



Nutritious tissue in petals of Annonaceae and its function in pollination by scarab beetles

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ABSTRACT

The feeding of pollinating dynastid-scarab beetles on nutritious tissue of Annonaceae flowers results in macroscopically visible gnawing marks on petals. In the present paper, we present and discuss examples of such gnawing marks on Annonaceae from the Cerrado and the Amazon Forest in Brazil. The localization of gnawing marks on the petals and the histochemistry of the nutritious tissues are emphasized. In some species, nutritious tissue is apparently distributed among all petals, while in other species it is more or less diffusely localized. There are also cases in which nutritious tissue occurs only on clearly localized regions of the inner petals. Petals of selected Amazon species were stained, and studied by light and scanning electron microscopy. The nutritious tissue consists of cells with mucilage-rich walls, which contain starch, lipids and/or tannins. Starch and lipids are not only energy-rich food for the beetles but are apparently also “fuel” for metabolic heating of the flowers, which is a further benefit for the pollinators inside the pollination chamber.

Keywords: cerrado and Amazon forest Annonaceae, electron microscopy, histochemistry, lipids, mucilage cells in petals, starch, tannins

Introduction

The visiting of flowers by beetles usually is a long-lasting procedure. It is not at all a short contact as it is for example by bees, butterflies, birds or bats, which rapidly collect nectar or pollen and leave the flowers afterwards. Conversely, beetles may remain on or in a flower or inflorescence for hours and days. Among basal angiosperms, the dominant specialist pollinator groups are beetles followed by flies. Since beetles and flies are both sedentary pollinators, which often remain for many hours on one flower or inflorescence, pollen transfer to the stigmas is most effective at their arrival and pollen-uptake shortly before they leave. Thus, protogynous dichogamy probably was the answer of basal

angiosperms for a successful cross-pollination by sedentary pollinators like beetles or flies (Gottsberger 2016a).

The beetle flowers of the basal angiosperms have several devices that apparently are adaptations to the foraging of voracious insects; among others, these are thick tepals or petals. The floral organs are not only thick or leathery, but they also curve over the flower center or cover it partly, and thus form a more or less dark interior. Such a “closed” flower, typical for cantharophilous species, provides a so-called pollination chamber for the beetles. “A pollination chamber is able to keep the beetles inside a flower as long as it is necessary to make them effective cross-pollinators. The closed, dark interior of the flower also shields the beetles from light during the day hours, the chamber may be warm and scented, promoting the activities of the beetles, such

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as feeding, digesting and mating, and at the same time it protects the beetles against predatory birds and lizards” (Gottsberger 2016a).

The majority of Annonaceae is pollinated by small beetles (e.g. Nitidulidae, Chrysomelidae, Curculionidae), while other species, some in the same genera, have diversified being pollinated by large beetles (e.g. *Annona*, *Asimina*, *Cymbopetalum*, *Duguetia*, *Fusaea*, *Malmea*, *Porcelia*, *Uvariadendron*). The earliest divergent genus of the Annonaceae, *Anaxagorea* (Scharaschkin & Doyle 2005; 2006), is pollinated by small nitidulid beetles (Webber 1996; Armstrong & Marsh 1997; Teichert *et al.* 2011; Braun & Gottsberger 2011; Gottsberger 2012; 2016b). The family Annonaceae itself seems to have originated in the Cretaceous (Pirie & Doyle 2012) and supposedly was initially associated with small nitidulid beetles. Saunders (2012), in his attempt to reconstruct the ancestral pollination system in the family, identified “small beetles” (but not specifically nitidulids) as pollinators. Small beetles as pollinators apparently have accompanied the evolutionary history of the family. On the other hand, scarab beetles (Scarabaeidae) are apparently late comers in the evolutionary history of beetles and seem not to have developed before the Tertiary (Ratcliffe *et al.* 2005; Krell 2006; Ahrens *et al.* 2014; McKenna *et al.* 2015). It appears probable that as late as during the Tertiary, several Annonaceae species have become adapted to pollination by large scarab beetles (mainly Dynastinae, but also Rutelinae, Cetoniinae, Trichiinae). Thus, there is strong indication that scarab beetle pollination in Annonaceae is a derived and relative modern phenomenon.

Flower morphological characters associated with adaptations to large and voracious scarab beetles are large pollination chambers and concomitantly overall larger flowers than in flowers pollinated by small beetles, as well as extremely thick petal tissue. In several species, thick petal tissue is associated with nutritious tissue at the inner side of the petals. This petal tissue provides the necessary nutrition for the attracted beetles.

The first report about pollinating dynastid scarab beetles gnawing on the surface of the inner side of Annonaceae petals was by Webber (1981). He described the phenomenon for *Annona muricata*, *A. montana* and *A. nitida*. In a short note about the chemistry of nutritious tissues, Gottsberger *et al.* (1998) described the microscopically detectable substances starch, lipids and tannins in nutritious tissue cells with remarkable pectin-rich cell walls. Further studies on scarab beetle-pollinated Annonaceae came from Norman & Clayton 1986, Gottsberger 1989, Schatz 1990, Webber 1996, Momose *et al.* 1998 and Gottsberger *et al.* 2011. From these studies and others, we know that *Cyclocephala* and other flower-visiting Cyclocephalini species have a strong link to the flowers they visit. The adult beetles seem to feed exclusively on tissue and pollen of the flowers that attract them, also because the scent of these flowers not only attracts but stimulates the beetles to settle and to

initiate mating (e.g. Gottsberger & Silberbauer-Gottsberger 1991). For those beetles, the flower is not only the place for nourishment but also a rendezvous and mating place and thus essential for their proper reproduction. Scarab flower beetles are thus not generalist feeders, but very specific pollinators, not only of flowers of Annonaceae, but also of Nymphaeaceae, Magnoliaceae, Araceae or Cyclanthaceae species.

The present paper shows the variability of nutritious tissues in different genera and species of Annonaceae. Especially, it is emphasized which chemicals are involved in the so-called “nutritious tissues” and how far these chemicals serve as food for the beetles.

Materials and methods

Previous results of our studies of scarab beetle-pollinated Annonaceae indicated that nutritious tissues in Annonaceae flowers may have different topographic extensions on the petals (examples shown in the present publication for the cerrado species *Annona cornifolia* St. Hil., *A. coriacea* Mart., *A. aurantiaca* Barb. Rodr. and *A. crassiflora* Mart.; for pollination of these species see Gottsberger 1989). Also the depth of these special tissues is variable, viz., it can be epidermal or parenchymal but mostly it is both. The nutritious tissues may be without any topographical differentiation or may form distinct elevations, being among others papillate or warty.

A detailed anatomical and histochemical approach was used to show that deposited chemicals in the cells of different species have a certain variation and an unequal concentration. Flowers of five rainforest Annonaceae (*Annona montana* Macfad., *Cymbopetalum euneurum* N.A. Murray, *Duguetia riparia* Huber, *D. ulei* (Diels) R.E. Fries, and *Malmea manausensis* Maas & J.M.S. Miralha), all being pollinated by scarab beetles (see Webber 1981; 1996), were collected in the lowland forests of the Reserva Adolpho Ducke, close to Manaus (2°55' - 3°08'S 59°57'W, altitude 70 m). The flowers were preserved in ethanol with glycerine (70 %). The fixed material was prepared for investigation with the light microscope, either by cutting by hand or with a microtome. For electron microscopy the stored flowers were dehydrated through an ethanol series. After dehydration, the samples were embedded in paraffin wax and cut (7 µm) with a Leitz microtome. After dehydration, the samples were critical point dried and sputter-coated with gold (Balzer Union, Liechtenstein). Detailed observations occurred using a scanning electron microscope (Zeiss DSM 240, Germany).

The histochemical study sought to reveal the presence in ± concentrated form of four groups of chemicals in the nutritious tissue area: Starch, staining dark blue with Lugol's Solution or showing the characteristic cross under polarized light, Lipids, staining dark blue with Sudan Black or bright red with Red Oil, Tannins (including polyphenols) in a compact polymerized form; if not stained, they show their



typical brownish, red, or yellowish color, Polysaccharide Mucilage (Pectin) cells, the cell wall staining blue with Alcian Blue.

Results

Annona cornifolia has relatively small flowers (about 2 cm diam.) as compared with the other, mostly larger-flowered cerrado *Annona* species pollinated by scarab beetles. Its main pollinator was found to be *Cyclocephala quatuordecimpunctata* (Scarabaeidae: Dynastinae), besides another much less common species, *C. atricapilla*, in a relation of about 10:1. The nocturnal beetles approached the semi-open flowers and started to eat from the inner sides of the external and internal three petals (Fig. 1A, B). When a larger number of beetles was visiting a flower, they ate on all petals of a flower and finally destroyed them (Fig. 1C), indicating that the entire corolla, viz. the outer and inner petals as a whole, consist of nutritious tissues appreciated by the

pollinators. The large-flowered (about 5 cm diam.) *Annona coriacea* was visited by *Cyclocephala atricapilla* (Fig. 1D), its main pollinator. The diffuse two gnawing marks at the base of the inner sides of the inner petals indicated a nutritious tissue, apparently preferred by beetles to the rest of the tissue. In the previously mentioned *A. cornifolia*, even if only one beetle was present in a flower, it started to gnaw on the entire inner side of petals, apparently not distinguishing between well tasting nutritious and normal petal tissues. On the other hand, in *A. coriacea* the entire petals were only eaten, if a larger number of beetles was inside the pollination chamber. In the two species *Annona crassiflora* and *A. aurantiaca* (both species with flowers of about 4 cm diam.), the nutritious tissues were exactly delimited pairwise at the basal part of the three inner petals (Fig. 2A, B). In flowers of *A. aurantiaca*, even if there were up to ten beetles, the gnawing marks were the only signs of the beetles' voracity; this was strong indication that beetles probably gnawed exclusively at that special spots.



Figure 1. *Annona* species, their pollinating *Cyclocephala* beetles and gnawing marks. **A.** Nodding, semi-open flower of *A. cornifolia* (ca. 2 cm diam.) with several individuals of *C. quatuordecimpunctata* and *C. atricapilla* inside. **B.** Two individuals of *C. quatuordecimpunctata* inside a flower of *A. cornifolia*, gnawing on the inner side of petals. Note that all six petals, the outer and inner ones have extensive gnawing marks. **C.** *A. cornifolia* with six individuals of *C. quatuordecimpunctata* and one individual of *C. atricapilla* gnawing on petal tissue. Note, that the tissue of all six petals is in large part destroyed by the beetles. **D.** A flower of *A. coriacea* (ca. 7 cm diam) in the pistillate stage (with brilliant stigmas) held open to show one individual of the pollinating beetle, *C. atricapilla* (ca. 1.7 cm length), in the interior. Note the brown spots at the base of the three inner petals, where the beetle has been feeding on the specialized nutritious tissue. The roundish holes in one external petal are the result of predatory, non-pollinating *Conotrachelus* (Curculionidae) beetles.

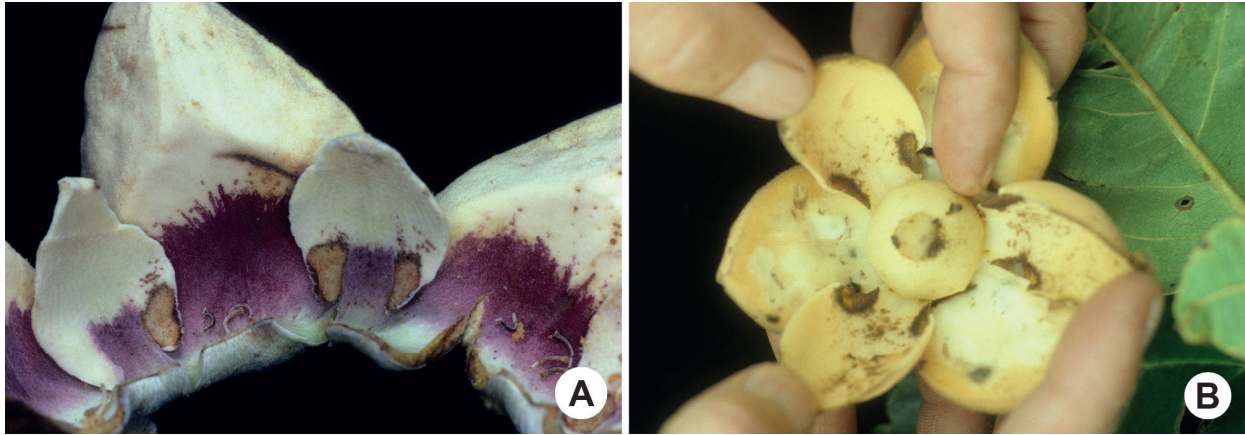


Figure 2. *Annona* flowers with strictly localized gnawing marks. **A.** A dropped sympetalous flower of *A. crassiflora* showing one or two localized gnawing marks at the lateral inner sides of inner petals only. The larger and much thicker outer petals apparently do not have nutritious tissues (length of outer petal ca. 3 cm). **B.** *A. aurantiaca* (ca. 6 cm diam.) also has localized gnawing marks at the base of the smaller inner petals only, done by the pollinator *C. atricapilla*.

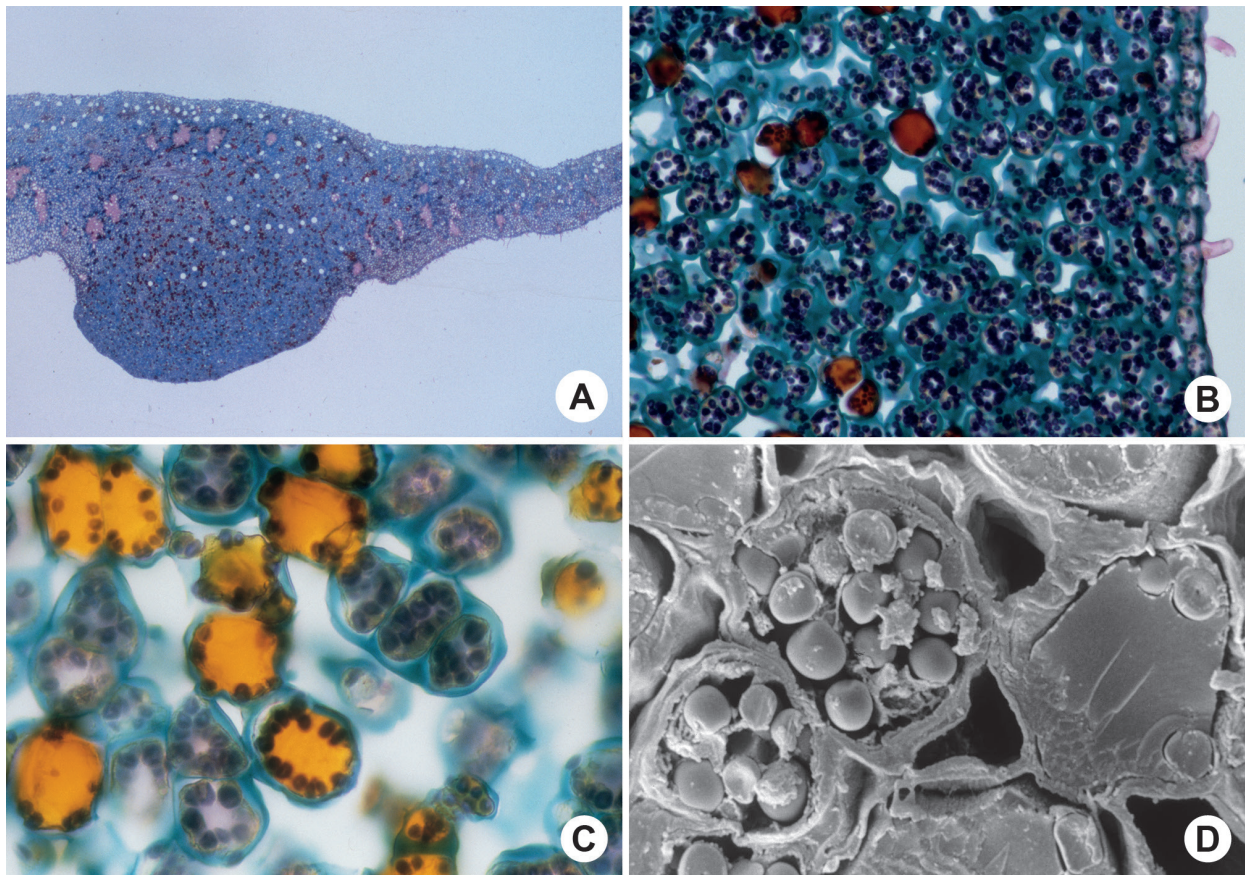


Figure 3. Anatomy and histochemistry of *Annona montana* petals. **A.** Inner petals have two wart-like structures at the basal lateral side of the inner petals. The cut shows one of the wart-like structures, which has a more intense blue than the surrounding tissue, indicating cell walls with a high content of mucilage (magnification 4:1). **B-C.** Closer views show the blue thick mucilage cell walls. Many cells contain dark-blue stained starch granules and a few number of cells are filled with orange-colored tannins mixed with starch granules (magn. of B is 80:1 and of C is 160:1). **D.** A scanning electron microscope picture shows the thick cell walls of polysaccharide mucilage (pectin), cells filled with roundish starch granules and further cells filled with tannins and a few starch granules (magn. ca. 400:1). The pollinating beetle of *A. montana* is *C. undata*.



In *Annona montana*, the nutritious tissue was localized at the base of the internal petals in two wart-like structures (Fig. 3A). These structures consisted of cells with tannins (brown) and starch (dark blue); most cells contained only starch. The cell walls were full of mucilage (Fig. 3B-D). The two studied species of *Duguetia*, *D. riparia* and *D. ulei* also had structured tissues, but on the internal and external petals, in form of papillae in the former (Fig. 4A, B) and in a corrugated way in the latter species (Fig. 4C, D). The histochemical study revealed mainly tannins and starch as content of *D. riparia* (Fig. 4B) and mainly lipids (and starch) in *D. ulei* (Fig. 4C, D). The visiting beetle species, *Cyclocephala undata*, ate only the special nutritional tissues but not the other parts of the petals. *Cymbopetalum euneurum* had a thickening at the margin of the inner petals, which were nutritious tissues (Fig. 5A). The cells contained starch and probably lipids (Fig. 5B). In *C. euneurum*, as also in *A. montana*, there was only indirect proof of the presence of lipids, as was inferred from central holes in cells; these holes appeared to be artifacts by preparation and might have been filled with lipids in the living cell. In *Malmea manausensis* there was no topographical differentiation of the tissue but only a different coloration at the internal side of the internal petals (Fig. 5C, D). In *A. montana*,

C. euneurum and in *M. manausensis*, the pollinating beetles are also *C. undata*, which were seen to eat exactly only from the nutritious tissues without destroying the rest of the petal tissue (compare Fig. 5D with Fig. 5E and 5F). The analysis of the petals of *M. manausensis* before and after the visit of *C. undata* showed that the beetles distinguished very well between the nutritious and the “normal” tissue of a petal.

Discussion

Nutritious tissues on tepals, petals or other floral organs are not limited to flowers of Annonaceae pollinated by scarab beetles, but occur also in other families. For example, *Victoria* (Nymphaeaceae) species have starch-containing carpellary appendages, eaten by the large beetles (Prance & Arias 1975). In *Philodendron* (Araceae) species, scarabs not only nourish themselves on the large amounts of pollen but also on entire nutritious sterile and fertile staminate flowers (Gottsberger *et al.* 2013). In *Magnolia ovata* (Magnoliaceae), the visiting scarabs start eating at the nutritious tissue at the base of the inner petals and after consumption of these regions extend their gnawing to the whole petals (Gottsberger *et al.*

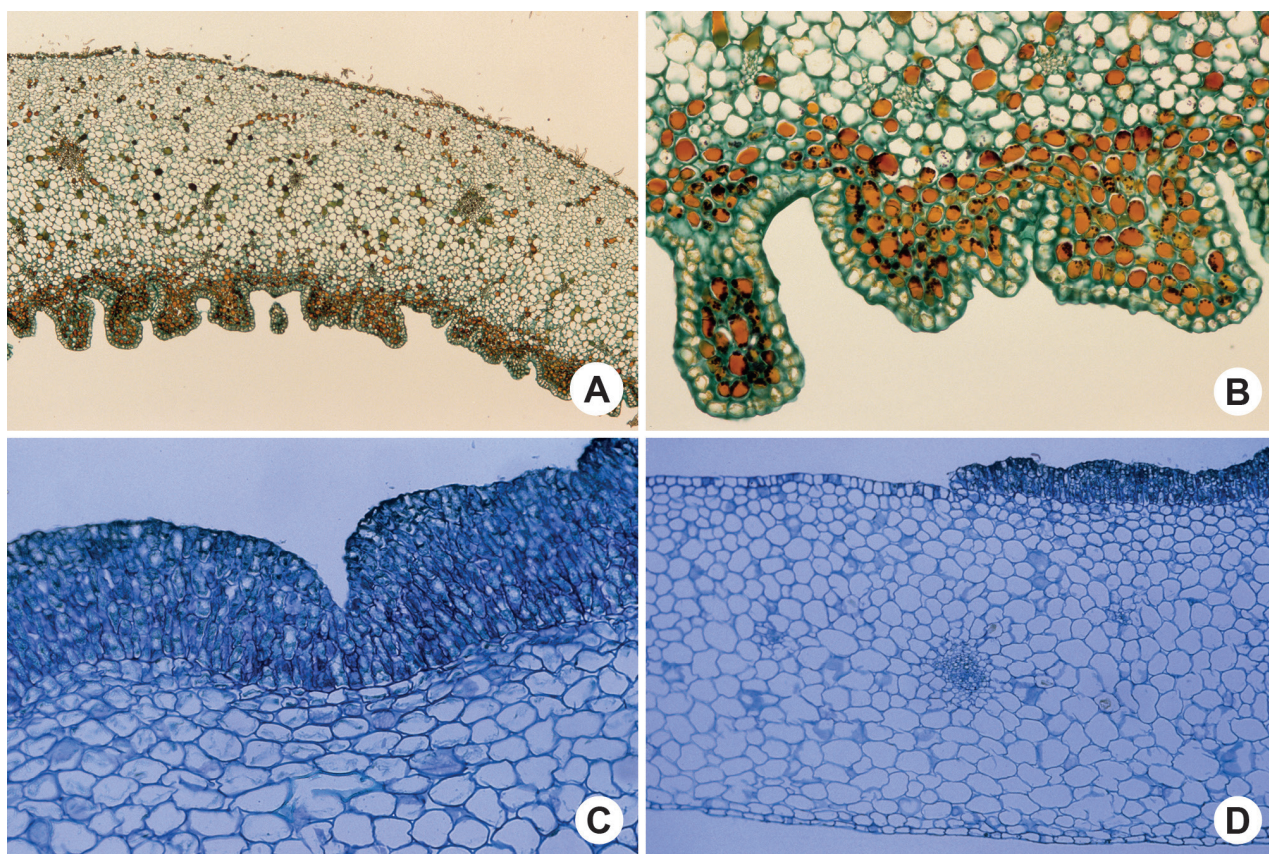


Figure 4. A-B. *Duguetia riparia*. Papillate structured inner sides of petals with nutritious tissue. Orange-colored tannin cells, with some of them containing starch granules. The outer cells of the papillae have mucilage-rich cell walls (magn. of A is 10:1 and B is 40:1). **C-D.** *Duguetia ulei*. Corrugated inner sides of petals with nutritious tissue, containing mainly dark blue-colored lipids and a low quantity of starch (magnif. of C is 40:1 and of D is ca. 30:1). The pollinating beetles of both *Duguetia* species are *C. undata*.



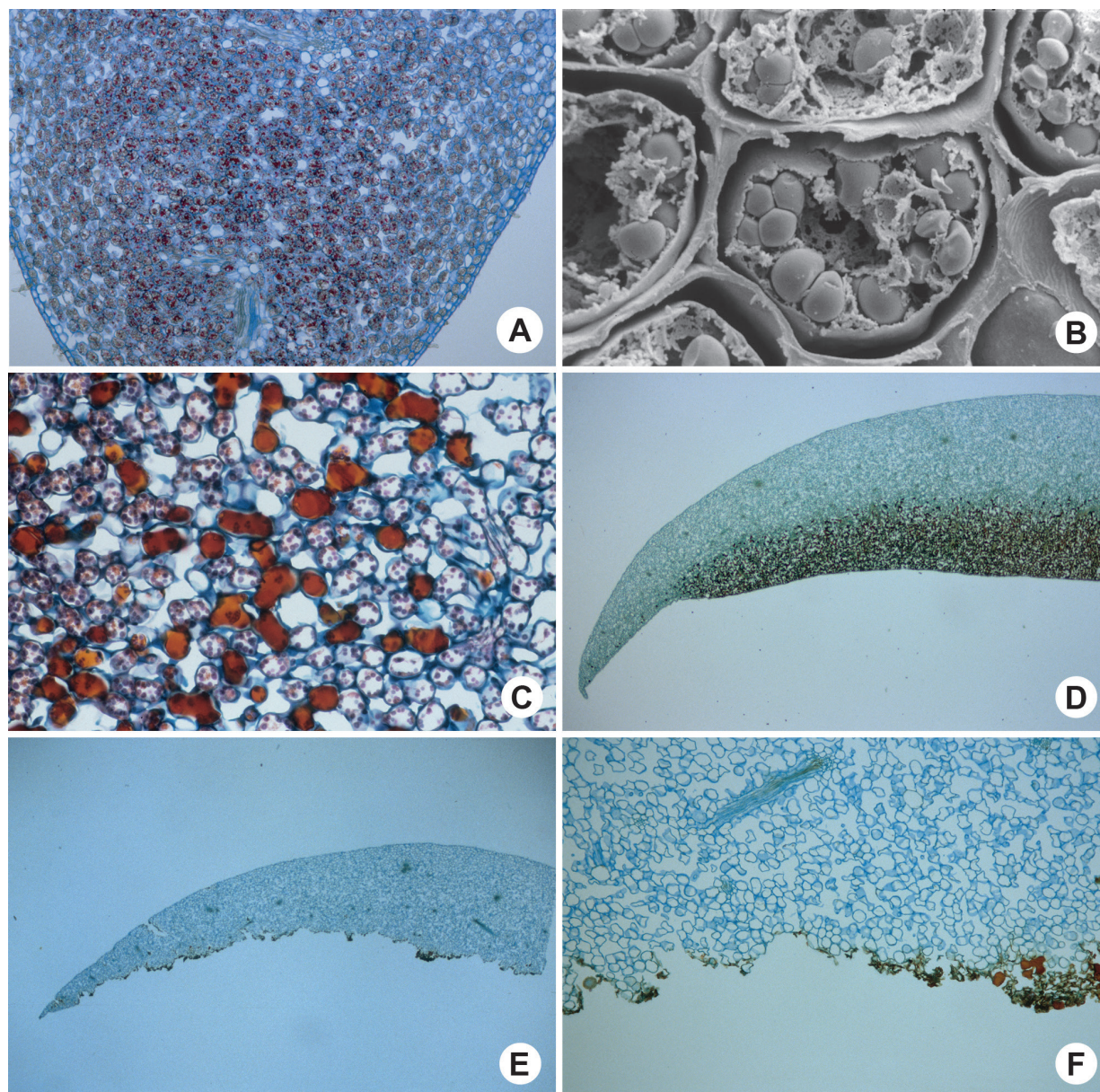


Figure 5. **A.** *Cymbopetalum euneurum*. Nutritious tissue at the basal margin of the inner petals, with cells containing starch and probably lipids (magn. 20:1). **B.** *C. euneurum*. A scanning electron microscope picture shows cells with starch granules and holes in the center of cells; the holes appear to be artifacts by preparation, which perhaps have been full with lipids in the living cell (magn. ca. 400:1). **C-F.** *Malmea manausensis*. **C.** Nutritious tissue with cells containing starch, tannins or both (magn. 58:1). **D.** Nutritious tissue at the margin of an internal petal indicated by a darker coloration (magn. 4:1). Situation before the presence of the pollinating beetle, *C. undata*. **E.** A petal after the presence of pollinators. The nutritious tissue entirely eaten, with only the non-nutritious part of the petal left (magn. 4:1). **F.** Close-up of gnawed petal. Remains of the nutritious part of the petal left by the pollinators (magn. 20:1).

2012). *Cyclanthus bipartitus* (Cyclanthaceae) plants have on the adaxial surface of the inner bracts of an inflorescence a 2-3 mm thick layer of homogeneous, mealy tissue (almost 50 % lipids by dry weight), on which pollinating scarabs forage (Beach 1982).

In the Neotropics, the flower-visiting and petal-gnawing scarab beetles belong mainly to the Dynastinae, and to a great percentage to the genus *Cyclocephala*. On the other hand, the flower-visiting scarabs of the North American

annonaceous genus *Asimina* are members of the subfamilies Trichiinae and Cetoniinae (Norman & Clayton 1986); the nutritious tissues of *Asimina obovata* contain 50 % carbohydrates and 8 % lipids. The pollinating species of the African genus *Uvariadendron* were scarabs of the subfamilies Trichiinae and Rutelinae (Gottsberger *et al.* 2011).

Not only scarab beetle-pollinated species but also flowers of species pollinated by small-sized beetles can have nutritious floral tissues. The Annonaceae *Sapranthus*

palanga, pollinated by small beetles, has nutritious tissues, which contain starch and lipids (Olesen 1992). Thien *et al.* (1990) found densely packed polysaccharide granules at the inner surface of several species of *Zygogynum* (Winteraceae) pollinated by weevils (*Palontus* spp., Curculionidae), and interpreted them as “food-bodies”, which function as pollinator rewards. Pollinating *Elleschodes* (Curculionidae) species feed on staminodes of *Eupomatia* (Eupomatiaceae) flowers (Hamilton 1897; Diels 1916; Hotchkiss 1958). A very sophisticated case of food-providing to small beetles occurs in the genus *Calycanthus* (Calycanthaceae). In the North American species *C. occidentalis* and *C. floridus*, the innermost tepals, the stamens and inner staminodes have whitish food bodies on their tips, which contain high levels of protein and which are eaten by *Colopterus* and *Carpophilus* (Nitidulidae) species during their visits to the flowers (Grant 1950; Rickson 1979).

Schatz (1987) classified the nutritious tissues in Annonaceae and mentioned several categories: “...petal specialization in response to scarab visitors has evolved in two directions.” For him either the entire fleshy petal serves as food reward without further modification, or only a part of the petal is modified and functions as a specialized “food area”. The genus *Sapranthus* to Schatz illustrates a different type of petal specialization for pollination, to which he referred to as “food body”. “A ‘food body’ is distinguishable from a ‘food area’ by virtue of additional structural (=topographical) modification, entailing the elevation of the region above the surface of the rest of the petal, and/or a change in the texture of the surface of the region from that of the rest of the petal.” However, based on our experience, there appears to exist a continuum between food areas and food bodies, such that the term nutritious tissue or food tissue probably would suffice.

When beetles gnaw on petals and take up nutritious tissues, they ingest cells containing all three or four substances consisting of starch, lipids, tannins and mucilage. It is remarkable that beetles appreciate tannins, because tannins are deterrent substances, largely used by plants as protection against herbivory. How the scarab beetles have overcome this anti-feeding barrier, even appreciating tannins, apparently is unknown.

Starch and lipids in the nutritious tissues of Annonaceae, besides providing food for the attracted beetles, have another great importance for the pollination process. A considerable number of Annonaceae has the ability to raise flower temperature above ambient temperature through metabolic heat production, resulting in a warm floral chamber. This is an aid in strongly volatilizing the floral scent compounds for attracting the pollinators. The warming of flowers also helps beetles, which are facultative endotherms and need a great deal of energy for reducing thermoregulatory costs, to increase their activity levels in mating, locomotion, digestion and growth (Seymour & Schultze-Motel 1997; Seymour *et al.* 2003; 2009; McCallum *et al.* 2013). Starch and lipids are

the known “fuel” in plants for metabolic heating of flowers and inflorescences. During anthesis, the content of these substances in the cells, therefore, will diminish or almost completely used up (Vogel 1963).

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