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ORIGINAL ARTICLE

Are the floral morphology and anatomy of *Galphimia australis*, an atypical neotropical Malpighiaceae, associated to a new pollination syndrome?

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Abstract
The flowers of the species of Malpighiaceae in the Neotropical Region are relatively uniform in their morphology due to their dependence on oil-collecting bees as their main pollinators. However, many species of the genus *Galphimia* seem to have acquired a different floral syndrome, lacking markedly zygomorphic flowers and developed elaiophores in the calyx. Likewise, these species present anthers with great development, probably in response to the selection of pollinators that collect pollen. *Galphimia australis* incorporated some of these traits but also retained some residual characteristics typical of species pollinated by oil bees. This leads to many questions on how these flowers ensure their pollination. Inquiring about the reduction or modification of these characteristics allows us to understand how *G. australis* achieves a different pollination syndrome. In this research, we carry out a detailed morphological and anatomical study of the flowers and pollen grain devolvement of *G. australis* and floral visitors were observed and captured. Results were analyzed in order to determine how this species changed from the oil-floral syndrome, typical of neotropical Malpighiaceae, to one syndrome with pollen as the main reward.

Keywords Malpighiaceae · *Galphimia* · Flower morphology · Flower anatomy · Sexual plant reproduction

Introduction

Malpighiaceae is a family of trees, shrubs, and vines that grow in tropical and subtropical forests, and savannas of the New and Old Worlds. Most of the genera and species are found in the Neotropical Region, with a total of 1300 species belonging to 77 genera. On the other hand, in the Old World, they are poorly represented with only 150 species in 17 genera (Davis and Anderson 2010). Most of its diversity is concentrated in South America, which is considered one of the centers of origin and diversification of the family (Anderson 1979; Cameron et al. 2001; Davis et al. 2001). The species of this family show great variation in growth forms, fruit types (Anderson 1979), and pollen grain morphology (Anderson 1990; Lobreau-Callen 1983; 1984; Lowrie 1982); however, in the species of the Neotropical Region, the flowers are relatively uniform in their morphology, especially their attraction, orientation and reward to pollinators, due to their dependence on oil-collecting bees, which are their main pollinating agent (Anderson 1979, 1990; Davis et al. 2014; Vogel 1990). These flowers are bilaterally symmetrical, and are oriented with the posterior petal erect and at the back of the flower from a bee's point of view (Anderson et al. 2006). In this oil bee syndrome (Malpighiaceae species and oil-collecting bees), the pollinators exhibit an stereotyped foraging behavior (Torretta et al. 2022; Vogel 1990); the bee females oriented the head to posterior petal, grasped the thickened claw with its mandibles, and foraged by floral oil with fore- and middle legs (Avalos et al. 2020; Sigrist and Sazima 2004; Vogel 1990), in a four-legged pattern of the oil-collecting organs (Neff and Simpson 1981). Although, the concept of pollination syndromes has been criticized

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(Waser et al. 1996), they represent convergent floral adaptations to specific functional pollinator groups (Fenster et al. 2004; Dellinger 2020), that have similar behavior and exert comparable selection on flowers (Ashworth et al. 2015). Most neotropical Malpighiaceae exhibit a marked floral syndrome, characterized mainly by rewarding to their pollinators with floral oil (Vogel 1990), a reward that requires morphological (e.g., oil-collecting combs of highly modified setae) and behavioral adaptations of pollinators to be collected (e.g., stereotyped movement of four legs) (Neff and Simpson 1981).

Floral oil is produced in glands called elaiophores located in pairs in the abaxial epidermis of all or only four sepals, except of anterior sepal that is opposite to the posterior petal (Simpson and Neff 1983; Vogel 1974, 1990). However, in the Neotropical Region, some species present glandular and eglandular flowers (Sazima and Sazima 1989; Cappellari et al. 2011) while others have completely lost the elaiophores and possibly are pollinated by pollen-collecting bees (Cappellari et al. 2011). In contrast, only few of the Old-World species produce floral oil, and are pollinated by pollen—or/and, to a lesser extent, nectar-collecting bees (Aliscioni et al. 2019; Qian et al. 2016; Vogel 1990; Zhang et al. 2016).

Galphimia Cav. is a neotropical genus of Malpighiaceae that comprises 26 species, most of which are found in Mexico, and only 4 species grow in South America (Anderson 2007): *G. amambayensis* C.E. Anderson, *G. australis* Chodat, *G. brasiliensis* (L.) A. Juss., and *G. platyphylla* Chodat. The main floral characters that differentiate this genus from most neotropical Malpighiaceae are the corolla varying from moderately bilaterally symmetrical to nearly radial, and the tendency to the absence of oil glands in the calyx, that when present, are limited to a single one at base of sinus between some or all adjacent sepals. In some species of *Galphimia*, these small glands have been observed in some or all the sepals, like those present in the leaves, which acts as extra nuptial nectaries (Vogel 1974). According to Castro et al. (2001), these calyx glands have structures homologous to those of the leaf glands, but exudates few lipids with traces of polysaccharides. However, this has not been verified with field observations if pollinators foraging for this exudate.

Anderson (1977) mentions that species of *Galphimia* with eglandular flowers have large anthers, probably in response to selection pressures exerted by pollen-collecting bees. This suggests that some *Galphimia* species acquired a different pollination syndrome than most neotropical Malpighiaceae. This tendency to the loss of oil-pollination syndrome is also observed in other neotropical genera such as *Thryallis* Mart, *Lasiocarpus* Liebm. (Anderson 1979), and species such as *Pterandra pyroidea* A. Juss. (Cappellari et al. 2011), which are not closely phylogenetically related to *Galphimia*. These data suggest that such pollination shift within neotropical Malpighiaceae could have originated more than once within the family as another strategy for the sexual reproduction.

Galphimia australis is a widespread species, reaching the southernmost distribution for the genus (Aliscioni and Torretta 2017; Anderson 2007). Its flowers present very small glands in the calyx with intra-floral and inter-floral variations in size, number, and position (Castro et al. 2001, cited as *G. brasiliensis*). The reduction or modification of the typical characteristics of oil flower syndrome leads to many questions about the changes that have occurred in the flowers to ensure their pollination. Inquiring about these characteristics will allow us to understand how *G. australis* achieves a different pollination syndrome. Likewise, until now, there are no morphological and anatomical studies on all the floral whorls that can bring a better comprehension on this scenario.

In this research, a detailed morphological and anatomical study of the flowers of *G. australis* is carried out. The aims of the present studies were (1) to study the presence of secretory tissues in the calyx and corolla, (2) to analyze pollen grain development and morphology concerning anther modifications, (3) to analyze the morphology and anatomy of the gynoecium in relation to pollinators and previous studies on the family, and (4) to investigate which flower visitors are potential pollinators of *Galphimia australis* and observe their foraging behavior. We hypothesized that morphological and anatomical features in different floral pieces will allow *Galphimia australis* to change from the oil-floral syndrome, typical of neotropical Malpighiaceae, to one syndrome with pollen as the main reward.

Material and methods

Studied species and sampling sites

Galphimia species are small flowering subshrubs, shrubs, or treelets with yellow flowers, often with red markings, with calyx 5-merous, corolla 5-merous, and ten fertile stamens surrounding a tricapellar ovary with three subulate styles (Anderson 2005, 2007). *Galphimia australis* is a subshrub, with leaves linear to narrowly elliptical, with 2 glands (sometimes only 1 gland or absent) on the margin near the base of the lamina, with flowers arranged in terminal inflorescences. This species grows from southern Brazil, Bolivia, Paraguay, and western Uruguay to northeastern of Argentina, from the province of Misiones to the west of Entre Ríos, habiting open woodlands and grasslands (Aliscioni and Torretta 2017; Anderson 2005; 2007; Castro et al. 2001).

Natural populations to collect material were visited from Provincial Park Profundidad (27°33′49″S, 55°42′06″W) in Misiones province (2–3 December 2019), Colonia Garaví (28°14′03″S, 55°46′45″W) in Corrientes province (6–7

December 2021), and National Park El Palmar (32°26′48″S, 58°15′57″W) in Entre Rios province (7–8 December 2019), all sites in Argentina. Vouchers of the plant specimen (Torretta 119, 126, 138) were deposited in herbarium form the Instituto de Botánica Darwinion (SI).

Morphological and anatomical analysis

Floral buds at pre-anthesis ($n = 100$ buds), opened flowers ($n = 100$–200 flowers), and senescent flowers (recognized by changed color and turgidity of the petals, empty anthers and/or not receptive stigmas; $n = 50$ flowers) of *Galphimia australis* were collected from different individuals and fixed in formalin, alcohol, acetic acid (FAA) for further morphological examination.

Later, in the laboratory, selected samples of flowers (in anthesis and without damage) were chosen to examine them through a stereomicroscope Wild M5. For bright-field microscope studies flowers at different stages of development, ranging from the smallest buds up to opened flowers that had been pollinated were fixed in FAA, dehydrated in an ethanol series, transferred to xylene, and then embedded in paraffin wax. Longitudinal and transverse Sects. 10 µm thick were cut and stained with safranin combined with fast green, following the Zarlavsky (2014) technique, and mounted in Spurr low viscosity resin (Ted Pella Inc). In order to analyze the presence of secretory tissues, some sections of the calyx glands and sections of the limb of posterior ("flag") and lateral petals were deparaffinized and hydrated to make the following histochemical tests: Sudan IV in saturation for lipids (Pearse and Hess 1961), Lugol reagent (1gr potassium-iodide in 100 ml distilled water and 0.3 g of sublimated iodine) for starch grains (Johansen 1940), 0.5 ml naphtol + 0.5 ml dimethyl–paraphenylene–diamine in 49 ml of buffer 0.05 mol (NADI) reagent for terpenes (David and Carde 1964), 5% (*w*/*v*) aqueous solution of ferric trichloride 5% to identify tannins (Zarlavsky 2014) and Cresil Blue 1% to find mucilage (Zarlavsky 2014). Periodic acid–Schiff (PAS) reaction was used in sepal glands to locate insoluble polysaccharides (Jensen 1962). All slides were observed and photographed with a bright field microscope Labomed LX400.

Styles with stigmas and mature anthers previously fixed in FAA (formalin, alcohol, acetic acid) were processed following Zarlavsky's (2014) technique for observations with scanning electron microscopy. These were first dehydrated in an ethanol series (70, 80, 90, 100%). Then, they were critical point dried with liquid CO_2 and sputter-coated with gold–palladium for 3 min using a metallizer Termo VG Scientific SC 7620. Scanning photomicrographs were taken with a Philips XL 30 microscope (Philips, Amsterdam, The Netherlands).

Pollinators

In all three populations, we observed flowers and captured species of floral visitors of *G. australis*, at different times of day (between 8:00 and 19:00 h). To achieve this, we conducted direct observation on flowers (cumulative time = 240 min in each population) and we captured all floral visitors. In each population, we realized a total of twelve 10-min censuses (six censuses for day in the following time ranges; 8:00–9:00, 9:30–10:30, 11:00–12:00, 14:00–15:00; 16:00–17:00; and 18:00–19:00) on two patches (ca. 2×2 m) with high abundance of flowers (one observer by patch). During the censuses, we observed the foraging behavior of flower visitors. Moreover, in all three populations, we randomly walk in an area of 1 ha (ca. 100×100 m) to register all oil-rewarding species of Malpighiaceae and other plant families (cumulative time = 240 min in each population) and to observe their floral visitors, discriminating between oil-collecting bees and others. The captured insects were sacrificed in situ and preserved to be determined to genus or species level (if possible). Taxonomic determination was carried out in the laboratory (Roig-Alsina 2013; Dalmazzo et al. 2014). All captured specimens are preserved in the Entomological Collection of the General Botany Unit (FAUBA) at the Faculty of Agronomy, University of Buenos Aires.

Results

Flower morphology

Flowers are down facing, pedicelled, and grouped on erect, terminal racemes (Fig. 1a). They are yellow during anthesis and turn red in post-anthesis. Flowers are slightly zygomorphic (Fig. 1b). Calyx 5-merous, with sepals of 1.7–2 mm long and 0.8–1 mm wide (Table 1), glabrous except at the apex with a tuft of hairs; with one to five glands of circular aspect of 0.1–0.2 mm wide and long (Fig. 1c). The variation in the number of glands between flowers of the same inflorescence was observed in all studied populations. The corolla is 5-merous, with subequal petals, the limb of the posterior petal measure 5–6 mm long and 2.5–3 mm wide, and the limbs of the other petals 3–5 mm long and 1.5–2 mm wide (Table 1); all deciduous in post-anthetic flowers, with a claw between 1 and 1.5 mm long. The limbs are triangular-ovate with the margin slightly denticulate; the posterior petal ("flag") is poorly differentiated by the wavy and finely toothed margin and a slightly larger limb (Fig. 1b). Androecium consists of 10 stamens, in 2 whorls, with subulate filaments, fused at the base; the outer whorl with filaments of 1.9–2 mm long and 1–1.2 mm wide while the inner whorl

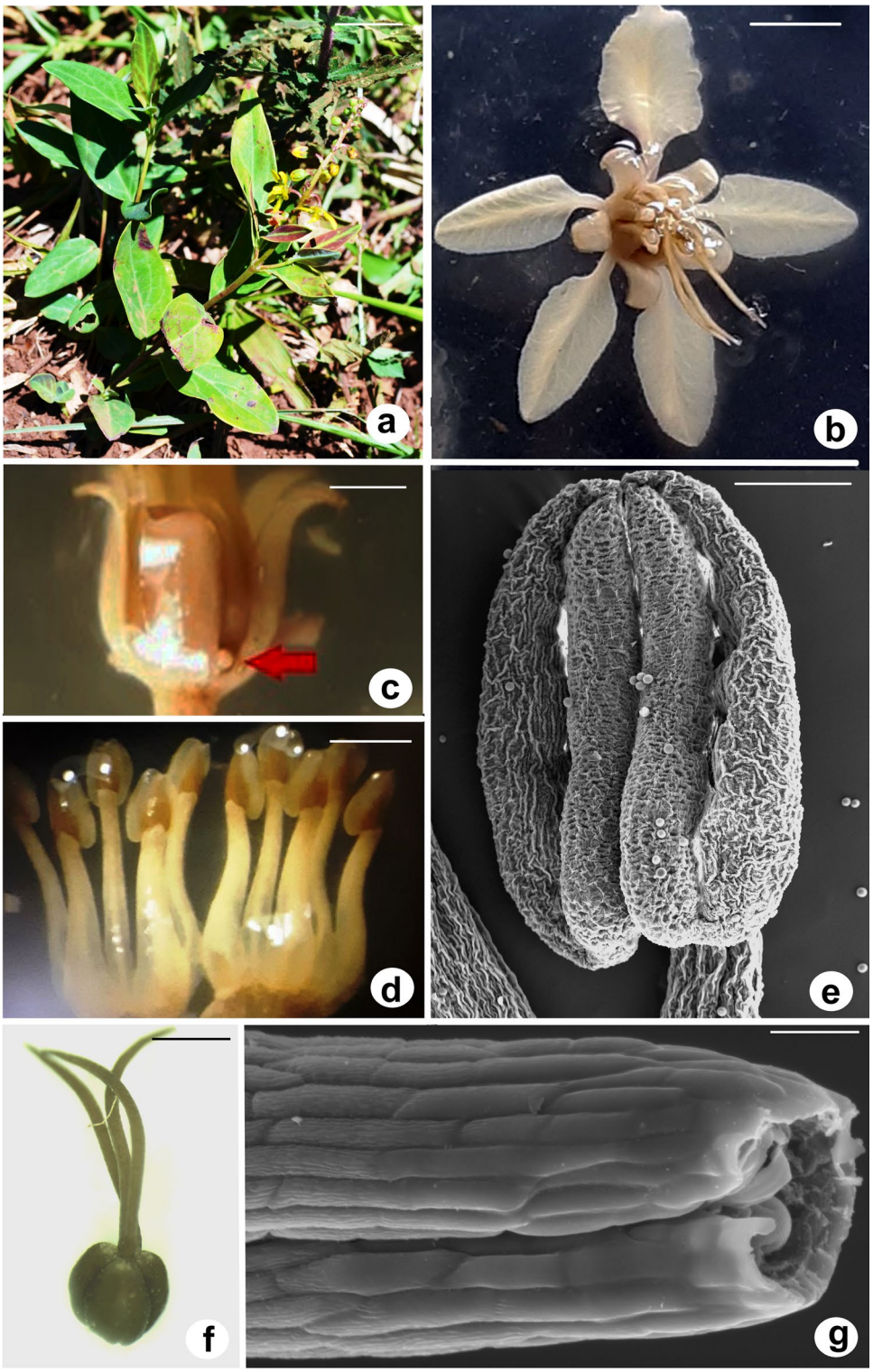

Fig. 1 **a** General aspect of a subshrub of *Galphimia australis*, with narrowly elliptical leaves and a terminal inflorescence. **b**–**g** Stereoscopic microscope. **b** Detail of the slightly zygomorphic flower of *Galphimia australis* with subequal petals, the posterior one with a wavy and finely toothed margin and a larger limb. **c** Detail of the calyx with residual glands (arrow), corolla removed. **d** Picture taken with stereoscopic microscope showing the 2 whorls of stamens. **e** Detail of the stamen with a bitheca introrse anther, longitudinally dehisced photographed with SEM. **f** General aspect with stereoscopic microscope showing a t3-carpellary ovary and three equal free styles. **g** Detail of a style without evident stigma photographed with SEM. Scale bars: **a** 2 cm; **b** 3 mm, **c**, **d**, **f** 1 mm; **e**, **g** 200 µm

has filaments slightly shorter (1.5–1.7 mm) and thinner (0.8–0.9 mm; Table 1). Each stamen has a bitheca anther which is dehiscent in pre-anthesis. The inner whorl of stamens is opposite to the sepals and has smaller anthers. In both whorls, these are oblong, glabrous, longitudinally dehisced, and introrse (Fig. 1d, e). The gynoecium is formed by 3 fused carpels. It has a superior ovary with 3 locules and one ovule each. There are three equal free styles, 4 mm long with terminal stigma without cuticle (Fig. 1f, g), that remain over the androecium.

Table 1 Results of morphological and histochemical analysis in *Galphimia australis*. N/A: not assessed; +: some positive reaction; ++: intense positive reaction; −: no reaction

	Histochemical reactions						Measurements (mm)	
	Lugol	Ferric trichloride	NADI	Sudan IV	Cresil Blue	PAS	Long	Wide
Sepals	NA	NA	NA	NA	NA	NA	1.7–2	0.8–1
Sepal glands	−	+	−	+	+	++	0.1–0.2	0.1–0.2
Lateral Petals	+	+	+	+	+	NA	3–5	1.5–2
Posterior Petal	++	+	++	+	+	NA	5–6	2.5–3
Outer anther whorl	NA	NA	NA	NA	NA	NA	1.5–1.7	0.8–0.9
Inner anther whorl	NA	NA	NA	NA	NA	NA	1.9–2	1–1.2

Flower anatomy

Calyx

In cross section, sepals have similar thickness, although they are slightly wider at the zone of the central and larger vascular bundle giving them a subtriangular aspect (Fig. 2a). Cells of the adaxial epidermis are larger than those of the abaxial one (Fig. 2b). Epidermal cells are filled with dark content and stomata are present mainly in the abaxial face

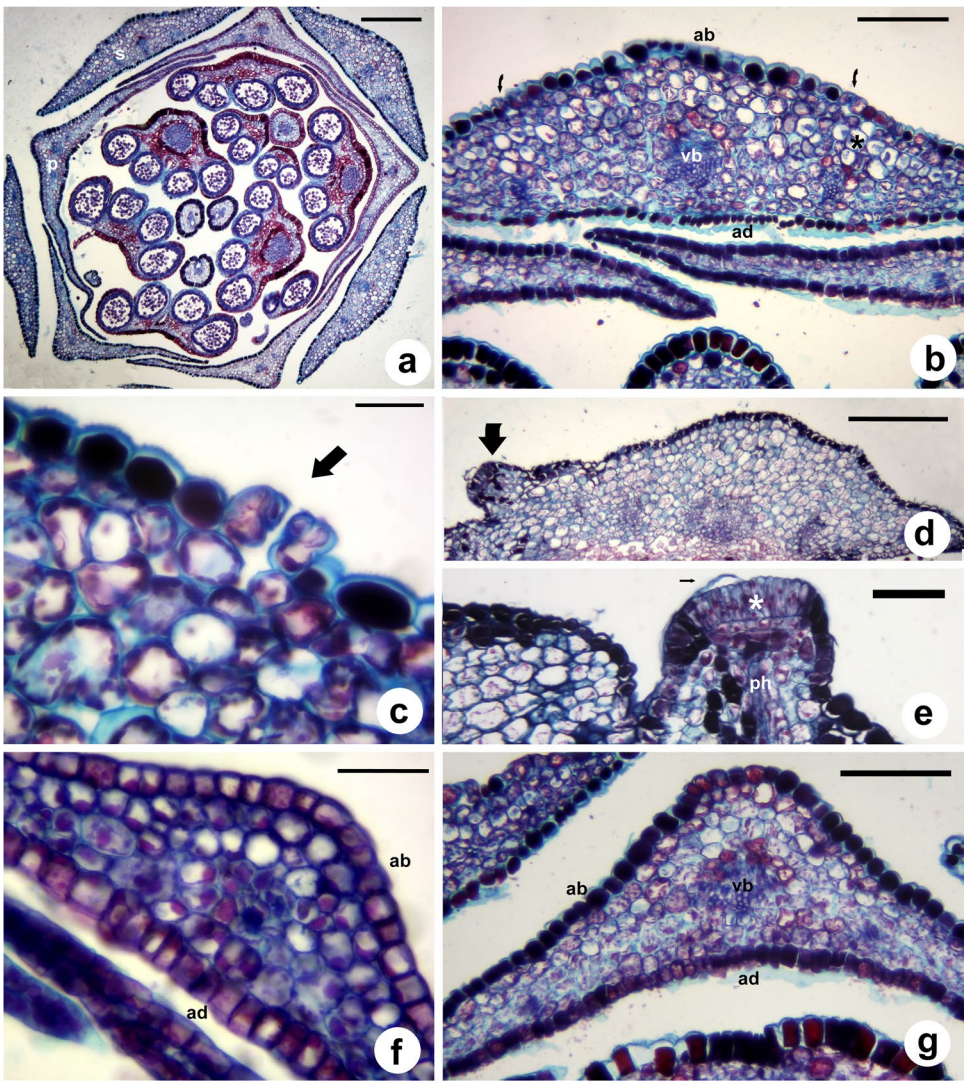

Fig. 2 *Galphimia australis*. Bright field-microscope. **a** Transverse section of a flower showing the sepals (s), petals (p), anthers, and three free styles. **b** Detail of the transverse section of a sepal with a subtriangular aspect, with stomata (arrows) in the abaxial epidermis (ab), vascular bundle (vb), and cell near the abaxial epidermis larger (asterisk) than those next to the adaxial epidermis (ad). **c** Epidermal cells filled with dark content and a stoma (arrow). **d** Gland (arrow) at the base of the calyx. **e** Detail of the gland with a secretory epithelium (asterisk), a subepidermic dense tissue and traces of phloem (ph). **f** Detail of a petal during pre-anthesis with the cytoplasm of epidemic cells restricted to the periphery and no differences between abaxial (ab) and adaxial (ad) mesophyll. **g** Detail of a petal during anthesis with the cells of the abaxial epidermis (ab) and some of the adaxial epidermis (ad) filled with dark content, vb: vascular bundle. Scale bars: **a** 250 µm; **b** 125 µm; **c** 25 µm; **d** 315 µm; **e** 50 µm; **f** 135 µm; **g** 100 µm

(Fig. 2c). In this side, the parenchyma is formed by larger cells (Fig. 2a, b). Mesophyll cells have a large central vacuole, and the cytoplasm is restricted to the periphery (Fig. 2c).

A transverse section of the glands, present near the base of the calyx, in the union zone between sepals, shows an epithelium formed by large longitudinal cells with a dense cytoplasm (Fig. 2d), and a subepidermic dense tissue with traces of phloem (Fig. 2e).

Corolla

Posterior and lateral petals have a large central vascular bundle (Fig. 2a). Traces of other minor vascular bundles are observed in the thinner areas. During pre-anthesis, adaxial and abaxial epidermic cells have a central vacuole and the cytoplasm restricted to the periphery (Fig. 2f). During anthesis, the cells of the adaxial epidermis and some of the abaxial epidermis, fill with dark content. The mesophyll is formed by a dense tissue that is not differentiated into palisade and spongy parenchyma and is restricted to a single layer of cells in the thinner areas (Fig. 2g). Stomata are present in adaxial and abaxial epidermis.

Histochemical analysis of calyx and corolla

Calyx glands

Cells of the epidermis and some parenchymatic ones without any staining are observed filled with brown content (Fig. 3a). These cells reacted positive with Ferric trichloride and Cresil blue suggesting this color is caused by the

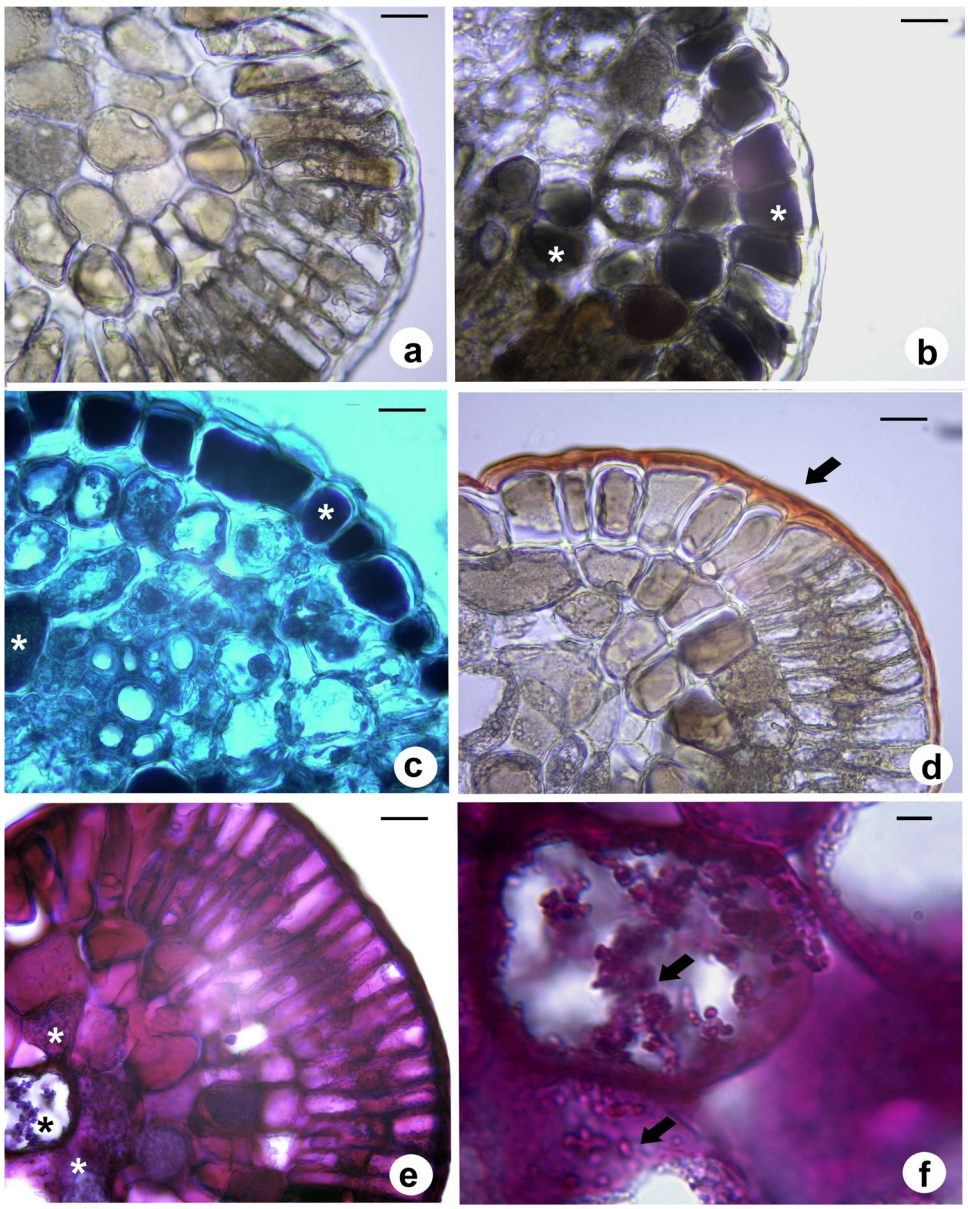

Fig. 3 Histochemical analysis of the calyx glands of *Galphimia australis*. **a** Control, epidermal, and some parenchymatic cells with brown content. **b** Ferric trichloride stained some epidermal and parenchymatic cells (asterisks). **c** Positive reaction with Cresyl Blue in epidermal and few parenchymatic cells (asterisks). **d** Cuticle stained red with Sudan IV (arrow). **e** Positive reaction with PAS in some parenchymatic cells (asterisks). **f** Detail of the positive reaction with PAS, showing vesicles with polysaccharide content (arrows). Scale bars: **a–e** 15 µm; **f** 3 µm

presence of tannins and mucilage respectively (Fig. 3b, c). The cuticle stained red with Sudan IV revealing its lipidic composition (Fig. 3d). Abundant polysaccharides in the cytoplasm of parenchymatic cells were detected with PAS (Fig. 3d, e). There was no staining with Lugol or NADI (Table 1).

Corolla: lateral petals

Cells of the adaxial epidermis without any staining are observed filled with brown content (Fig. 4a, b). These cells positively reacted with Ferric trichloride suggesting this color is caused by the presence of tannins (Fig. 4c). Cresil Blue evinced the existence of mucilage in the epidermic cells (Fig. 4d). Lugol reaction indicated the presence of starch in the cytoplasm of cells of the adaxial and abaxial epidermis and in the mesophyll cells (Fig. 4e). NADI reagent showed some blue areas in the mesophyll and the abaxial epidermis, which suggests the secretion of terpenes (Fig. 4f, f', g). The Sudan IV technique produced a dull red coloration in the cuticle revealing the presence of lipids (Fig. 4h). Results of histochemical tests are summarized in Table 1.

Corolla: posterior ("flag") petal

The epidermal cells of the flag petal that was not stained are observed with brown content, darker in the adaxial

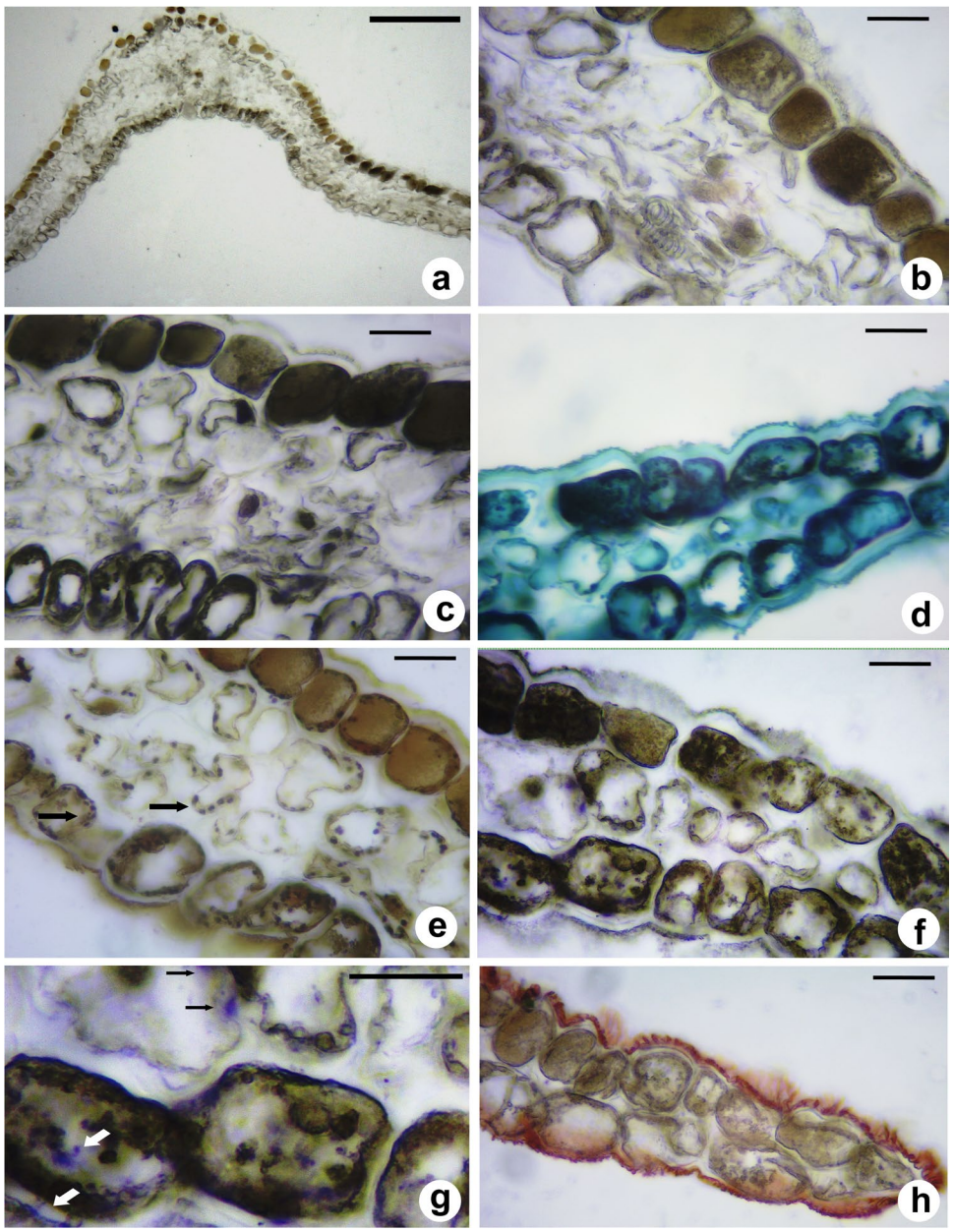

Fig. 4 Histochemical analysis of the corolla—Anterior and lateral petals. Of *Galphimia australis*. **a**, **b** Control, epidermal cells with brown content. **c** Ferric trichloride stained mainly epidermal cells. **d** Positive reaction with Cresyl Blue, mainly in epidermal cells. **e** Positive reaction in cytoplasm of epidermal and mesophyll cells (arrows) with lugol. **f** General aspect of the petal with a positive reaction to NADI. **g** Detail showing the positive reaction with NADI in cells and cuticle (arrows). **h** Only the cuticle stained with Sudan.. Scale bars: **a** 250 µm; **b**–**h** 20 µm

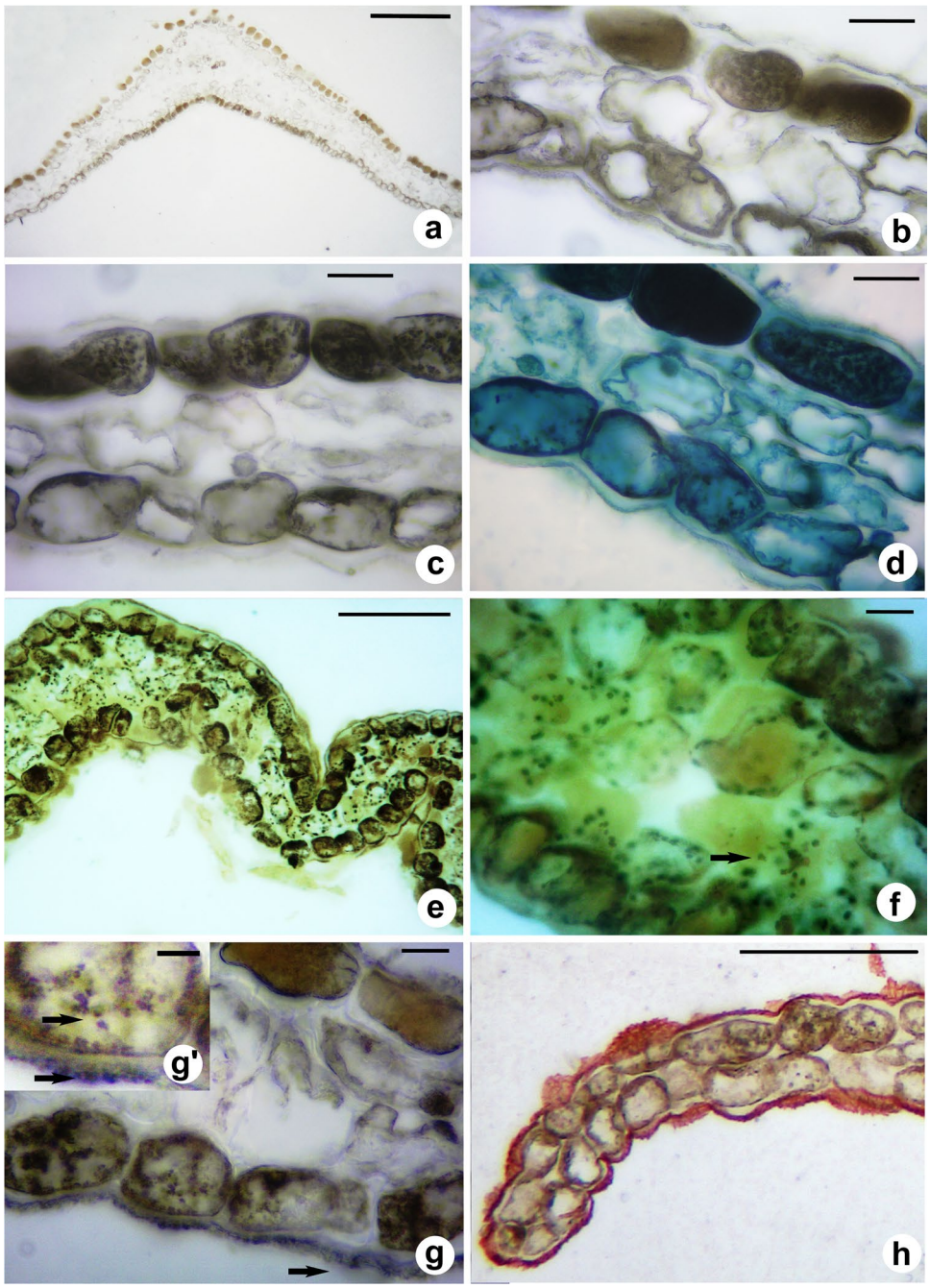

Fig. 5 Histochemical analysis of the corolla; posterior petal of *Galphimia australis*. **a**, **b** Control, epidermal cells with brown content. **c** Ferric trichloride positive reaction in epidermal cells. **d** Positive reaction with Cresyl Blue in epidermal cells. **e** Intense positive reaction with lugol in cytoplasm of epidermal and mesophyll cells. **f** Detail of abundant positive reaction in the cytoplasm of mesophyll cells, suggesting the presence of amyloplasts (arrow). **g** General aspect of a petal stained with NADI arrow showing the positive reaction in the cuticle. **g'** Detail showing the positive reaction with NADI in cells and cuticle (arrows). **h** Only the cuticle stained with Sudan. Scale bars: **a**, **h** 250 µm; **b** 25 µm; **f** 120 µm; **c**–**e** 20 µm, **g** 15 µm, **g'** 5 µm

epidermis (Fig. 5a, b). Presence of tannins in the abaxial epidermal cells was revealed by positive reaction with Ferric trichloride (Fig. 5c) and presence of mucilage in adaxial and abaxial epidermal cells by the positive reaction to Cresil blue (Fig. 5d). According to the reaction with Lugol, it seems that mesophyll and epidermal cells have more starch than the other petals (Fig. 5e, f). NADI reagent evidenced some blue areas in mesophyll cells and the cuticle of the abaxial epidermis (Fig. 5g, g'). The reaction with Sudan IV showed similar results than in the rest of the petals (Fig. 5h). Results of histochemical tests are summarized in Table 1.

Androecium and pollen grain development

There were no anatomical differences between different anthers of both whorls. Anthers were dehiscent with mature pollen grains in all of them before anthesis.

Microspore mother cell stage

At microspore mother cell stage, the anther consists of epidermis, endothecium, two middle layers, a secretory type tapetum, and a sporogenous tissue (Fig. 6a). This tissue is

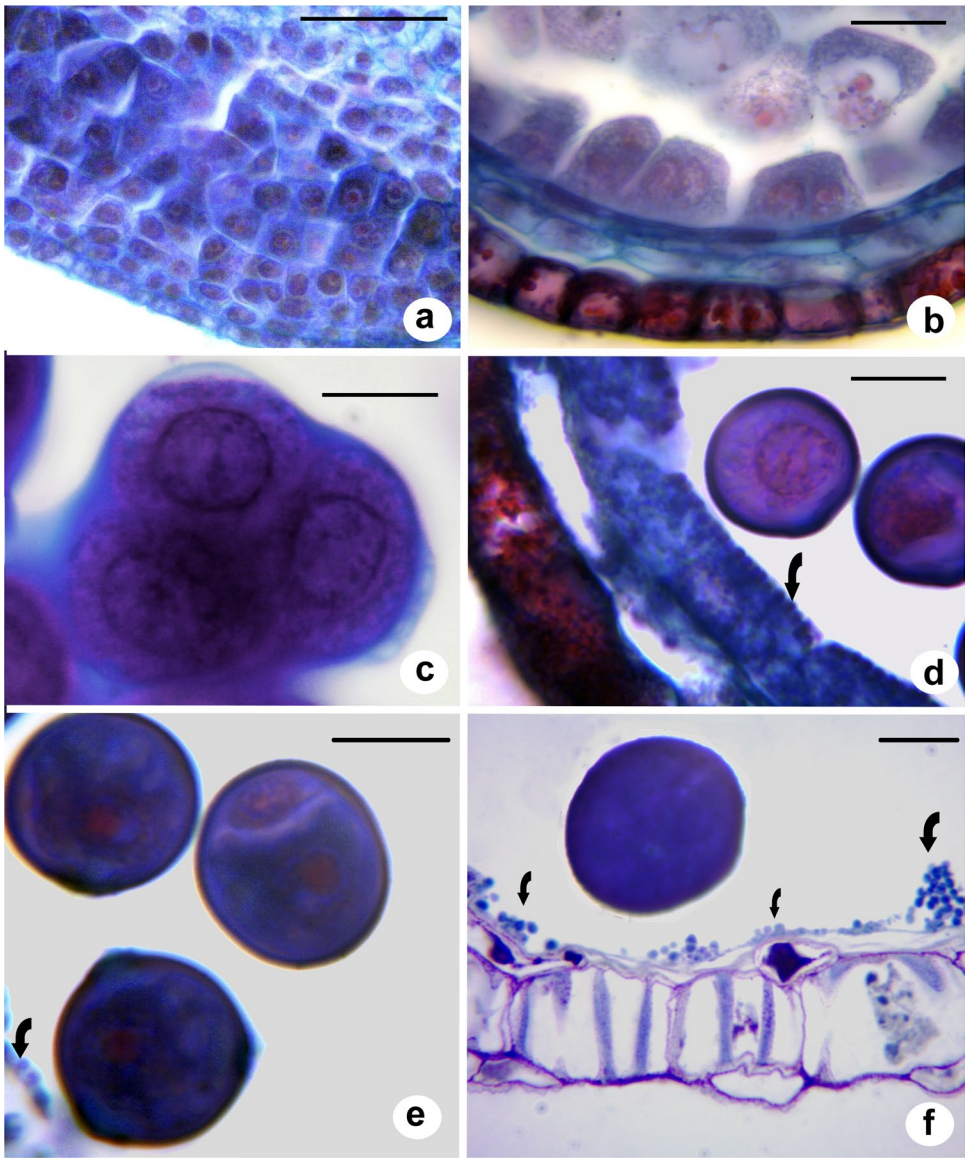

Fig. 6 Androecium and pollen grain development of *Galphimia australis*. Bright field-microscope. **a** Anther with epidermis, endothecium, two middle layers, a secretory type tapetum, and a sporogenous tissue. **b** Microspore mother cells with callose and binucleate tapetal cells. **c** Tetrad with tetrahedral arrangement. **d** Free microspore stage, the anther wall consists with dark red stained epidermis, endothecium and the tapetum with numerous orbicules. **e** Young pollen grain. Tapetal cells start to degenerate and orbicules are clearly observed in their walls. **f** Mature pollen grain. Endothecium with U-shaped pattern and basal anastomosis of fibrillar thickenings, cytoplasm of tapetal cells is no longer observed and the enormous number of orbicules on their walls can be better appreciated. Scale bars: **a** 30 µm; **b** 10 µm; **c**, **d** 5 µm; **e** 8 µm; **f** 9 µm

distinguishable by the isodiametric cells with prominent nuclei and dense cytoplasm and presence of few intercellular spaces (Fig. 6a).

Once microspore mother cells differentiate, the anther wall changes its aspect. The epidermal cells appear larger and stain red, the endothecium and middle layers are more visible, and the tapetal cells enlarge and appear binucleate (Fig. 6b). Microspore mother cell walls become thicker because of the deposition of callose between the plasmalemma and the primary wall. Subsequently, they come apart by the dissolution of the middle lamella and primary walls that keep the sporogenous tissue together (Fig. 6b).

Microspore tetrad stage

The anther wall shows similar characteristics than in the previous stage. Each microspore mother cell undergoes simultaneous reductive divisions and gives rise to microspore tetrads with tetrahedral arrangement (Fig. 6c).

Free microspore stage

In the free microspore stage, the anther wall consists of a dark red stained epidermis, endothecium, and the tapetum that begins to show a contracted cytoplasm in some areas.

Middle layers are no longer observed. Numerous orbicules are seen on the inner tangential and radial walls of the tapetal cells (Fig. 6d). Each individual microspore separates from the tetrad by the dissolution of the callose wall. The free and mature microspores present a conspicuous nucleus in a central position and a slightly vacuolized cytoplasm, surrounded by an exine wall (Fig. 6d).

Young pollen grain stage

In this stage, the cytoplasm of tapetal cells starts to degenerate and orbicules are clearly observed in their walls (Fig. 6e). The fibrillar thickenings of the endothecium start to develop. The generative cell that is formed by a mitotic division of the microspore nucleus can be seen occupying a parietal position. The vegetative cell is larger. Both have central nuclei and a slightly vacuolized cytoplasm (Fig. 6e).

Mature pollen grain stage

During this stage, the cytoplasm of the tapetal cells is no longer observed and an enormous number of orbicules on their walls can be better appreciated. The endothecium presents fibrillar thickenings in its walls with a mainly U-shaped pattern and basal anastomosis (Fig. 6f). The generative cell migrates toward a central position and appears surrounded by the dense cytoplasm of the vegetative cell (Fig. 6f). The pollen grain is released in the bicellular stage.

Morphology of the mature pollen grain and orbicules

The orbicules measure between 1 and 2 µm, and are spherical to subspherical. They can be observed isolated or forming aggregates of 3, 5, or more orbicules (Fig. 7a). The pollen grain is isopolar, isodiametric, spheroidal, with a circular outline in polar view. It is small, between 15 and 25 µm. It

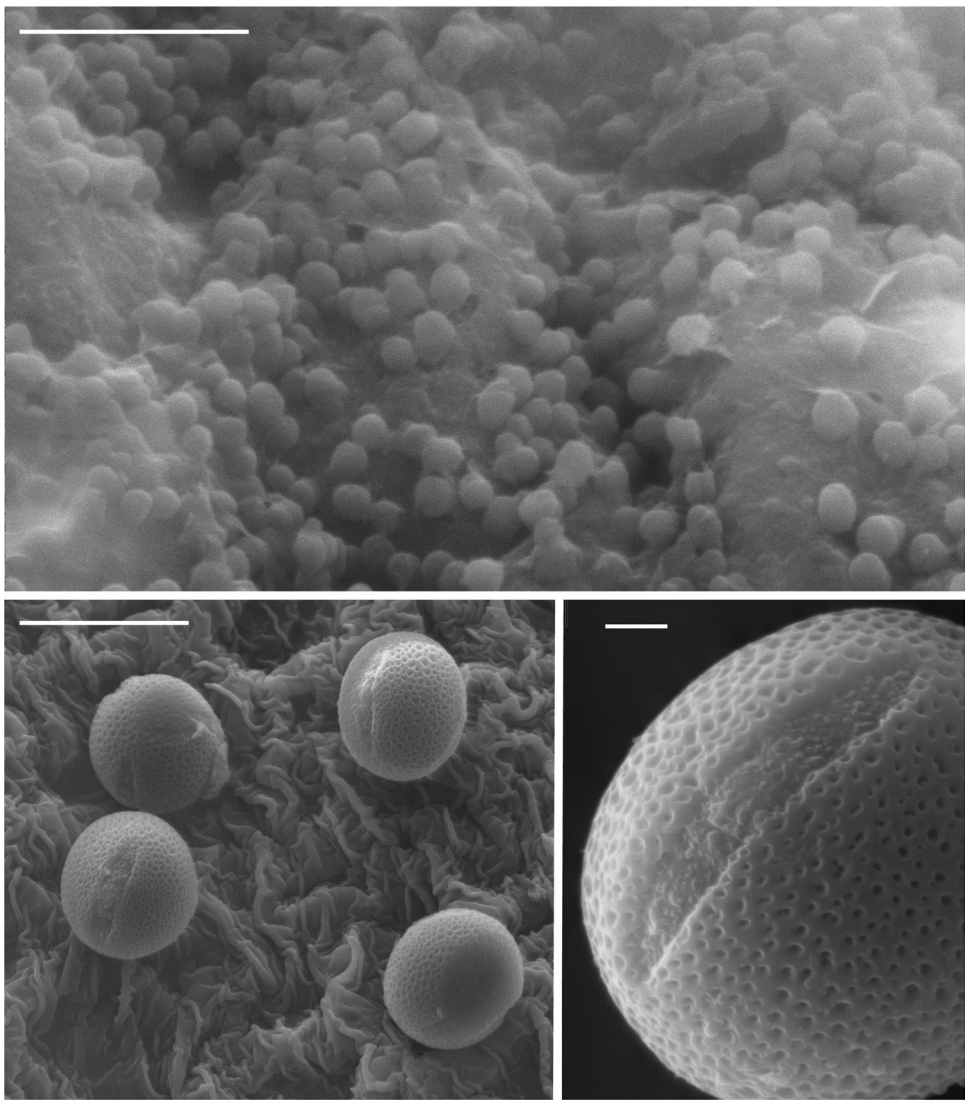

Fig. 7 SEM. *Galphimia australis.* **a** Orbicules. **b** Pollen grains. **c** Detail of a pollen grain. Scale bars: **a** 5000 nm; **b** 20,000 nm; **c** 2000 nm

has three openings in the form of colpores. The exine is reticulate, except at the height of the colpore where it is granular (Fig. 7b, c).

Gynoecium

The stigma is terminal with no cuticle. Each of the three style presents a small invagination facing the center of the flower, which gives the style a horseshoe-shaped aspect in cross section (Fig. 8a). Styles are slender tapered distally to a minute stigma. Style consists of an epidermis, a cortical parenchyma with tannic cells, a central vascular bundle and a small canal surrounded by denser cells, located sub-epidermically in the zone of the invagination (Fig. 8b). Styles get close to each other (Fig. 8c) and fuse between each other first by the sides keeping the invagination of each carpel toward the center (Fig. 8d). Downwards all canals fuse into one, which is surrounded by secretory epithelium. In this way, a compitum that connects the three locules is formed (Fig. 8e). The central canal is no longer observed in cross sections of the upper zone of the ovary (Fig. 8f). Cross sections of the middle of the ovary show three lysigenous canals and carpels

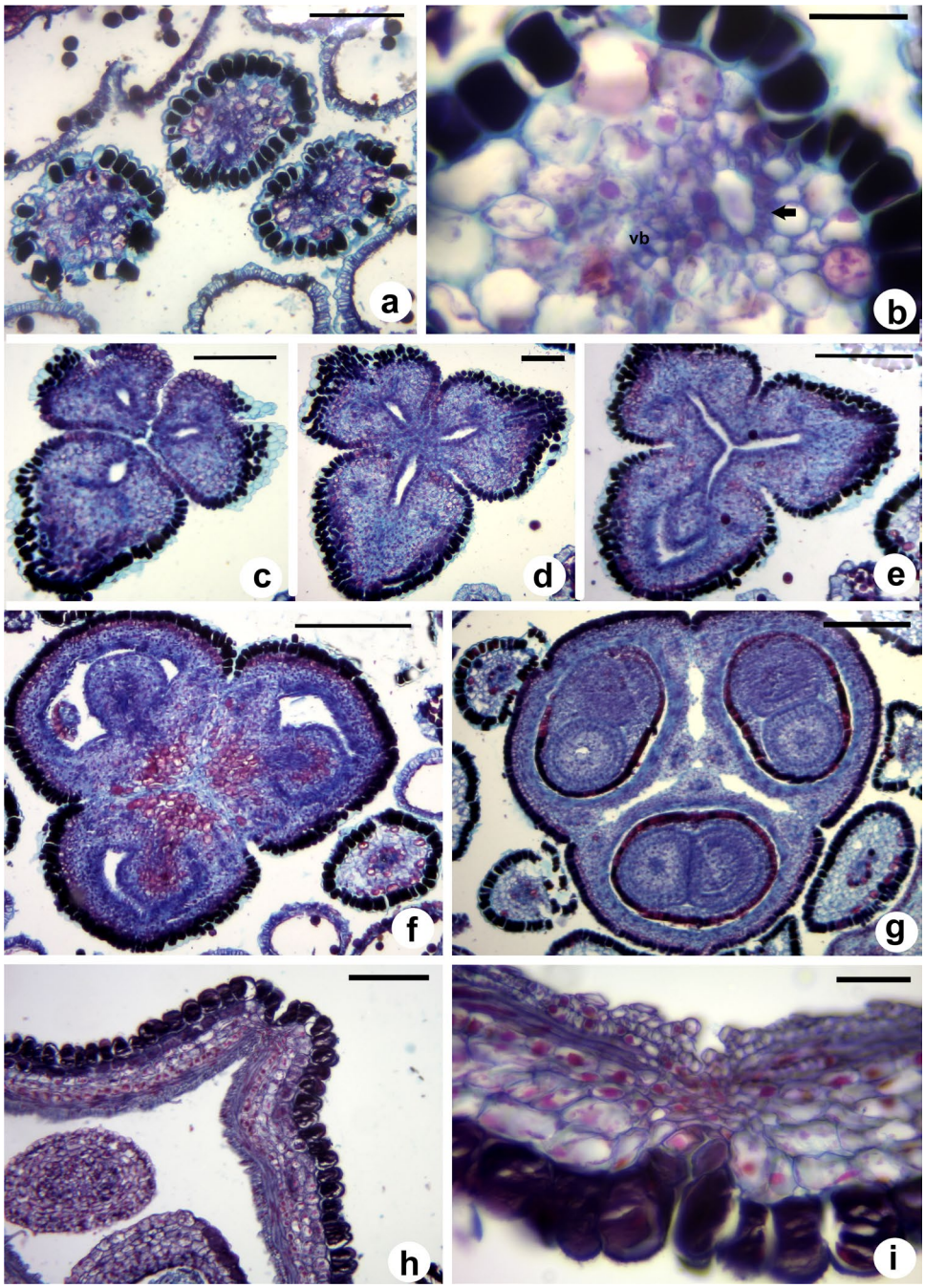

Fig. 8 Bright-field microscope. Cross sections of gynoecium of *Galphimia australis*. **a** Styles with horseshoe-shaped aspect. **b** Detail of a style with small canal surrounded by dense cells. **c** Syles getting closer to each other. **d** Styles fused. **e** Canals fused into one, surrounded by a secretory epithelium and forming a compitum that connects the three locules. **f** The central canal is no longer observed in cross sections of the upper zone of the ovary. **g** Cross sections of the middle of the ovary show three lysigenous canals and carpels remain fused only by their vascular bundles. **h** After anthesis, carpel walls also get constricted at the zone of the middle vascular bundle. **i** Detail of **h**. Scale bars: **a**, **c**: 125 µm; **b**, **i**: 25 µm; **d**: 80 µm; **e**: 200 µm; **f**: 250 µm; **g**: 225 µm, **h:** 100 µm

remain fused only by their vascular bundles (Fig. 8g). After anthesis, carpel walls also get constricted at the zone of the middle vascular bundle (Fig. 8h, i).

Potential pollinators

In two studied populations, we observed and captured only individuals of pollen-collecting bees. In N.P. El Palmar population, we collected individuals of three species of sweat bees (Halictidae: Augochlorini) as floral visitors: three individuals of *Augochlorella ephyra*, two females of *Augochloropsis acis*, and one female of one undetermined species of *Augochloropsis*. For the other hand, in the Colonia Garaví population, we captured two species of small-carpenter bees (Apidae: Ceratinini) and one species of sweat bees as potential pollinators: two females of *Ceratina* (*Neoclavicera*) *asunciana*, one female of one undetermined species of *Ceratina* (*Ceratinula*) and one female of one undetermined species of *Augochloropsis*. All observed visits were carried out by females, and it were very fast (1–3 s), which made it difficult to accurately observe the foraging behavior of these females. However, it was possible to observe that during the pollen collection, these females contacted the fertile whorls suggesting that these species could be efficient pollinators of *Galphimia australis*. The presence of *G. australis* pollen grains on plumose hairs scopal and/or basal metasomal sterna of several of captured females ($n = 6$) corroborates the effective collection of this reward by these bees (one female of *A. ephyra*, one of *A. acis*, and one of *C. asunciana* did not carry pollen on their bodies). In the third population (Provincial Park Profundidad), no visits were recorded during the censuses.

Discussion

The genus *Galphimia* belongs to a proven monophyletic clade (Anderson et al. 2006; Davis et al. 2020) along to other four American genera that commonly present oil glands in all or four sepals, showing a more stereotyped oil-collecting pollination syndrome (e.g., presence of elaiophores, zygomorphic flowers). *Galphimia* seems to be an exception into this linage and our results (e.g., reduction of elaiophores, tendency to actinomorphy) support the hypothesis that *G. australis* flowers have undergone modifications that allowed them to change from the typical floral syndrome of the family. As we mentioned, most neotropical Malpighiaceae species have zygomorphic flowers and oil glands in the abaxial epidermis of sepals (Vogel 1974; Davis and Anderson 2010), associated to the stereotyped oil-collecting behavior of *Centris*, *Epicharis* and *Monoeca* bee females in a "four-legged" pattern of the oil-collecting organs (Neff and Simpson 1981; Torretta et al. 2022). Lobreau-Callen (1989) mentioned that genus *Galphimia* has actinomorphic flowers; however, Anderson (1979) reported that in this and other genera without oil-rewarding glands, there is a tendency toward zygomorphy. Later, Castro et al. (2001) and Anderson (2007) characterized the *Galphimia australis* flowers as bilaterally symmetrical flowers. However, our observations of flowers in the three studied populations show all petals are similar (the posterior petal slightly larger) to each other and regularly distributed, with a somewhat greater separation between the posterior petal and the lateral ones, than among the four remaining petals, suggesting the flower is slightly zygomorphic.

In all three studied population, the number of the calyx glands vary among flowers of the same or distinct inflorescences of the same individuals of *Galphimia australis*, as was already registered in other studies (Castro et al. 2001; Anderson 2007). Most neotropical Malpighiaceae have eight elaiophores, since the anterior sepal lacks these glands (Aliscioni et al. 2022; Anderson 1990; Vogel 1990). However, this number may show variations in the flowers of the same individual, or between individuals in different species (Aliscioni et al. 2022; Gates 1982; Sazima and Sazima, 1989). In our focal species, the glands, when present, are very small, between 0.1 and 0.2 mm width occupying a small area of sepals, compared with the elaiophores of oil-rewarding Malpighiaceae species, which vary between 0.5 mm width in smaller flowers and 1.45 mm width in larger flowers (Aliscioni et al. 2022; Cocucci et al. 1996; Possobom et al. 2015; Vogel 1974). Moreover, in these latter species, in each sepal the elaiophores are present in pairs occupying a great area of the sepals (Aliscioni et al. 2022; Vogel 1974). The anatomy of these glands is like that of other species of Malpighiaceae (Aliscioni et al. 2022; Araújo and Meira 2016; Cocucci et al. 1996; Possobom et al. 2015; Possobom and Machado 2017, 2018; Subramanian et al. 1990; Vogel 1974). The glands of *G. australis* were studied with bright-field, transmission, and scanning electron microscope by Castro et al. (2001). Anatomical traits observed with bright-field microscope were similar to ours: an epidermis with a secretory aspect, parenchymatic cells with dense cytoplasm, and the presence of phloem reaching the subepidermal parenchyma. The presence of phloem is important for the provision of nutrients necessary for secretion (Evert 2006). Castro et al. (2001) ultrastructural analysis showed few lipid bodies and reported the presence of a viscous and translucent exudates composed of lipids and small amounts of polysaccharides. Our results from histochemical reactions showed mainly the presence polysaccharides in the cytoplasm of cells, and lipid content was reduced to the cuticle. Therefore, we consider these glands as residual elaiophores. Possibly, this species (and other species of *Galphimia*) could be in a transitional state toward the definitive loss of these "elaiophores." Our consideration can be validated by the

absence of oil-collecting bees on the flowers of *Galphimia australis*. In all three studied populations, numerous species of oil-collecting bees were observed foraging for these resources in many species of other genera of Malpighiaceae (Avalos et al. 2020; Torretta et al. 2017; 2022), and in other genera of other families of oil-rewarding species (Iridaceae: *Cypella*, *Sisyrinchium*; Plantaginaceae: *Angelonia* Solanaceae: *Nierembergia*) (Torretta and Roig-Alsina 2017, Torretta pers. obs.), but never in flowers of *Galphimia australis*. Flowers of this species received few floral visitors (inclusive, in one of the studied populations, we have not observed any floral visitor) and possibly our sampling was temporally limited, however, observations carried out in other populations and/or other sampling periods of our focal species (Torretta pers. obs.) show similar results.

Other important trait in *Galphimia australis* are its downfacing flowers. Although flowers are yellow, its orientation and size make them little conspicuous, and possibly the odor plays an important role as floral attractive. Anatomical and histochemical analysis of the corolla showed secretory characteristics in all petals during anthesis that suggest whole petal would act as osmophores. These traits include the presence of vascular bundle reaching the entire petal, few intercellular spaces, cells with a dense cytoplasm, and the presence of tannins, mucilage, terpenes, and starch. Tannins are one of the most important secondary metabolites in plant cells (Hassanpour et al. 2011). They are common in secretory cells producing phenolics or phenolics associated to other compounds, such as osmophores (Castro and Demarco 2008). Although the secretory tissue is similar between petals, the posterior one stained more intensively with NADI suggesting a more conspicuous secretion of terpenes (David and Carde 1964; Marinho et al. 2014). Likewise, these petals also presented greater amount of starch. This polysaccharide is a source of energy for intensive metabolic cellular processes and is one characteristic of osmophore cells (Pacek and Stpiczynska 2007; Adachi and Machado 2020). It is known that starch grains accumulate in osmophores since it is related to fragrance production (Stern et al. 1987; Vogel 1990; Curry et al. 1991; Kowalkowska et al. 2015). Therefore, it is probable that in *G. australis*, petal act as osmophores, which seem to be more developed in the posterior petal. This could be a retained trait, which is more distinctive of oil-bee pollinating Malpighiaceae species. As we mentioned earlier, the typical number of elaiophores by flower is eight, and its legitimate pollinator have a four-legged pattern of the oil-collecting organs, accordingly posterior petal secrets more volatile substances. Thus, the pollinator oriented the head to this petal, grasped with its mandibles, and foraged by floral oil with fore- and middle legs (Avalos et al. 2020; Sigrist and Sazima 2004; Vogel 1990). This anatomical variability among petals was also observed in *Diplopterys pubipetala* (A. Juss.) W.R. Anderson & C. Davis, in which the degree of development, including the number, size, and vasculature of the petal glands was different between posterior and lateral petals suggesting the increased production of secretions in the former (Possobom et al. 2015). According to these authors, little is known about the structure of osmophores and the emission of floral scents in Malpighiaceae (Possobom et al. 2015). Other reports of petal osmophore in the family include *Heteropterys chrysophylla* (Lam.) Kunth (Vogel 1974), *Burdachia prismatocarpa* Mart. ex A. Juss., and *Glandonia macrocarpa* Griseb. (Lobreau-Callen 1989). Moreover, some authors mentioned that connective glands (absent in *Galpimia australis*) in some species could play the role as osmophores (Avalos et al. 2020; Possobom et al. 2015).

Stigmas of *Galphimia australis* are terminal with no cuticle. In most studied species of Malpighiaceae, the stigmatic surface is covered by a thick cuticle that helps to limit the self-pollination (Aliscioni et al. 2018; Sigrist and Sazima 2004). The mechanic rupture of this cuticle by legitimate oil-collecting bees during the stereotyped foraging behavior exposes the stigmatic surface, and promotes the secretion of exudates that allow adherence, hydration, and germination of pollen grains (Aliscioni et al. 2018; Possobom et al. 2016; Sigrist and Sazima 2004). In *Galphimia australis* flowers, the absence of cuticle could favor pollination by small pollen-collecting bees, with quick visits, instead of those that collect oil. *Galphimia* belongs to galphimiod clade (Davis and Anderson 2010), along to others four American genera: *Andersoniodoxa* C. Davis & Amorim, *Lophanthera* A. Juss., *Spachea* A. Juss., and *Verrucularia* A. Juss. In all genera, except *Spachea*, the stigmas are minute, and possibly this stigma type is a plesiomorphy for this clade, and for the subfamily Byrsonimoideae (Anderson 1977). In *Spachea*, the stigmas are large and reniform or bilobed (Davis et al. 2020), and the flowers of *Spachea membranacea* Cuatrec. are oil-rewarding and presumably pollinated by *Paratetrapedia calcarata* and *Centris longimani* (Steiner 1985). Also, there are reports of oil-collecting bees in flowers of *Lophanthera lactescens* Ducke (Gaglianone 2003). The species of the other two genera *Andersoniodoxa* and *Verrucularia*, because of the conserved floral morphology (including functional elaiophores), are most probably pollinated by oil-collecting bees but we have not obtained data in the literature.

This is the first report of microsporangium and pollen devolvement in *Galphimia australis.* The anther structure and development were studied in few species of the family Malpighiaceae including *Thryallis glauca* (Cav.) Kuntze (Singh 1959), *Malpighia glabra* L. (Miyashita et al. 1964), *M. coccigera* L. (Siddiqui 1968), and *Stigmaphyllon bonariense* (Hook. &Arn.) C.E. Anderson and *S. jatrophifolium* A. Juss. (Avalos et al. 2020). In *Galphimia australis*, and both *Stigmaphyllon* spp. (Avalos et al. 2020) anther wall development follows the basic type (Davis 1966), and tetrads present a tetrahedral

arrangement. All species of Malpighiaeae studied until now have pollen grains that are shed at the two-celled stage (Singh 1959; Miyashita et al. 1964; Siddiqui 1968; Johri et al. 1992; Avalos et al. 2020). Mature pollen grains of several species of the family were described by Lobreau-Callen (1983) as tricolporate, sometimes syncolporate, or parasyncolporate. He claims that colporate pollens are reticulate or microreticulate. This is in concordance with our observations, since *Galphimia australis* presents tricolporate pollen grains with reticulate exine.

Orbicules were described by Ubisch (1927) as small bodies coating the interior of the anther locule. These vary in size between 0.14 and 20 µm and are resistant to acetolysis (Huysmans et al. 1998; Galati 2003). The function of orbicules is not clear. However, smooth orbicules are usually observed in species actively pollinated by animals while anemophilous and "buzz pollination" species have microechinate orbicules and anther loculus surface coated by pointed structures. This increases the repulsion force of the pollen grains which is an advantage when pollen is released by shaking the anther and not by presentation as in other pollination modes (Galati et al. 2019). Orbicules in *Galphimia australis* are spherical to subspherical, and with no spicules. Therefore, our observations support the theory that claims orbicules are related to pollen dispersal (Galati et al. 2019). Anthers are dehiscent in the bud stage, as occurs in two species of *Stigmaphyllon* (Avalos et al. 2020). As we have not carried out stigmatic receptivity analysis, we cannot ensure the existence of protandry or that the species does not exhibit dichogamy.

In *Galphimia australis*, an incomplete postgenital carpel fusion occurs by the concrescent development of the ventral surface of the epidermal invagination and similarly as in *Tricomaria usillo* Hook. & Am., it forms a compitum (Aliscioni et al. 2019). According to Sokoloff et al. (2017), this is an area where transmitting tracts of each carpel unite allowing the transition of pollen tubes from one carpel to another and is a precondition to the emergence of carpel dimorphism. However, in *Galphimia australis* flowers, there is no evidence of carpel dimorphism nor a tendency toward the specialization of the functions of the carpels for labour division (i.e., some carpels bear fertile ovules and other carpels bear functional stigma, Sokoloff et al. 2017) as in *T. usillo*, which is a species of Malpighiaceae associated to oil-bee pollination (Aliscioni et al 2019).

Conclusions

This is the first report on the complete morphology and anatomy of the flower of *Galphimia australis.* Several floral traits show that this species shifts its pollination syndrome from oil flower system to other with the pollen as only reward: down-facing and slightly zygomorphic flowers, residual elaiophores, high amount of orbicules that could allow a rapid release of pollen from the anthers, and absence of stigmatic cuticle. Our observations in two of the three studied population of *Galphimia australis* confirm bees foraging for pollen as the only flower visitors and potential pollinators.

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Authors' contributions MG, SA and JPT contributed to the study conception and design. Data collection and analysis were performed by JPT. Morphological observations were made by PTK and SA. Flowers were processed for microscope observations and analyzed by SA, PTK and MG. The first draft of the manuscript was written by MG, SA and JPT; all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Declarations

Ethics approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

Conflict of interest The authors declare no conflict of interest.

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