Nectary Structure in Dichogamous Flowers of *Polemonium caeruleum* L. (Polemoniaceae)

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The flowers of *Polemonium caeruleum* are protandrous. The nectary is in the form of a rim encircling the ovary. Secreted nectar accumulates in a chamber located at the bottom of the floral tube and is protected by dense staminal hairs. The nectary tissue is not vascularized, but is supplied by vascular strands that occur near the base of the nectary and which directly supply the stamens. Nectar is secreted via modified stomata located on the upper part of the rim, particularly on the adaxial surface. The number of stomata and the volume and sugar concentration of nectar are greater during the female stage than during the male stage. In both stages, however, the nectar is sucrose-dominant. This paper shows that in *P. caeruleum* the nectar sugars are not a direct product of current photosynthesis, since plastids of nectary cells are devoid of chlorophyll. The main source of sugars in secreted nectar is the phloem sap, together with starch that accumulates in the nectary cells during the male stage and is then rapidly hydrolyzed during the female stage.

Key words: Nectar secretion, histochemistry, protandry, Polemoniaceae, red-listed plant.

INTRODUCTION

In *Polemonium caeruleum*, as in many other plants, floral nectar is the main floral food reward, containing a large number of constituents such as sugars, amino acids and many other secondary components that meet the energetic and dietary demands of pollinators (Nicolson and Thornburg, 2007). According to many authors, nectar composition varies in accordance with the type of pollinator, the environment and presentation type (Baker and Baker, 1982, 1983; Pacini and Nepi, 2007; Nepi et al., 2012). For example, high-sucrose nectars are found in flowers pollinated by bees, butterflies, moths and hummingbirds, whereas high-hexose nectars are produced by flowers pollinated by small unspecialized insects, passerine birds and bats. Some researchers, however, have suggested that it is phylogeny which determines nectar composition (Galetto and Bernardello, 2003; Chalcoff et al., 2006; Nicolson and Thornburg, 2007), whereas others have shown that nectar composition is relatively labile and can be influenced by abiotic factors such as habitat, temperature, humidity, solar radiation,

and CO_2 level (Davis, 2003; Nicolson and Thornburg, 2007; Nepi et al., 2012), together with biotic factors such as the presence of yeasts (Herrera et al., 2009). Moreover, nectar composition may be influenced by plant hormones (Nepi et al., 2012; Escalante-Perez and Heil, 2012) and also, at the intraspecific level, it can vary in accordance with the age of the flower, its position on the plant and on the inflorescence, as well as its sexual stage (Pacini and Nepi, 2007; Nepi et al., 2012; Nocentini et al., 2012). In some species, nectar sugars and nectar volume diminish, often as a consequence of resorption by floral organs. Resorption is generally recognized as a resource recovery strategy which allows at least some materials invested in nectar production to be recycled, but nectar resorption can occur concomitantly with nectar secretion. Nectar resorption can be selective, and is thus a homeostatic mechanism, all nectar constituents being resorbed relatively equally. The nectar homeostatic mechanism regulates the volume, concentration and thus viscosity of nectar by reducing the effect of water loss due to evaporation (Nepi, 2007; Nepi and Stpiczyńska, 2007, 2008; Stpiczyńska et al., 2012).

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In dichogamous plants, nectar constituents may change concomitantly as the flower passes from one sexual stage to the next. In protandrous *Carum carvi*, nectaries in female-stage florets secrete significantly more nectar sugar than male-stage florets, that of the male stage being hexose-rich, whereas nectar during the female stage is hexose-dominant (Langenberger and Davis, 2002). Also, the available nectar can be distributed unequally between male and female phases. Of the 41 dichogamous species Carlson and Harms (2006) studied, representing 18 families and 22 genera, the rate of nectar production was greater during the male phase in 21 species and during the female phase in 20 species. Thus the nectar production rate was unbiased. According to Zych et al. (2012), the flowers of *Polemonium caeruleum* are protandrous, with the period of pollen presentation being shorter than stigma receptivity. Protandry was not always complete, and for 2/3 of surveyed flowers a slight overlap between the pollen presentation and stigma receptivity stages was observed. Dichogamy was not completely synchronized at the inflorescence level, and usually the floral display of a given plant simultaneously contained both male and female stage flowers. Both the male and female stages vary significantly in terms of nectar volume and concentration, whereas the sugar composition remains unchanged. HPLC analysis indicated that the nectar in this species is sucrose-dominant, together with smaller and almost equal quantities of hexoses. The most important pollinators observed for the investigated population of *P. caeruleum* were bumblebees, hoverflies and honeybees. However, the relative proportions of the various insect visitors varied annually. Nectar secretion and the nectary structure of this species have been investigated (Chwil, 2010; Chwil and Chwil, 2011) but without consideration of the occurrence of dichogamy in the flowers, nor the differing secretory activity of nectaries during particular sexual stages.

In this work we investigated possible differences in nectary structure and histochemistry between the male and female stages. We consider how these may potentially affect nectary activity.

MATERIAL AND METHODS

Polemonium caeruleum L. (Polemoniaceae) is a herbaceous perennial plant. The plants used in this study came from a large natural population recently discovered near the village of Kleczkowo (Ostrołęka district, NE Mazovia, Mazowieckie Province, Poland). The population consists of several thousand plants in a damp meadow complex on the small Ruż River (central point N 53°02.9' E 21°51.8'; Zych and Werblan-Jakubiec, 2004). In Poland, *P. caeruleum* is red-listed in category VU and has been legally protected since 1983 (Rutkowski, 2000).

We studied nectary structure at the male stage on the first day of anthesis (when pollen presentation commences but stigma lobes are closed, Fig. 1a) and at the female stage (when anthers have completely dehisced and the stigma lobes are open and presented above the anthers, Fig. 2a). To examine the position and size of nectaries in fresh flowers we used a Nikon SMZ 1000 stereomicroscope (Nikon Corp., Tokyo, Japan). The nectaries were then prepared for histochemical study by light microscopy (LM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM). For bright field LM, hand-cut sections from fresh nectaries were stained with an alcohol solution of Sudan IV for lipids and IKI for starch (Jensen, 1962). The nectary stomata were counted in squashed material treated with IKI (ten nectaries from male and from female stages), then recalculated per 1 mm^2 nectary surface. Semithin sections of nectaries were prepared by fixing nectary tissue in 2.5% glutaraldehyde/4% formaldehyde in phosphate buffer (pH 7.4, 0.1 M) for 4 h at 4°C, followed by three careful washes in phosphate buffer. Samples were subsequently dehydrated in a graded ethanol series and infiltrated with LR White resin. Following polymerization at 60°C, the sections were cut to 0.9–1.0 μm thickness with a glass knife. For general histology, semithin sections were stained with 1% aqueous solution of methylene blue-azure II. The nectaries were also tested for starch and other polysaccharides using the periodic acid-Schiff reaction

Fig. 1. Nectary of *Polemonium caeruleum* during male stage. Macro, LM, SEM and TEM images. (**a**) Habit of flower showing presentation of pollen and closed stigma lobes (arrow), (**b**) Longitudinal section of flower with exposed ovary encircled by nectary rim. Nectar droplets indicated by arrows. Bar = 5 mm, (**c**) Hand-cut longitudinal section of nectary visible on both sides of ovary. Bar = 0.5 mm, (**d**) Abaxial surface of nectary rim seen by SEM with adhered pollen grains and no visible nectar. Bar = 200 μm, (**e**) Section of base of nectary stained with methylene blue-azure II showing vascular bundle running to the filament. Bar = 100 μm, (**f, g**) Section of nectary stained with IKI. Starch is present in secretory cells and within guard cells. Bars = 100 μm, (**h**) Thin, intact cuticle covering nectary epidermal cells staining yellow-green with auramine O. Bar = 10 μm, (**i**) Nectary tissue under UV. Note the absence of chlorophyll autofluorescence. Bar = 60 μm, (**j**) Amyloplasts, ER and small vacuoles in cytoplasm. Bar = 2 μm, (**k**) Nectary parenchyma cells with electron-dense cytoplasm containing ER, nucleus and amyloplasts. Plasmodesmata (arrows) occur in the thin cell walls. Bar = 5 μm. ER – endoplasmic reticulum; n – nectary; O – ovary; st – starch; Vb – vascular bundle.
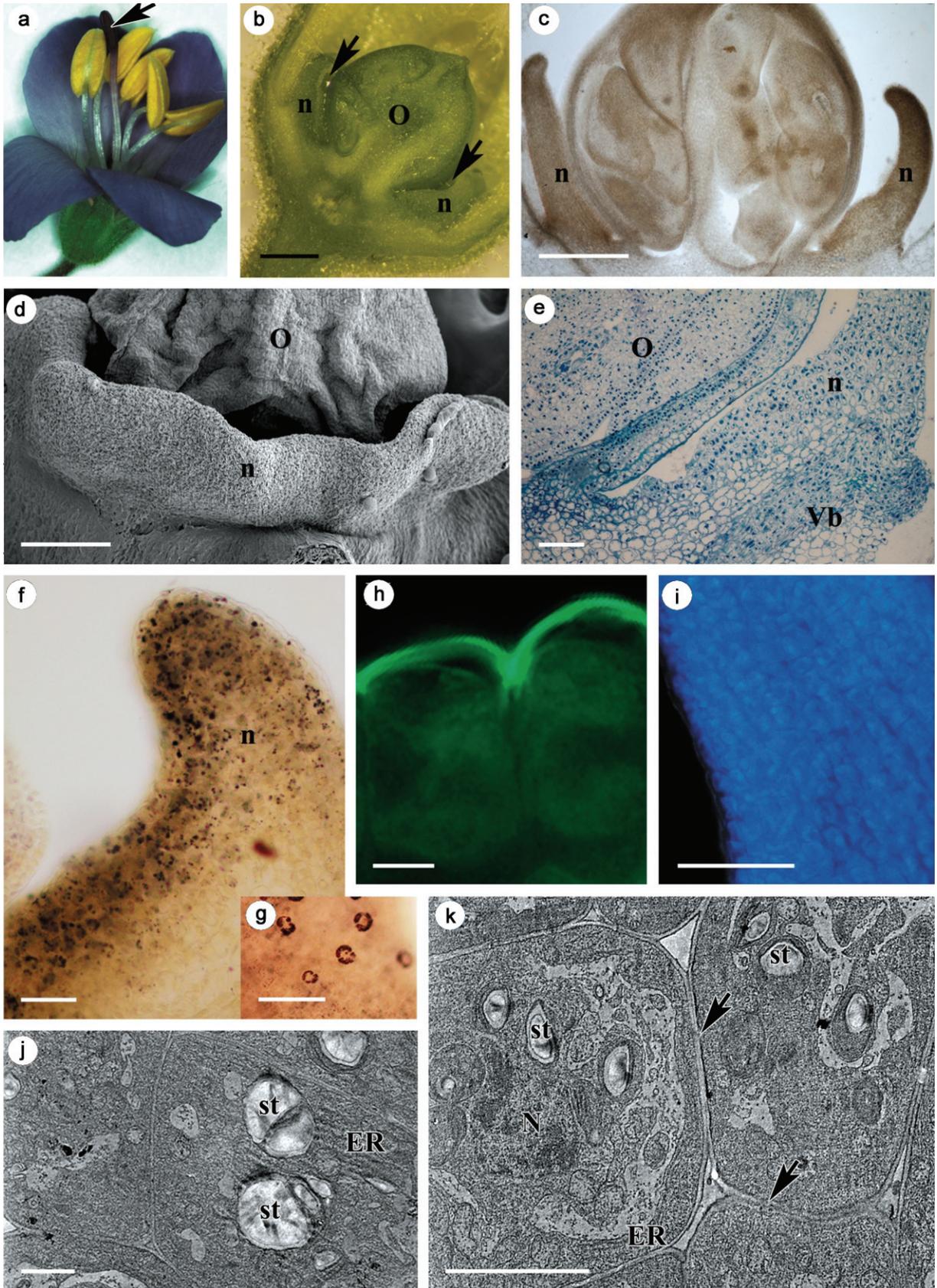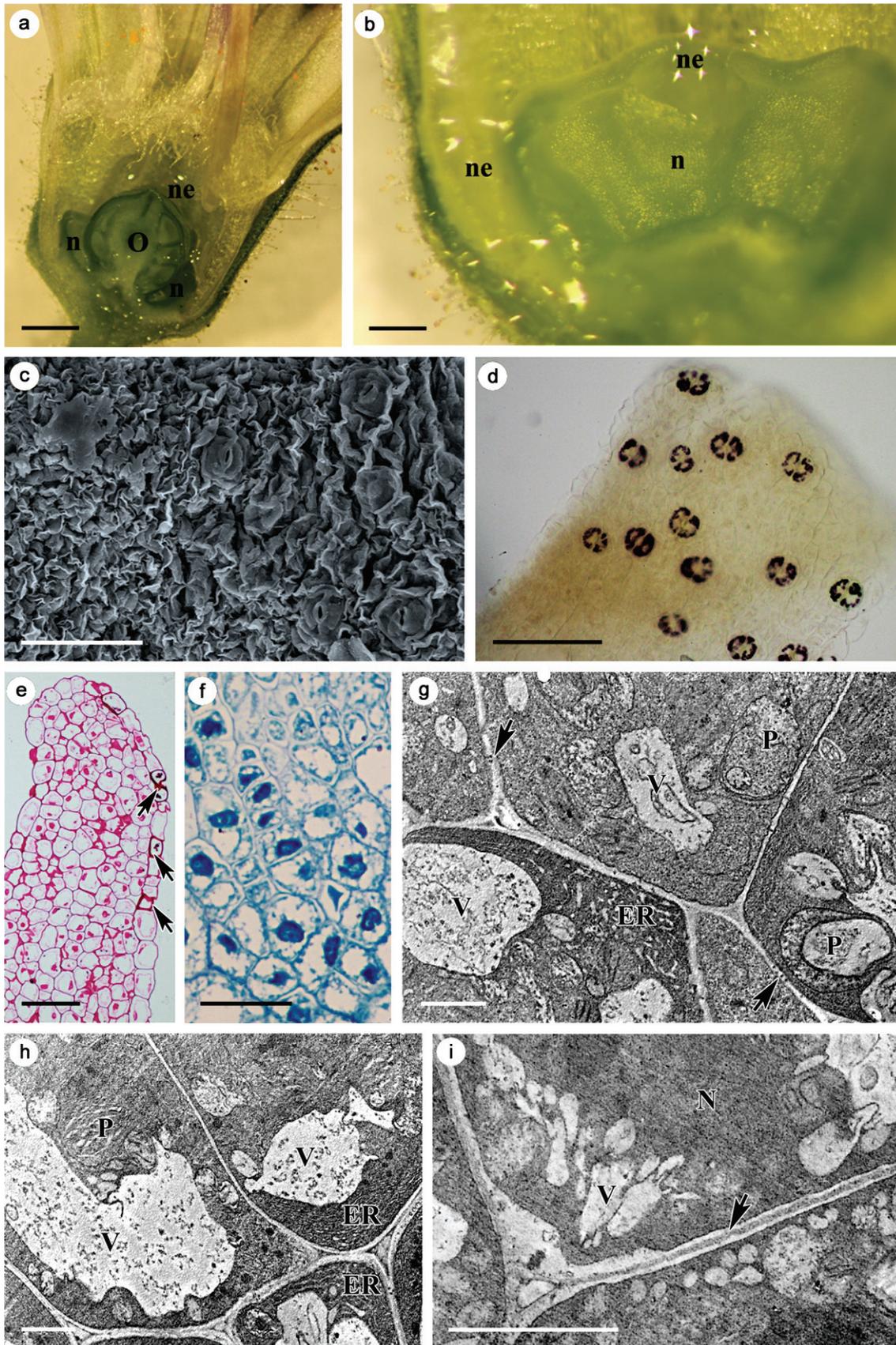
(Jensen, 1962). For this, prior to dehydrating and resin-embedding the nectaries were fixed in 70% ethanol to avoid false-positive PAS reactions due to the presence of aldehydes in the fixative. LM observations employed a Nikon Eclipse 400 microscope (Nikon Corp., Tokyo, Japan), and measurements were made with NIS-Elements Br 2 imaging software (Nikon Corp., Tokyo, Japan).

The sections were also examined by means of fluorescence microscopy. To test for the presence of cuticle and suberized cell walls, hand-cut sections were stained with auramine O (Gahan, 1984) and studied with a Nikon 90i fluorescence microscope fitted with an FITC filter (EXP. 465–495, DM 505, BA 515–555). Autofluorescence of hand-sectioned material under UV illumination was used to detect the distribution of chloroplasts and lignified nectary cells. Photomicrography was done with a Nikon 90i fluorescence microscope with a digital camera (Nikon Fi1) and NIS-Elements Br 2 software.

Material for TEM was fixed as above but then post-fixed in 1% osmium tetroxide solution at 0°C for 1.5 h, washed in distilled water and dehydrated using a graded ethanol series and embedded in LR White resin. Ultra-thin sections were cut with a glass knife at 60 nm using a Reichert Ultracut-S ultramicrotome (Reichert Jung, Wetzlar, Germany), stained with uranyl acetate and lead citrate (Reynolds, 1963) and examined with a Zeiss Leo EM 912 TEM (Zeiss SMT GmbH, Göttingen, Germany) at accelerating voltage of 90 kV.

For SEM observations, nectaries fixed as above were dehydrated in acetone, critical-point dried using liquid CO_2, sputter-coated with gold and examined with a TESCAN/VEGA LMU SEM (TESCAN, Brno, Czech Republic) at accelerating voltage of 30 kV.

RESULTS

In *P. caeruleum*, nectar secretion commences in flowers at the male stage and increases significantly during the female stage (Figs. 1a,b, 2a,b). The nectary encircles the base of the ovary and is rim-like, slightly lobed and thickened at the top. The rim does not adhere to the ovary wall (Fig. 1b–e). The ovary together with the nectary rim is located within a nectary chamber formed by the fused bases of the perianth segments and stamen filaments (Fig. 2a,b). The entrance to this chamber is protected by numerous hairs (Fig. 1a). The nectaries of the male and female stages are similar in size; the height of the nectary rim ranges from 910.81 to 1030.01 μm, the width at its base ranging from 407.23 to 436.53 μm. Nectar is released onto the surface via modified stomata (Figs. 1f,g, 2b–e). These are located on the highest part of the nectary, especially upon the adaxial surface. There are fewer nectary stomata at the male stage (average 136.71 stomata per 1 mm², SD=21.35) than at the female stage (156.3, SD=34.64). The nectary rim is not vascularized; the vascular bundles that supply the stamens run at its basal part.

The nectary is composed of a thin-walled epidermis, its outer cell-walls coated with a thin cuticle (Fig. 1h). Several layers of secretory parenchyma are also present (Fig. 1e–k). The nectary cells have very thin cellulosic cell walls with numerous plasmodesmata, only small intercellular spaces are present between the secretory cells during each of the sexual stages investigated, and the protoplasts of the cells are electron-dense (Figs. 1j,k, 2g–i) and also stain intensely with methylene blue-azure II solution (Figs. 1e, 2f). They have large nuclei and small vacuoles. The vacuoles enlarge during the female stage (Fig. 2g,h). Rough endoplasmic reticulum dominates the cytoplasm, and the Golgi apparatus is relatively rare in both stages (Figs. 1j, 2g,h). During the male stage, leucoplasts containing a single large starch grain and a few internal membranes are typically present in nectary cells (Fig. 1j,k). Leucoplasts also occur within the secretory parenchyma and epidermis, particularly in modified stomata (Fig. 1 f,g). The amount of starch in nectary cells diminishes significantly after the first day of anthesis, and during the female stage is found exclusively in guard cells (Fig. 2d,e). Simultaneously, large deposits of starch are present in the ovary wall and in the ovules. Although the nectary appears green, the plastids did not autofluoresce, indicating the absence of chlorophyll (Fig. 1i).

Fig. 2. Nectary of *Polemonium caeruleum* during female stage. Macro, LM, SEM and TEM images. (**a**) Nectary chamber filled with nectar and protected by hairs. Bar = 5 mm, (**b**) Adaxial surface of nectary coated with nectar (ovary removed). Bar = 0.25 mm, (**c**) Nectary stomata with nectar upon abaxial nectary surface, Bar = 50 μm, (**d**) Nectary stained with IKI solution. Starch is present exclusively in the guard cells. Bar = 50 μm, (**e**) Nectary stained with PAS. Here also, starch is detected only in guard cells (arrows). Bar = 50 μm, (**f**) Nectary tissue stained with methylene blue-azure II. Nectary cells contain large nuclei and vacuoles. Bar = 50 μm, (**g**) Starchless plastids irregular in outline, vacuoles and ER in cytoplasm. Arrows indicate plasmodesmata. Bar = 2 μm, (**h**) Vacuoles and plastids with dilated internal membranes. Bar = 2 μm, (**i**) Nucleus and numerous vacuoles in parietal cytoplasm. Arrow indicates plasmodesma. Bar = 5 μm. ER – endoplasmic reticulum; ne – nectar; n – nectary; N – nucleus; O – ovary; P – plastid; V – vacuole.

DISCUSSION

Although nectar is present in *P. caeruleum* during both the male and female stages, the flowers differ significantly in the amount of nectar secreted. During the female stage, nectar production is significantly higher (Zych et al., 2012). Similar differences in the quantity of available nectar in dichogamous plants have also been observed for florets of *Echinacea*, where nectar production per floret was highest at the female stage during the first day of stigma receptivity (Wist and Davis, 2006). In our work, nectary structure did not differ significantly between sexual stages and generally resembled that described by Chwil and Chwil (2011), who also showed that modified stomata are located in regions at the top and on the lateral wall of the abaxial surface of the nectary projection, which concurs with our observations. Stomatal nectar secretion has also been observed in the nectary disc of *Polemonium reptans* and the nectaries of other representatives of Polemoniaceae (Schönenberger, 2009; Schönenberger et al., 2010). Stomata are frequently noted at the top of the nectary rim/projection not only in Polemoniaceae but also in other dicotyledons (Davis and Gunning, 1992; Wist and Davis, 2005); such an exposition of stomata probably facilitates access to released nectar. More rarely, modified stomata are sunken in epidermis covering a flat nectary, as in *Fatsja japonica* (Nepi. 2007) or in some *Ribes* species (Stpiczyńska, unpubl. data). During the female stage, which displays greater secretory activity, there were more stomata than in the male stage. In general, however, stomata are regarded as unable to regulate nectar flow and, as indicated for *Vicia faba* and *Echinacea purpurea* (Davis and Gunning,1992; Wist and Davis, 2005), stomata at various stages of development are present on the nectary regardless of its secretory activity. The latter authors noted stomatal apertures fully open before secretion began (even wider than at the stage of maximum secretion) and immature stomata still occurring at pistillate stage in *Echinacea*, where probably they never reach maturity. Moreover, apertures of modified stomata can be occluded by material of unknown structure, irrespective of the developmental stage or nectary activity, indicating that nectar passage is not controlled by the stomatal pores (Davis and Gunning, 1992; Gaffal et al., 1998; Wist and Davis, 2005).

In this investigation we observed no traces of vascular bundles penetrating nectary tissue. Vascular bundles (composed of xylem and phloem elements) nearest the nectary (at the base of the nectary projection) supplied the stamens. Nectaries lacking a vascular supply have been reported for the disc-shaped nectaries of tobacco (Ren et al., 2007). In *P. caeruleum*, nectar sugars probably are uploaded from phloem sieve elements present at the nectary base and then transported to the nectaries and stored temporarily as starch in amyloplasts. Transport of sugars may be facilitated by the presence of thin cellulosic cell walls with numerous plasmodesmata. During the female stage, when the rate of secretion is high, starch is hydrolyzed, and sucrose partially metabolized by cell wall invertases which serve to produce nectar containing both sucrose (dominant sugar in nectar of *P. caeruleum*) and hexoses. Although nectary structure and most histochemical characters tested were similar for the two sexual stages, we noted a significant difference in the starch content of secretory cells, and this may affect nectar secretion. In *P. caeruleum*, starch was abundant at the beginning of secretion (male stage) but had disappeared almost entirely by the female stage, when the rate of nectar secretion is considerably higher. This may indicate that starch serves at least partly as a source of nectar sugars. A similar reduction of starch content, suggesting that it is converted to nectar sugars, has been reported for *Nicotiana* (Ren et al., 2007) and in nectaries of other species (Nepi, 2007 and references therein). Starch accumulation is not a universal feature of nectary cells, however. According to Heil (2011 and references therein), the nectar sugar of extrafloral nectaries is derived directly from sieve elements and/or photosynthesis, and starch does not generally accumulate in such nectaries. Since the floral nectaries are derived from extrafloral nectaries, direct transport from the phloem probably represents the archetypal condition. Accumulation of starch may be regarded as a derived strategy, supporting the intensive secretion of large amounts of sugar during the peak activity of floral nectaries (Heil, 2011). This process is sufficient to attract pollinators, particularly to flowers with a relatively short life-span, such as *P. caeruleum*. Elimination of soluble sugars (sucrose) from nectary cells at the presecretory stage and the formation of starch grains avoids establishment of an equilibrium between the sucrose concentration within nectariferous tissue and that of the phloem sap, and this in turn facilitates the uploading of sucrose from the phloem. Conversely, starch hydrolysis during the stage of intense secretion increases the osmotic pressure within nectary cells, resulting in the influx of water along the sugar concentration gradient, together with a concomitant enlargement of vacuoles, as recorded for *P. caeruleum* and the nectary cells of *Cucurbita pepo*, for which starch depletion during copious secretion was noted (Nepi et al., 1996).

Secretory activity frequently is associated with plastid differentiation in nectary cells. Floral nectaries of numerous plant species contain chloroamyloplasts during the presecretory stage.

Following secretion, these organelles often differentiate, forming chloroplasts or chromoplasts (Nepi, 2007 and references therein). We did not observe chloro-amyloplasts nor detect chlorophyll autofluorescence in *P. caeruleum*, however, and TEM observations confirmed the absence of chloroplasts with a granal system sufficiently developed to contribute effectively to photosynthesis.

ACKNOWLEDGMENTS

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