most common, and probably the ancestral condition in cactus family (Anderson 2001, Mandujano et al. 2010, Schlumpberger & Renner 2012), whereas pollination by birds, bats and moths would be derived conditions common to some tribes, such as Pachycereeae and Trichocereeae (Grant & Grant 1979, Gibson & Nobel 1986). The phenotypic specialization indicating bird pollination has been developed independently in three of the four subfamilies recognized in Cactaceae, except for the small subfamily Maihuenioideae (Anderson 2001, Gorostiague & Ortega-Baes 2016). For Cactoideae, the highest proportion of reddish-flower species was observed in the Pachycereeae tribe, followed by Cacteae, Cereeae, and Trichocereeae (Gorostiague & Ortega-Baes 2016). With regard to Trichocereeae, it has been observed within *Echinopsis s.l.* and related genera that reddish flowers are a recurrent trait through its phylogeny (Schlumpberger & Renner 2012). Although phenotypic specialization to bird pollination has been frequently recorded in cacti, this condition is not always predictive of the specialization

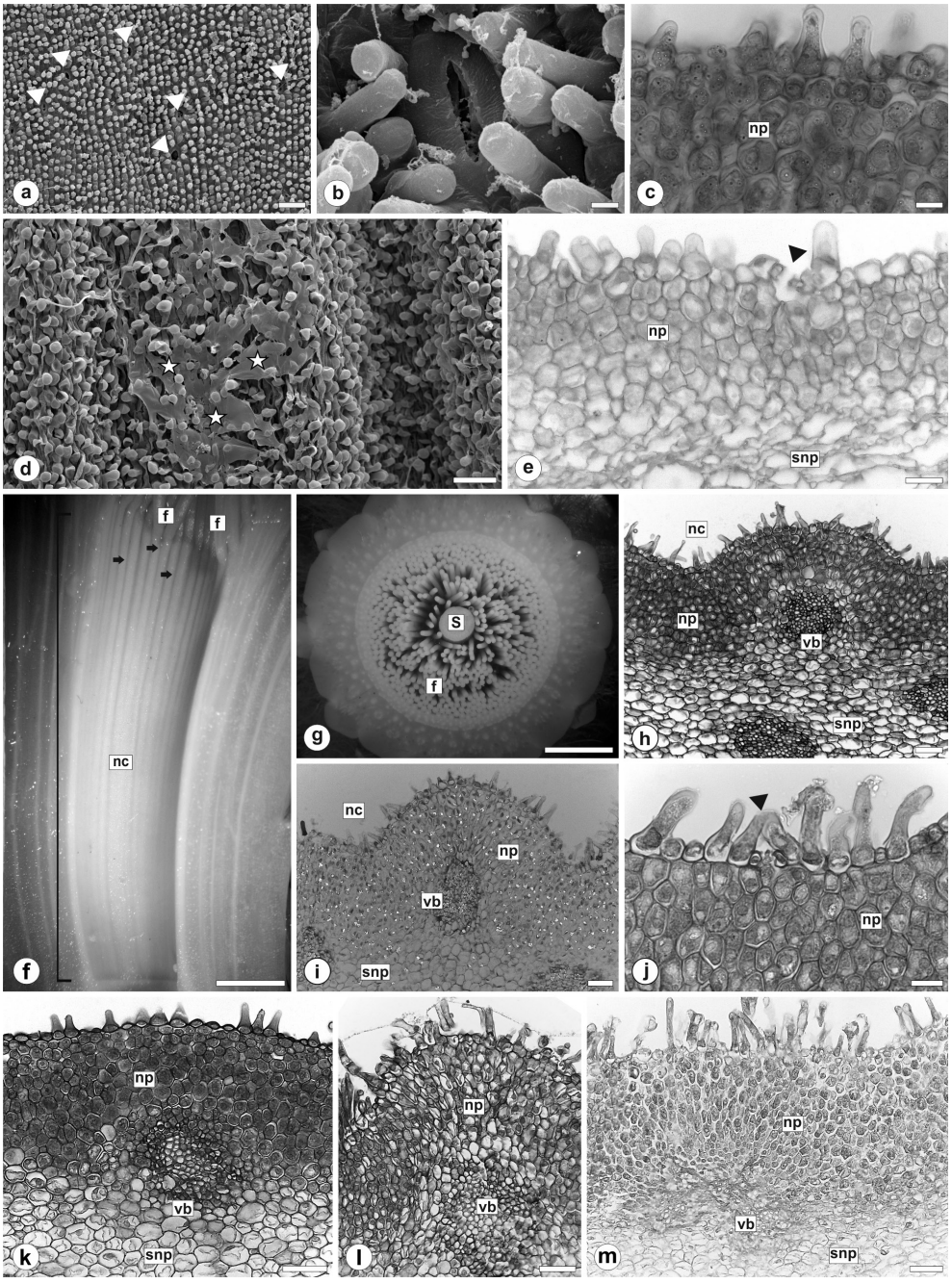

Figure 4. Floral nectary morpho-anatomy of *Echinopsis*. a-c *E. albispinosa*. a-b SEM. a Superficial view of nectar chamber wall, note nectarostomata (white arrowheads). b Detail of nectarostoma. c Light micrographs of cross section of floral nectary. d-e *E. ancistrophora*. d Superficial view of nectar chamber wall, note nectar secretion (white stars). e Light micrographs of cross section of floral nectary. f-j *E. atacamensis*. f Nectar chamber in longitudinal section, note free filaments of the internal series at the top. g Transversal section of the flower above the nectar chamber, note the barrier formed by the filaments surrounding the style. h, k-m Light micrographs of transversal sections of floral nectary. i Floral nectary with polarized light, note druses and starch grains in nectariferous parenchyma. j Detail of nectarostoma with possible secretion remains (arrowhead), observe unicellular trichomes inclined towards the opening. k *E. leucantha*. l *E. schickendantzii*. m *E. terscheckii*. Abbreviations: (f) filament; (nc) nectar chamber; (np) nectariferous parenchyma, (S) style, (snp) sub-nectariferous parenchyma, (vb) vascular bundles. Scales: a, d, h-i, k-m 50µm; b 5µm; c 10µm; e, j 20 µm; f 3mm, g 5mm.

at functional or ecological level, since in most cases other pollinating groups were additionally recorded (Eggli & Giorgetta 2015, Gorostiague & Ortega-Baes 2016). However, pollinator efficiency was not measured, therefore the importance of birds as major pollinators cannot be ruled out.

Within Trichocereeae, *Cleistocactus*, *Denmoza, Oreocereus* and *Matucana* were traditionally recognized as ornithophilous genera, strictly base on floral attributes (Anderson 2001, Hunt 2006, Gorostiague & Ortega-Baes 2016). According to Van der Pijl (1960), the floral bird pollination syndrome includes a series of characteristics (i.e. diurnal anthesis, scentless flowers, conspicuous reddish colour, long tubular corollas, deep nectaries and abundant and diluted nectar) that would respond to selective pressures exerted by pollinators. Additionally, the presence of nectar chambers that allow nectar accumulation is a characteristic typically associated with hummingbird pollination (Brown & Kodric-Brown 1979, Stiles 1981, Díaz & Cocucci 2003, Agüero et al. 2018). The presence of conspicuous nectar chambers in *Cleistocactus* and *Denmoza* species here study confirm the latter assertion.

On the other hand, floral moth pollination syndrome includes floral traits such as odor heavy-sweet, white corolla, resistant tepals, many anthers with high pollen amount, shape not necessarily zygomorphic, mostly with narrow tube, nocturnal anthesis, and high nectar production (Van der Pijl 1960). On the basis of these floral traits, all *Echinopsis* species here studied were traditionally interpreted as sphingophylous (Anderson 2001). Regardless of the columnar or globose habit, *Echinopsis* species here studied share these floral characteristics associated with moth pollination. However, evidence based on field studies indicates generalized pollination system in these species, with diurnal insects and birds being complementary floral visitors (De Viana et al. 2001, Schlumpberger & Badano 2005, Ortega-Baes et al. 2011, Gorostiague & Ortega-Baes 2017). These cases demonstrate that birds can pollinate species that are not phenotypically adapted to ornithophily (Maruyama et al. 2013).

Nectar chamber morphology

In angiosperms, some cases of similar nectary type within a family have been recorded, however, one of the reasons for the great diversity observed in nectary structure and nectar constituents depends largely on the type of pollinator (Baker & Baker 1983, 1990, Pacini et al. 2003 and reference there in).

In cactus family, it has been found that the floral nectary type appears to be constant within most of the genera analyzed so far. For example, in *Pereskia* and *Rhipsalis* nectar is secreted via an annular receptacle (Zandonella 1977, Barthlott & Hunt 1993, Almeida et al. 2013). Whereas the nectar chamber type has also been described for species of *Disocactus, Epiphyllum, Hylocereus, Selenicereus, Weberocereus* (Almeida et al. 2013), *Polaskia* (Gudiño et al. 2015), *Opuntia elata* (Agüero et al. 2018) and *Strombocactus* (Camacho-Velázquez et al. 2019). As result of these works, we can say that the nectar chamber type is the most frequent nectary type registered among the family, even in different subfamilies.

The hypanthial nectary of all the species studied here share some traits in the overall structure. However, they present morphological differences that allow differentiation of some species, it is difficult to infer evolutionary trends. These chambers may appear more or less closed, due to the formation of a dense ring of filaments (e.g. *Echinopsis* species), or to the presence of filamentous or hypanthial appendages (e.g. *Cleistocactus* species here analyzed), and in some species of *Opuntia*, due to an annular or even cup-shaped excrescence

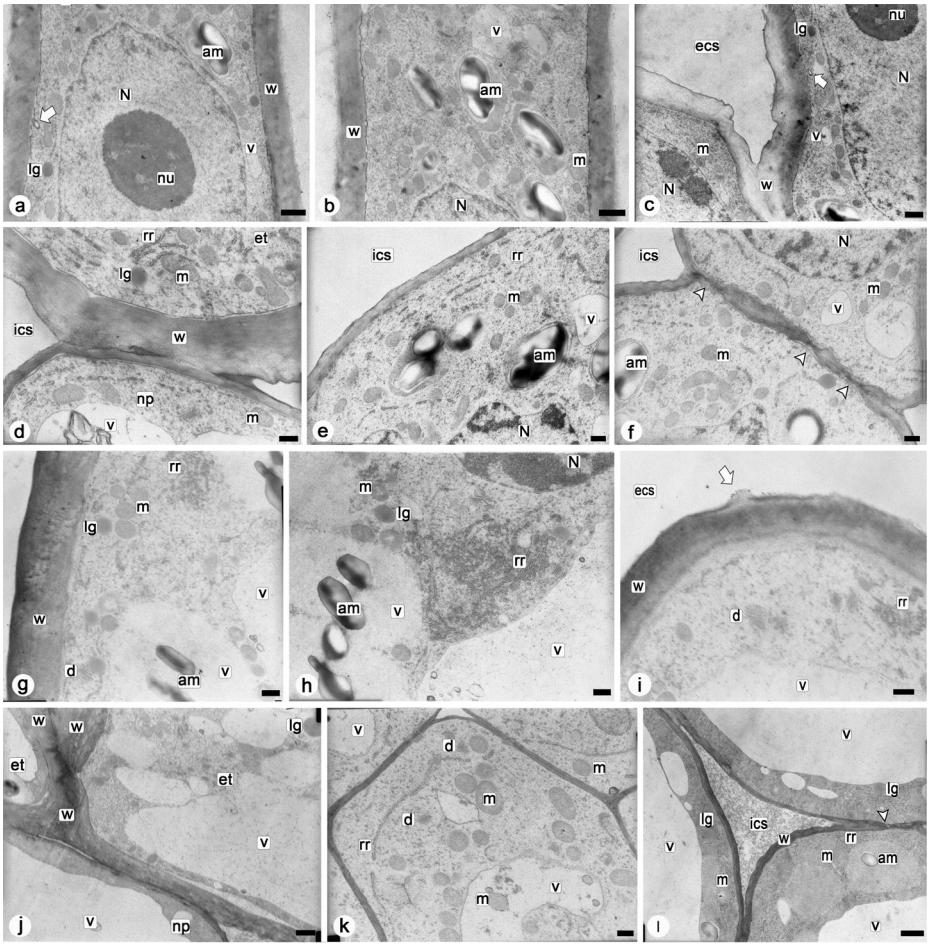

Figure 5. Transmission Electron Microscope (TEM) of floral nectary of *E. terscheckii*. a-f Pre-anthesis. (**a**) Proximal portion of an epidermal trichome, showing a large nucleus and a prominent nucleolus, white arrow indicates a small vacuole fusing with the cell membrane; note cell walls flared towards the base of the trichome. (**b**) Detail of dense granular cytoplasm of the trichome, note abundant mitochondria, vacuoles of small dimensions, abundant compound amyloplasts and few lipid globules. (**c**) Connection between two epidermal trichomes, showing portions of the nuclei of both cells, occupying a basal position; the white arrow indicates a vacuole fusing with the cell membrane. (**d**) Connection between epidermal trichome (upper position) and nectariferous parenchyma (lower position), observe cell walls of different thicknesses, and the absence of plasmodesmata between both cells. (**e**) Detail of dense cytoplasm of a parenchymal nectariferous cell, showing plastids with several grains of starch, small vacuoles, rough endoplasmic reticulum; note the intercellular space. (**f**) Detail of connection between two cells of the nectariferous parenchyma, white arrowheads indicate plasmodesmata that communicate the cytoplasm of secretory tissue cells. (**g-l**) Anthesis. (**g**) Portion of epidermal trichome, observe the cytoplasm less electrodense than in pre-anthesis, with larger vacuoles. (**h**) Detail of epidermal trichome cytoplasm, plastids with partially degraded starch grains; note the accumulation of rough endoplasmic reticulum in the cytoplasm. (**i**) Detail of the apical portion of the epidermal trichome with secretion released by rupture of the cuticle (white arrow). (**j**) Connection between two epidermal trichomes and an underlying nectariferous parenchyma cell; in both tissues, large vacuoles relegate the cytoplasm to the periphery of the cells. (**k**) Detail of nectariferous tissue cells, observe less dense cytoplasm and mitochondria in apparent degeneration. l Detail of intercellular space in the nectariferous tissue, with homogeneous electrodense material, suggests that sugary secretion accumulates in these spaces; note partially degraded amyloplasts, and plasmodesmata connecting cytoplasms (white arrowhead). **Abbreviations**: (**am**) amyloplast; (**d**) dictyosome; (**ecs**) extracellular space; (**ics**) intercellular space; (**lg**) lipid globule; (**m**) mitochondria; (**N**) nucleus; (**nu**) nucleolus; (**w**) cellular wall; (**np**) nectariferous parenchyma; (**rr**) rough endoplasmic reticulum; (**et**) epidermal trichome; (v) vacuole. Scale: a-c, j, l 1µm; d-i, k 500nm.

at the base of the style (Buxbaum 1953, Barthott & Hunt 1993, Agüero et al. 2018). In *C. baumannii*, as in *Schlumbergera*, the top surface of the nectary resembles a vault-roof structure formed by non-secreting parenchyma and the primary stamens surrounding the style (Almeida et al. 2013). *Denmoza* is the most interesting case because it presents a conspicuous chamber closed by a "plug" of curled staminodes. Because of its position, morphology and histology, these structures are considered non-vascularized staminodes. Floral visitors must cross this barrier with their beaks in order to access the nectar.

Staminodes are uncommon structures occurring within angiosperms, but frequently fulfill important secondary floral functions (Walker-Larsen & Harder 2000). Little is known about the presence of staminodes in Cactaceae. They were recorded in *Pereskia* forming groups of curled trichomes between petaloid tepals and stamens (Leuenberger 1986), and in *Brasiliopuntia*, *Matucana* and *Denmoza*, where a ring of hair like staminodes is observed (Anderson 2001). We consider that the structures formed by the cohesion of the filament bases (*Cleistocactus*), by the formation of a staminodes plug (*Denmoza*), or by the barrier formed by the internal series of free filaments (*Echinopsis*) provide protection against the evaporation of the diluted nectar that characterizes most ornithophilous flowers (Baker 1975). These structures regulate the communication of the chamber with the exterior and visitors foraging for food. Thus, only certain groups of potential pollinators can reach the base of the flower to collect nectar, which is also protected from wind or rain. In regard to the pattern of peaks and valleys forming the walls of the nectar chamber in *Denmoza* it is considered that increases the secretory surface and contributes to the accumulation of nectar between the folds. A similar case was reported for *Opuntia* (Agüero et al. 2018).

We identified interesting morphological differences at the floral nectary level that do not follow with the external morphological differentiation of flowers between species with similar floral syndrome (e.g. hummingbirds-*Cleistocactus* vs. *Denmoza*). Although *Cleistocactus* and *Denmoza* species share the typical floral morphology of ornithophilous species, they differ in the characteristics of the nectariferous chambers, such as the presence of a plug of curled staminodes in the upper portion of the chamber, epidermal characteristics, development of the nectariferous tissue, and vascularization of the secretory tissue.

Nectar chamber anatomy

The basic anatomy of the studied floral nectaries is similar to that described for other cactus species (Fuentes-Pérez et al. 2009, Almeida et al. 2010, 2012, 2013, Agüero et al. 2018, Camacho-Velázquez et al. 2019). The species analyzed here also show similarities in the general structural characteristics (i.e. hypanthial nectary and structured type with three differentiated tissues). However, they show certain anatomical differences, specially related to nectariferous epidermis, that allow us to distinguish the three genera, but these differences would not be directly associated with their phenotypical specialization (see Table I).

The micromorphological traits detected in the epidermis of the different specimens analyzed correspond to that recorded in other cacti, reinforcing phylogenetic conservatism. The presence of one-layer epidermis in floral nectaries of *Cleistocactus* species is shared with species of *Opuntia* (Fuentes-Pérez et al. 2009, Agüero et al. 2018) and *Rhipsalis* (Almeida et al. 2012), whereas epidermal trichomes have been reported in other Cactoideae genera

such as *Epiphyllum, Disocactus, Hylocereus, Weberocereus,* and *Selenicereus* (Almeida et al. 2010, Stefano et al. 2001). Based on anatomical and macromorphological evidence gathered in the present work, the conserved pattern in the epidermis of *Cleistocactus* and *Echinopsis* species, in relation to other cactus taxa, suggests that we cannot rule out the potential diagnostic value of epidermal traits of floral nectaries at generic levels within Trichocereeae. However, it will be necessary to extend the taxonomic sampling to more distant phylogenetically Trichocereeae to accept or reject the last hypothesis.

Echinopsis s.s. species (*sensu* Hunt 2006) here analyzed typically share the trichomes shape of the epidermal cells of the floral nectary and can be easily differentiated from *Cleistocactus* (*sensu* Hunt 2006) species analyzed. This trait can be an important character of diagnostic value and a potential synapomorphy for this group.

It should be noted that the absence of tannins in epidermal cells is shared with *Strombocactus* species (Camacho-Velázquez et al. 2019). Contrary, the presence of tannins in epidermal cells was recorder for some *Opuntia* species (Fuentes-Pérez et al. 2009), *Epiphyllum phyllanthus* (Almeida et al. 2010), *Cephalocereus tetetzo*, *C. columna-trajani* (Torres-Sánchez 2013), *Polaskia chende, P. chichipe* and *Stenocereus quevedonis* (Gudiño et al. 2015). This attribute could indicate a synapomorphy at higher hierarchical levels.

Nectar secretion through modified stomata, which have lost the ability to close, is the most frequent and widely reported form of nectar secretion in angiosperms (Durkee et al. 1981, Bernardello 2007, Nepi 2007). In Cactaceae, the presence of stomata in the nectar chamber was recorded for several taxa (Zandonella 1977, Almeida et al. 2012, Gudiño et al. 2015, Agüero et al. 2018, Camacho-Velázquez et al. 2019). However, nectar secretion has been reported to occur only through secretory trichomes in epiphytic cacti *Selenicereus grandiflorus* (Stefano et al. 2001), *Epyphyllum phyllanthus* (Almeida et al. 2010), *Disocactus ackermannii*, *Epiphyllum guatemalense* and *Hylocereus undatus* (Almeida et al. 2013). Additionally, the presence of epidermal interruptions in *C. baumannii* on which crystallized material is observed, indicates a type of holocrine secretion, as in *Selenicereus grandiflorus*, where secretion is probably initiated by the mechanical action of pollinators (Stefano et al. 2001) and *Polaskia chende* in which case the secretion of nectar to the nectariferous chamber occurs directly through fissures in epidermis (Gudiño et al. 2015).

The general arrangement and features of the nectariferous cells are typical of the floral nectaries described in other cactus species (Fuentes-Pérez et al. 2009, Almeida et al. 2010, 2012, 2013, Torres-Sánchez 2013, Gudiño et al. 2015, Agüero et al. 2018, Camacho-Velázquez et al. 2019).

Before anthesis, nectar sugars can be stored as starch in amyloplasts, in the secretory parenchyma or in the non-secretory parenchyma (Pacini et al. 2003, Nepi 2007). In the studied species, the nectariferous parenchyma has abundant amyloplasts. The absence of chloroplasts in the nectariferous tissue can be attributed to the location of the floral nectaries, inside a highly developed floral tube, which provides shelter from sun exposure.

On the other hand, mineral inclusions, in the form of calcium oxalate druses, in the nectariferous parenchyma could play a functional role, immobilizing calcium in this tissue in which active sugar transport is presumed to occur (Nepi 2007), since calcium has been shown to inhibit plasma membrane ATPase involved in the transport of sucrose in plants (Guiaquinta 1979, Nepi 2007).

Among angiosperms, the vascular tissue that innervates the nectariferous parenchyma is composed of phloem, although cases of traces consisting of xylem and phloem have been also recorded (Fahn 1979, 2000, Bernardello 2007). Interestingly, *Denmoza* presents branched xylem and phloem bundles that penetrate the secretory tissue; this configuration of vascular tissue was reported for *Opuntia* species, and it has been suggested that the pre-nectar does not originate only in the sub-nectariferous parenchyma (Agüero et al. 2018).

In documented cases of absence of traces that directly irrigate the nectariferous tissue, the supply comes from vascular bundles of surrounding tissues (Fahn 1979, 1988, 2000, Bernardello 2007). In the studied *Cleistocactus* and *Echinopsis* species, the vascular bundles that supply the secretory tissue are located on the edge between the underlying parenchyma and the nectariferous tissue. The traditionally suggested correlation between the tissue irrigating the nectary and sugar concentration in the nectar (Frey-Wyssling & Agthe 1950) does not always exist (Bernardello 2007). Agthe (1951) found that in species in which the nectar was concentrated, the secretory tissue was innervated only by traces of phloem, whereas in species in which the nectar was diluted, xylem and phloem were present. These observations suggest that the nectar of *D. rhodacantha* is probably more diluted than that of the other analyzed species. Thus, the presence of a plug of hairs to help prevent nectar evaporation is reasonable. According to Nicolson & Thornburg (2007), nectar properties tend to be similar in plants with comparable pollination syndromes: flowers pollinated by insects produce concentrated nectars, whereas flowers pollinated by bats and birds generally produce diluted nectars (Baker 1975). Thus, at least within a group of closely related taxa, relatively similar nectar volume and concentration parameters of the ornithophilic species are expected in *Cleistocactus* and *Denmoza*. Gorostiague & Ortega-Baes (2016) found a similar amount of nectar in *C. baumanii* and *C. smaragdiflorus*, and with an intermediate value concentration compared to that recorded for other ornithophilous species. However, this information is only available for a limited number of species. Further studies of the chemical composition, concentration and volume of nectar in Cactaceae will be relevant to elucidate the relationship between these variables, the phenotypic specialization of flowers, and the effective pollinators within this group of plants.

Floral nectary ultrastructure of *E. terscheckii*

The ultrastructural characteristics observed in the floral nectary of *E. terscheckii* are similar to those found in other angiosperms (Fahn 1979, Nepi 2007). However, they differ from traits recorded for extrafloral nectaries in Cactaceae (Mauseth 1982). According to Nepi (2007), the cytoplasm that characterizes secretory nectar cells is usually rich in ribosomes and mitochondria at the time of secretion, indicating an increase in energy requirements for nectar production. In this case, both the nectariferous parenchyma and the epidermal trichomes that cover the nectariferous chamber show certain characteristics that correspond to metabolically active cells, associated with nectar transformation and secretion processes (Stefano 2001, Nepi 2007, Agüero et al. 2018).

The nectar is exudated mainly through stomata, as in other studied cacti and in most Eudicots (Bernardello 2007, Agüero et al. 2018). However, the ultrastructural analysis of *E. terscheckii* suggests that the secretion probably occurs by a mixed mechanism: following simplastic pathway and releasing by rupture of the cuticle of epidermal trichomes and, via

apoplastic, releasing through stomata that are distributed between epidermal trichomes. The secretion apparently begins in the stage of anthesis, while in pre-anthesis stage, the high metabolic activity observed in the cytoplasm of epidermal cells and nectariferous parenchyma is probably related to sugar mobilization processes prior to the secretion stage. The source of sugar in nectars can be of direct photosynthesis in nectaries or in any other part of the plant, or the sugars can come from the hydrolysis of starch temporarily stored in the secretory tissue (Pacini et al. 2003, Nepi 2007). The absence of chloroplasts in nectariferous parenchyma and epidermal trichomes of *E. terscheckii* suggests that sugar is not produced by photosynthesis in these tissues, but is mobilized and stored there in amyloplasts, to then be hydrolyzed and used during synthesis phase in floral pre-anthesis.

Concluding remarks and future prospects

This is the first study of floral nectary structure in bird-pollinated species showing different floral phenotypic specialization in the cactus family. It provides novel data on species that have received little attention and reveals that the species here studied share similar nectariferous chambers and differ in some morpho-anatomy traits. These differences are not directly associated with the phenotypic specialization of flowers, even though pollination by birds has been verified in these species. These results are important because they may contribute to the interpretation of the evolutionary trend of the floral nectary and to the understanding of its systematic value in Cactaceae. Mapping of selected floral and morpho-anatomical characters on a phylogeny would help to interpret their ancestral and derived states and to find synapomorphies within the family.

The morpho-anatomical characteristics of the different types of floral nectaries provide important information that can contribute to our understanding of phylogenetic relationships or to the support of relationships previously inferred with molecular data. Particularly those related to epidermal traits have the potential to be incorporated into molecular phylogenies and help to resolve uncertain phylogenetic relationships within this tribe. For example, the nectary epidermis surface appears to be an interesting character that allows us to distinguish between the typical ornithophilous genera *Cleistocactus* and *Denmoza*, and also from *Echinopsis* species (the latter genera share epidermic trichomes). This new anatomical information supports a subclade comprised of *Cleistocactus* species in Schlumpberger & Renner´s (2012) phylogeny of *Echinopsis s.l.* However, a wider taxonomic sampling with distantly related genera would likely yield distinctions among genera or species complexes and fruitful taxonomic information with phylogenetic significance.

Moreover, a deeper study of the nectar characteristics is necessary to elucidate the possible relationship between the morpho-anatomical characteristics of the floral nectary structure, the phenotypic specialization of flowers, and the effective pollinators of this lineage.

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VVG, PG and POB contributed to the study conception and design, and field work. VVG performed material preparation, data analysis, and wrote the manuscript. BG contributed to transmission electron microscopy analysis. MSF read the manuscript, revised and completed the information. All the authors commented on previous versions of the manuscript and approved the final version.