ORIGINAL ARTICLE

Nectar production in the pollen flower of *Anemone nemorosa* in comparison with other Ranunculaceae and *Magnolia* (Magnoliaceae)

Claudia Erbar · Peter Leins

Abstract The observation that the flowers of *Anemone nemorosa* offer nectar to pollinating bee-flies (*Bombylius major*) prompted this investigation into the site of nectar secretion and nectary tissue. To allow comparison on a broader basis, other nectar-secreting pollen flowers of the Ranunculaceae and *Magnolia* (Magnoliaceae) were included in the analysis. The contradictory information available on the function of the mouthparts of bee-flies during nectar and pollen feeding motivated us to investigate the proboscis structure in detail by SEM. Our investigations in *Anemone nemorosa* proved, for the first time, nectar secretion in the genus *Anemone* s.s. (i.e. other than the *Pulsatilla* group) and in addition, within the family, a new type of a carpellary nectary. The latter is an epithelial nectary involving the whole epidermis of the ovarian part of the carpel. The nectary of *Anemone nemorosa* resembles that of *Magnolia* (e.g. *M. stellata*), which we re-investigated. In both *Anemone nemorosa* and *Magnolia stellata*, nectar production is limited mainly to the female phase of the proterogynous flower. In this way, the attractiveness of the flower is also assured in the non-pollen presenting phase. Especially in *Magnolia*, with its numerous carpels arranged on a cone-like receptacle, the economic disadvantage of a choricarpous- compared to a coenocarpous-gynoecium is compensated for by nectar secretion by each carpel. When licking up the nectar droplets from the carpel surfaces, contact of the insect's body with each stigma may be achieved.

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Introduction

In April 2010, in a beech forest near Heidelberg (Reilingen), we observed bee-flies, *Bombylius major*, visiting flowers of the wood anemone, *Anemone nemorosa* (Ranunculaceae). This was not a singular event; several individuals of *Bombylius* visited eagerly different flowers of the population of *Anemone nemorosa*. In between, *Bombylius* visited *Vinca minor* (Apocynaceae), where the insect is rewarded by copious nectar. The long mouthparts of bee-flies reach deep into the corolla tube when collecting nectar. But what is the reward in the *Anemone* flower? We never expected a bee-fly on *Anemone*, because *Anemone* is thought to be a true pollen flower and, at first sight, the mouthparts of *Bombylius* do not seem well suited to true pollen flowers. Small beetles, flies and bees are often recorded as visitors of *Anemone nemorosa* (e.g. Knuth 1898; Hegi 1912; Proctor et al. 1996).

We never observed bee-flies taking the anthers of *Anemone nemorosa* between their labella (the two distal parts of the labium) or handling the anthers with their forelegs. Instead, the bee-flies probed at the bottom of the flower, and this behaviour prompted our search for nectar and the site of nectar production in *Anemone nemorosa*.

It is well-known that, within the Ranunculaceae, there are flowers that offer only pollen and others that offer both pollen and nectar as rewards to their pollinators. Nectar is either secreted by special nectary organs (staminodes/"petals", e.g. in *Ranunculus* and *Aquilegia*, e.g. Hiepko 1965; Erbar et al. 1999), or at the base of filaments in some members of *Clematis*

(Kratochwil 1988) and *Pulsatilla* (Daumann and Slavikova 1968) and in one case (*Caltha*) by the carpel flanks (patches of hairs on both flanks; Sprengel 1793; Petersen et al. 1979). For the purpose of comparison, we re-investigated members of the above-mentioned ranunculaceous genera as well as a species of *Magnolia* (*M. stellata*). To date, in some of the taxa mentioned, although the site of nectar presentation is known, detailed morphological-histological studies are lacking.

Materials and methods

Flowers from fresh or liquid-preserved material were studied. Vouchers of the collected material have been deposited in the herbarium of the Botanical Garden of the University of Heidelberg (HEID).

Flower buds were fixed in FAA (formalin, acetic acid, alcohol). For samples studied using SEM techniques, the buds were dehydrated in dimethoxymethane, critical-point dried using liquid CO_2, mounted on stubs, coated with gold and studied in a Leitz (AMR 1200B, Leitz, Wetzlar, Germany) scanning electron microscope (software: Digital Image Processing System 2.6). For sections examined using light microscopy (Zeiss, Software: AxioVision 4), the flowers were dehydrated in an alcohol series, transferred to infiltration medium and embedded in a methacrylate resin (Technovit 7100, Kulzer, http://www.kulzer-technik.de). The resin blocks were cut with a rotary microtome (using disposable blades) at a thickness of 6 µm, and the tissue sections were then stained with toluidine blue.

Material investigated is shown in Table 1. The mouthparts of *Bombylius major* (Figs. 10–15) were investigated from individuals sampled on 20 May 2012 at the border of a floodplain forest near Heidelberg (Ketsch, Germany); these individuals visited *Glechoma hederacea* (Lamiaceae).

Results

Bombylius major

In spring 2010 (19 April), between 12.30 noon and 2.30 pm, in a beech forest near Heidelberg (Reilingen) we observed the major bee-fly, *Bombylius major*, visiting flowers of *Anemone nemorosa* (Figs. 1–4). Several individuals of *Bombylius* moved rapidly from flower to flower in the relatively dense population of *Anemone nemorosa*. *Bombylius* usually hovers next to the plant, then approaches the flower and rests with its legs on the flower to stabilize its hovering when feeding (Figs. 3–4). When the insect holds onto the flower, it buzzes more or less constantly with its wings. Only occasionally, when flower-visits last some time, is the buzzing briefly interrupted (Fig. 1). The insects visited the white, proterogynous flowers in the female and the early male as well as in the late male phase of anthesis. In between, *Bombylius* visited the blue-violet flowering *Vinca minor* (Apocynaceae; Fig. 6) and *Viola riviniana* (Violaceae). In both, the insect is rewarded by copious nectar. The long mouthparts of the bee-flies reach deep into the corolla tube of *Vinca* and the spur of *Viola*. During the period of observation (between noon and early afternoon) no liquid nectar could be observed in the *Anemone* flowers. Nevertheless, occasionally glistening of nectar drops (in

Table 1 Material investigated by scanning electron microscopy (SEM)

| Ranunculaceae:			
Anemone nemorosa L.	Erbar 2010	Reilingen (Germany = G)	Figs. 1–5
	Erbar 26/2010 (HEID 207056)	Reilingen (G)	Fig. 19
	Erbar 24/2012 (HEID 206695) HEID 207057	Heidelberg, Mühlental (G) Hengstbachtal (G)	Figs. 8–9, 16–18, 20–23
	HEID 207058	Nonnweiler (G)	
Ranunculus aconitifolius L.	*Erbar 20/2006* (HEID 207112)	near Ballon d'Alsace, (France)	Figs. 30–35
Ranunculus acris L.	Erbar 2009	Botanical Garden of the University of Heidelberg = BGHD	Fig. 24
Pulsatilla turczaninovii Krylov and Serg. [= *P. grandis* Wend. var. *turczaninovii* Popov]	*Erbar 31/2012* (HEID 207046)	BGHD	Figs. 25, 36–37
Clematis alpina (L.) Mill.	Erbar 1999	Italy, Southern Alps, beneath Passo Croce Domini	Fig. 26
	Wolf s.n., 2012 (HEID 207076)	Austria, Steiermark, Aflenzer Staritzen	Figs. 38–42
Caltha palustris L.	*Erbar 26/2010* (HEID 207069)	BGHD	Figs. 27–28, 43–46
Magnoliaceae:			
Magnolia stellata (Siebold and Zucc.) Maxim.	*Erbar 23/2012* (HEID 207042)	BGHD	Figs. 29, 47–53
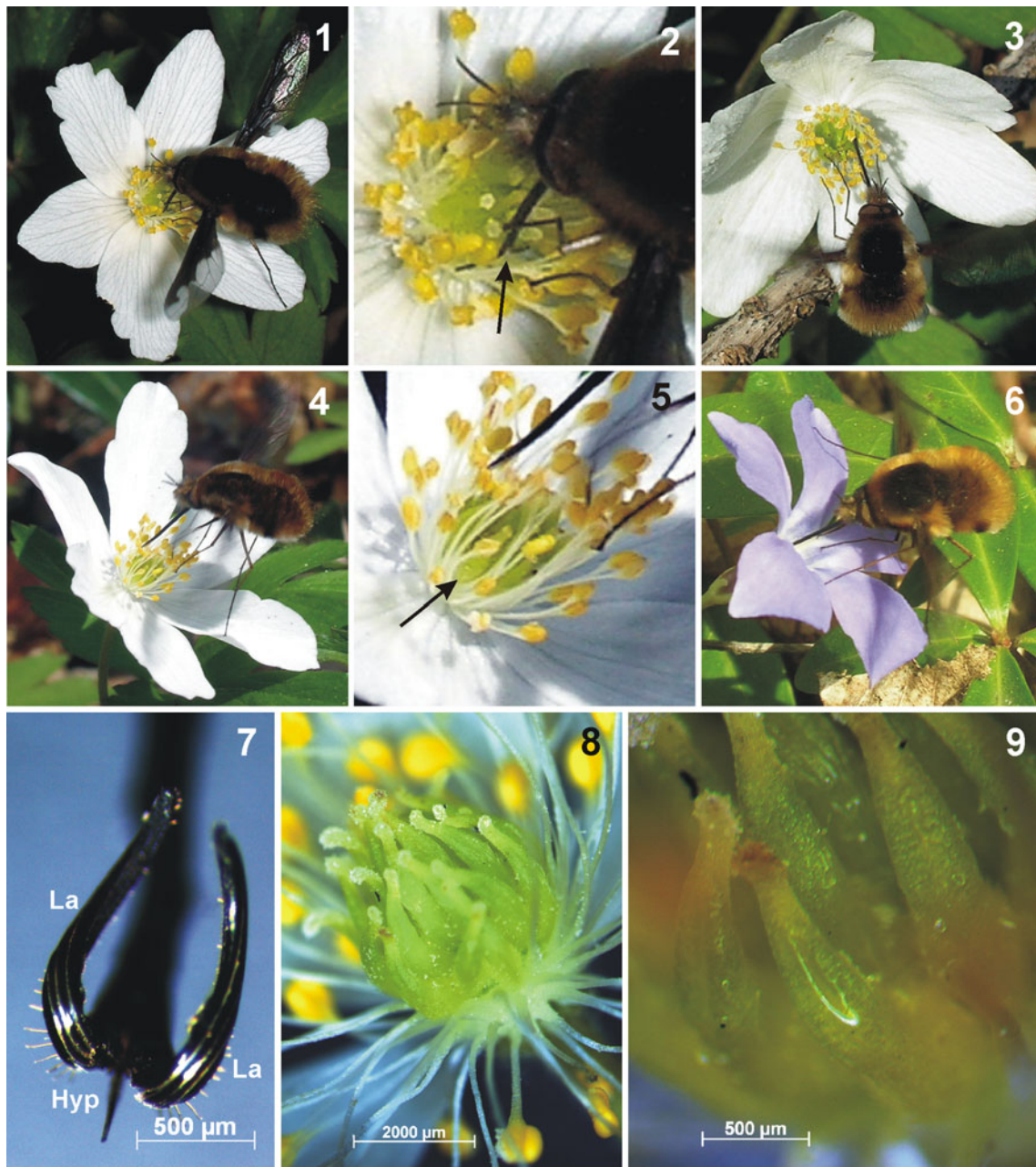
Figs. 1-9 Major bee-fly *Bombylius major* visiting flowers of *Anemone nemorosa* (Ranunculaceae). *B. major* is characterised by the dark patches on the anterior half of the wings. **Fig. 2** Enlarged section of **Fig**. **1** showing the spreading proboscis when dissolving solidified nectar. **Fig. 5** Enlarged section of **Fig. 4**, *arrow* points to solidified nectar. **Fig**. **6** *B. major* visiting *Vinca minor* (Apocynaceae). **Fig**. **7** Tip of the proboscis of *B. major*, spreading labella make the hypopharynx visible. **Figs**. **8–9** Nectar in a just-opened flower of *A. nemorosa*. *Hyp* Hypopharynx, *La* labellum

limited quantity) was visible (Fig. 5, arrow), which may occur when flowers have not been visited by insects for some time.

The bee-flies visiting *Anemone nemorosa* at our study site did not consume pollen out of the anthers. Instead, after probing at the bottom of the flower they spread the labella and, in doing so, they presumably dissolve solidified nectar in saliva (Figs. 1–2).

The rigid, needle-like proboscis (ca. 10 mm long, formed by labrum, labium and hypopharynx) is adapted for sucking nectar deep in flowers. It is projected forwards more or less horizontally and cannot be retracted. The paired labella, however, are flexible (Fig. 7) and make initial contact with substances to be ingested. If the labella are pressed tightly together, the proboscis is used to suck up liquid nectar (Fig. 10). If the slender
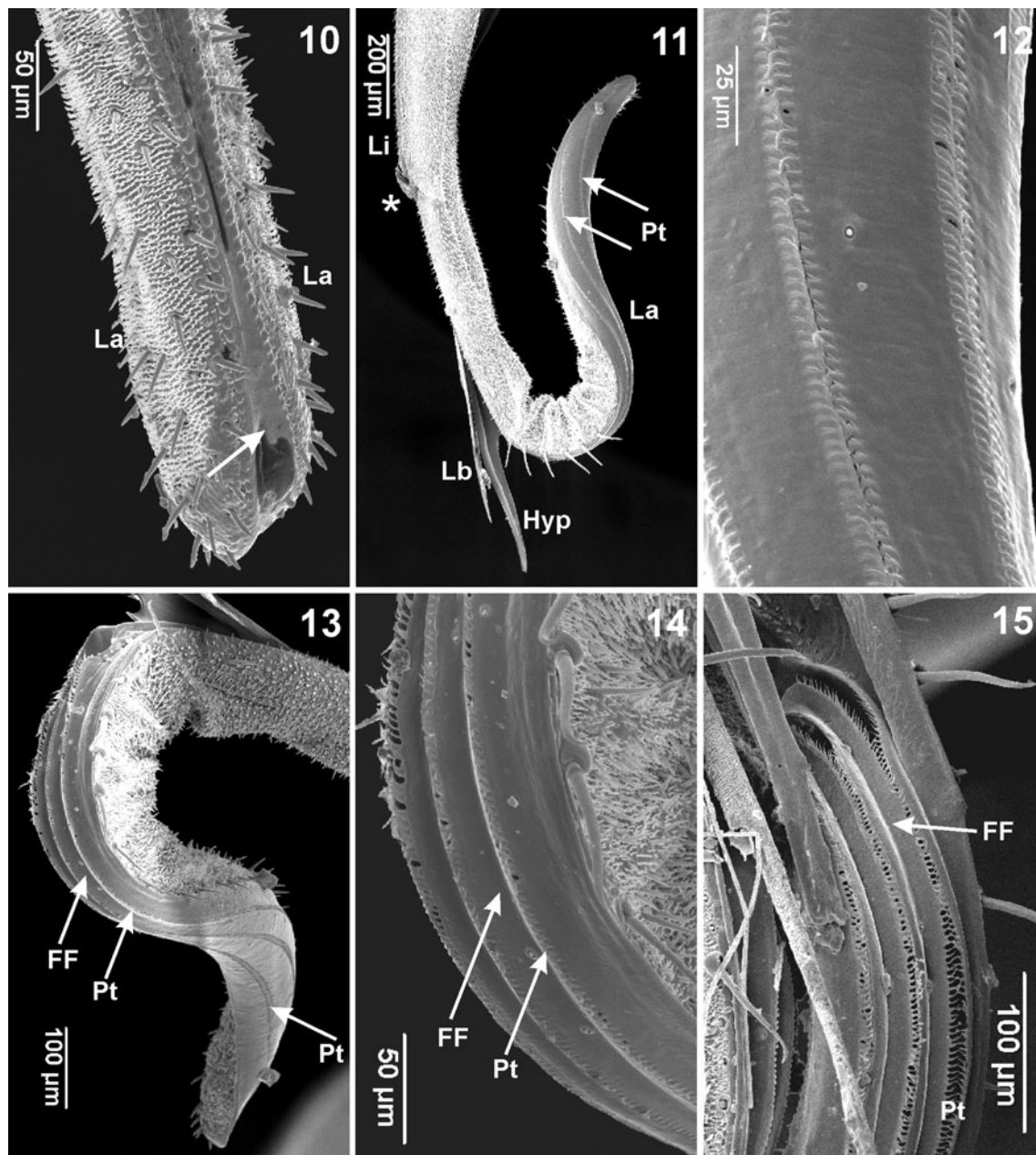
Figs. 10-15 Scanning electron microscopy (SEM) images of the proboscis of *Bombylius major*. **Fig**. **10** Both labella are tightly held together forming a "drinking straw" when feeding on nectar in tubular flowers, *arrow* points to solidified nectar (bee-fly caught shortly after nectar feeding). **Figs. 11–14** Same proboscis at different views. **Fig. 11** Tip of a proboscis with spreading labella (one labellum torn off, break-off point marked with *asterisk*. **Fig. 12** Detail of the distal part of a labellum showing the three pseudotracheae at higher magnification.

Fig. **13** Proximal part of one labellum. **Fig**. **14** Detail of the proximal part of the labellum showing the three convex pseudotracheae and the alternating food furrows at higher magnification. **Fig. 15** Three pseudotracheae bulged outwards with alternating food furrows, due to the handling of the insect the zipper-like closure of the pseudotracheae is partly broken up. FF Food furrow, *Hyp* hypopharynx, *La* labellum, *Lb* labrum, *Li* labium, *Pt* pseudotrachea

labella are spread at right angles to the axis of the proboscis as observed during the visits of *Anemone* flowers (Figs. 1–2), the labella are also involved in food uptake but, at the bottom of the *Anemone* flower, they come into contact only with dried up nectar. Each labellum is traversed on its inner side by three pseudotracheae, i.e.

canals that extend in the longitudinal axis of the proboscis (Figs. 7, 11–14). These tube-like structures have openings for the discharge of saliva. The outflowing saliva can dissolve the solidified nectar. The fluid is then conducted along furrows that are formed by interpseudotracheal folds (Figs. 13–15).

Flower of *Anemone nemorosa* and nectar secretion

Anemone nemorosa, the wood anemone or windflower, is a long-lived perennial, and is a common, often dominant, understorey spring herb of European (and also Asian) deciduous woodland, and naturalised in parts of North America. Its creeping rhizomes can make wide-spreading carpets. The solitary flowers are 2 cm in diameter, with mostly six (or seven) tepals with many stamens (mostly about 45) and a choricarpous gynoecium (about 10–20 carpels). The sepals are usually white inside, sometimes slightly tinged with pink outside. The flower is held erect during the day, but closes and droops at night and in bad weather.

Looking for nectar in the field may yield only limited success. At midday or in the afternoon, one may sometimes see some glistening of nectar at the base of the carpels (Fig. 5, arrow). Only with a dissecting microscope could we detect copious nectar in flowers that had just opened (Figs. 8–9; flowers collected on 13 April 2012, early in the morning).

The carpels are hairy all around their lower part (Fig. 16). The hairs are unicellular with a somewhat dilated base (Figs. 17–18). The epidermal cells between the hairs are striking. Sometimes they form a group around the base of the hair (Fig. 18). The epidermal cells in the area of the hairy part differ from those of the upper part of the carpel; they are smaller, more papillate and stain (with toluidine blue) deeper due to their dense protoplasm (Figs. 20–23). Nectar should be secreted by the epidermis in the ovarian part of the carpel; however, there is no underlying nectar tissue. We were unable to determine whether the hairs also secrete nectar. In any case, they hold the nectar. Stomata could be found only in the stylar region above the ovary (Figs. 19, 22), where they may serve in gas exchange. In some cases we found crystallized nectar in the SEM preparations due to nectar rising above the hairy region in flowers where much nectar was produced (Fig. 19). We obtained identical results in flowers of *Anemone nemorosa* sampled at four different sites in Germany (see Material and methods).

Nectaries in other Ranunculaceae

Flowers of many members of the Ranunculaceae offer nectar besides pollen. As examples, the different sites of nectar secretion were demonstrated in selected species (*Ranunculus aconitifolius*, *Pulsatilla turczaninovii*, *Clematis alpina*, *Caltha palustris*) and for comparison in *Magnolia stellata* (Magnoliaceae).

In many genera, nectar is secreted in special nectary organs, which are formed between perianth and androecium. These variously shaped nectary organs are often showy and take over petal function, as for example in *Ranunculus* (Fig. 24). In *Ranunculus acris*, like in most species of this genus, a scale covers a nectar-secreting pit, at the base of which nectar tissue can be found. In the white-flowering *Ranunculus aconitifolius* the scale is not a flat structure but tubular (Fig. 30). At the time when the stamen primordia have already differentiated into anthers and short filaments, the primordia of the nectary organs in *Ranunculus aconitifolius* are small and flat. The primordia may exhibit slight depressions (Figs. 31–32) due to pressure from contiguous stamens. Differentiation of the nectary organs starts with the formation of a horseshoe-shaped bulge at the ventral base of the young nectary organ (Figs. 31–32). Soon afterwards the upwards pointing ends come into contact, forming a ring-like structure (Fig. 33). By further unequal upgrowth the bowl-shaped structure changes into a obliquely tube-shaped scale whose longer part may be entire or slightly two-lobed (Fig. 30). A massive nectar-secreting tissue of small, plasma-rich cells lies at the base of the tubular scale (Figs. 34–35). Nectary slits could not be detected.

In the Siberian pasque flower, *Pulsatilla turczaninovii*, plenty of nectar is secreted, mainly during the early female phase of anthesis (flowers are proterogynous; Fig. 25). The nectar is secreted by the outer short, club-shaped sterile stamens (staminodes), either from the filament or from the entire staminode (Figs. 36–37).

Clematis alpina has outer spatulate staminodes (Figs. 26, 38). However, it is not these staminodes, but rather the fertile inner stamens that are the site of the nectary—strictly speaking the ventral side of their filaments (Fig. 39). Nectar is secreted by the epidermis in an oval area (Figs. 40–42). In this area the epidermis differs from that of the surrounding area: there are deep longitudinal furrows between the secreting cells (Fig. 41), perhaps capillary nectar holders.

Caltha palustris—the kingcup or marsh marigold (Fig. 27)—exhibits a carpellary nectary. At anthesis, flowers of *Caltha* have droplets of nectar between the carpels (Fig. 28). On either side of each carpel, there is a basal group of approximately 100 unicellular, clavate trichomes that are responsible for nectar secretion (Figs. 43–46).

In *Magnolia stellata*, nectar secretion is found only at the beginning of anthesis (the flowers are proterogynous; Fig. 29). Small droplets are produced in the region of the style and the ovary. There is no localised nectary tissue below the epidermis (Figs. 50–51), but the epithelial nectary covers the whole carpel from style (Fig. 52) to ovary (Fig. 53). In objects prepared for SEM investigation, the surfaces of the carpels are coated by a granular material, presumably solidified nectar (Figs. 47–49). Nectar secretion is through the epidermal cell wall. Stomata (Fig. 48) may only serve in gas exchange.
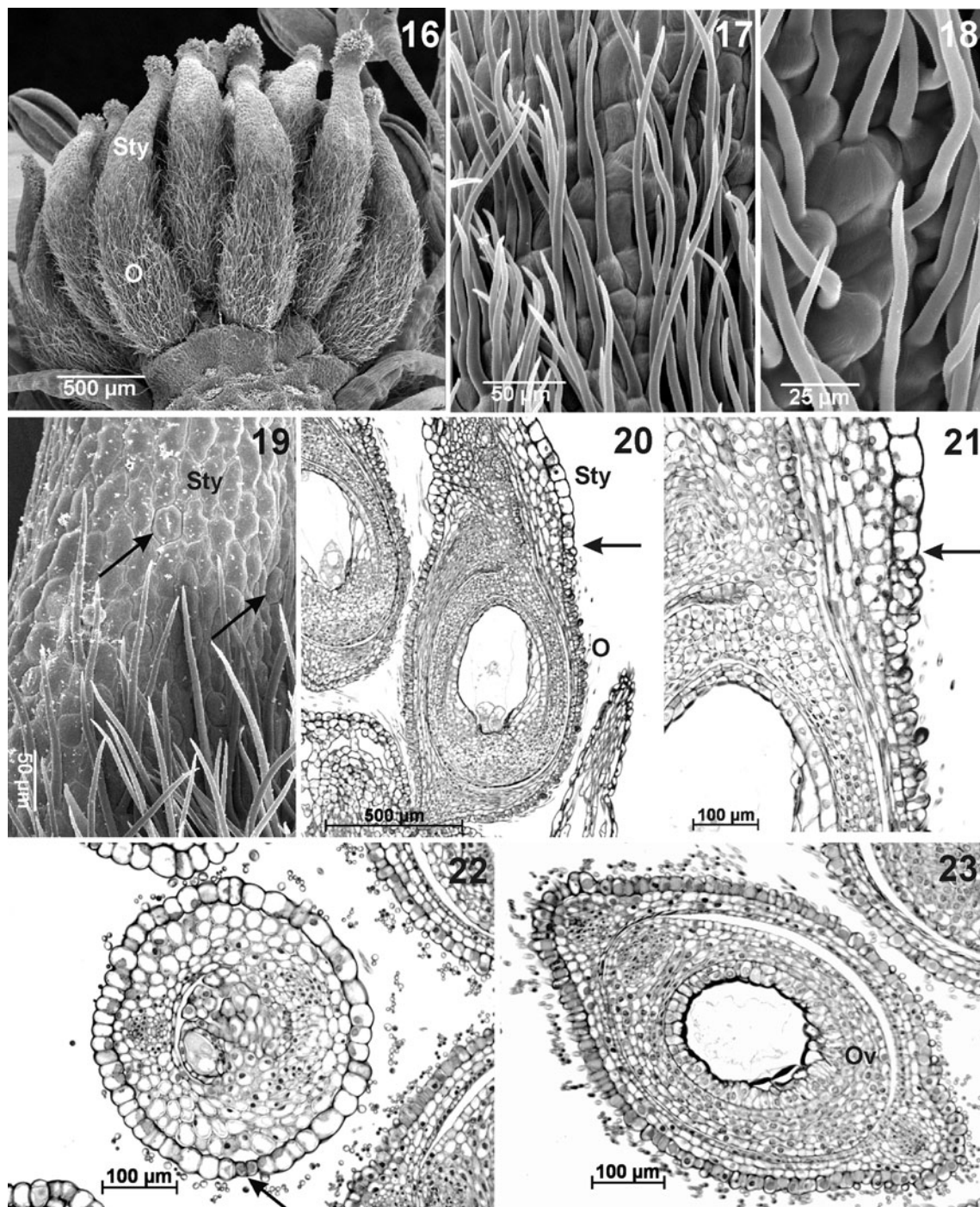
Figs. 16-23 *Anemone nemorosa*. **Figs**. **16–19** SEM images. **Figs**. **20–23** Histological sections. **Fig**. 16 Choricarpous gynoecium; note the basal hairy part of the carpels. **Figs**. **17–18** Hairs and epidermis at higher magnification. **Fig**. 19 Carpel surface above the basal hairy part; note the crystallised nectar and the stomata presumably for gas exchange (*arrows*). **Fig**. 20 Longitudinal section through a carpel; note the different size and staining of the epidermal cells in the ovarian and stylar area; *arrow* transition area of both parts. **Fig**. 20 Transition area at higher magnification. **Fig**. 22 Cross section through the style; note the large, ± unstained epidermal cells; *arrow* stoma. **Fig**. 23 Cross section through the ovary; note the cytoplasm-rich and thus more intensively stained epidermal cells (carpellary epidermal nectary). *O* Ovary, *Ov* ovule, *Sty* style
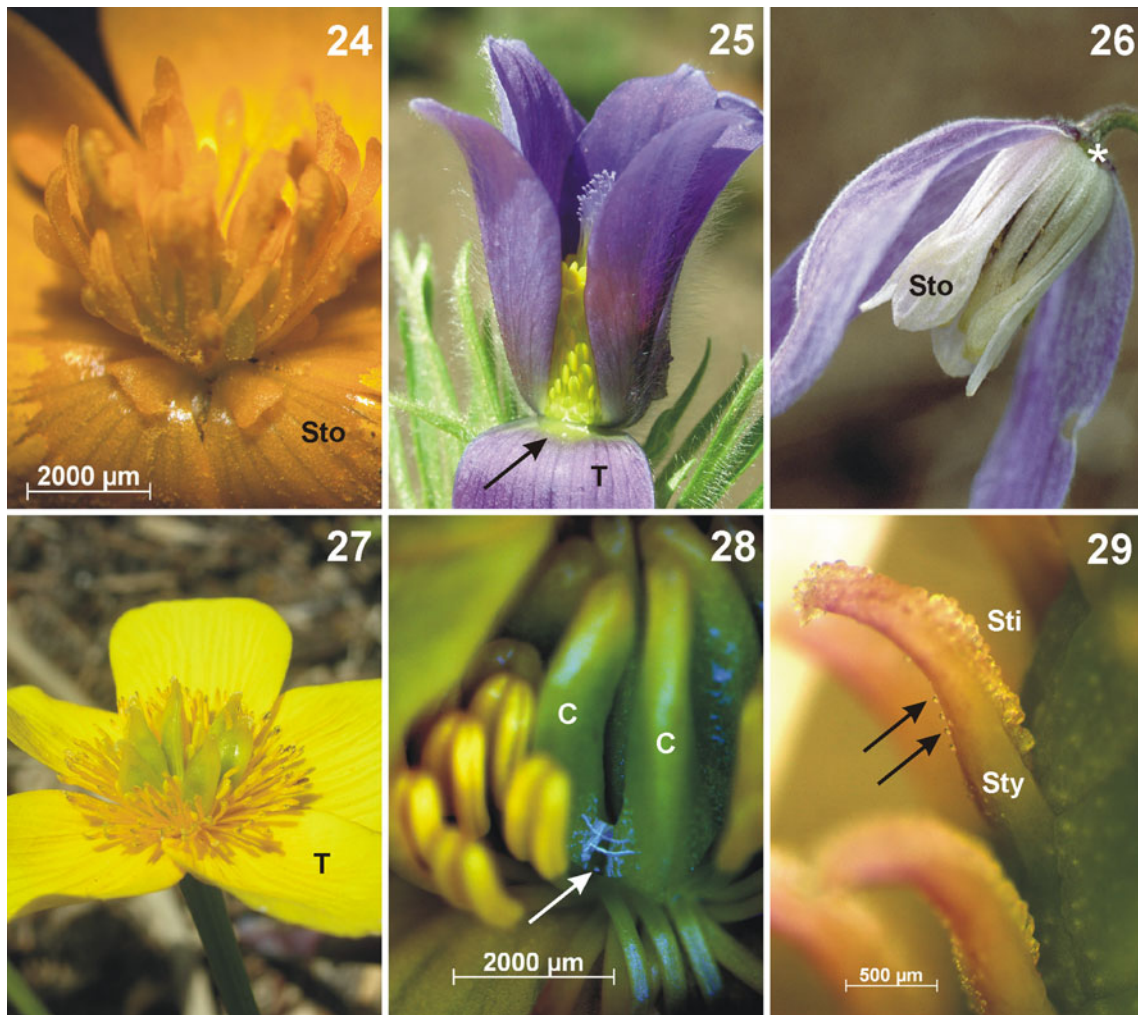
Figs. 24-29 Androecial nectaries in the Ranunculaceae. **Fig**. 24 *Ranunculus acris*, scales covering the nectary at the base of each petaloid staminode. **Fig**. 25 *Pulsatilla turczaninovii*, just-opening flower in the early female phase of anthesis presenting abundant nectar at the base of the androecium. **Fig**. 26 *Clematis alpina*, one tepal dissected (scar marked with *asterisk*). **Figs**. 27–29 Carpellary nectaries. **Figs**. 27–28 *Caltha palustris*, presenting a large nectar drop between the lateral flanks of the carpels. **Fig**. 29 *Magnolia stellata*, presenting small nectar droplet at the stylar surface; note that in the early female phase of anthesis the stigma is covered by a secretion. *C* Carpel, *Sto* staminode, *T* tepal. *Arrows* Nectar drops or droplets

Discussion

The mouthparts and their function in *Bombylius major*

Bombylius major (Bombyliidae) is found frequently in the whole northern temperate zone, from Europe to parts of Asia, and in North America. It is well-known as a nectar feeder and, with its long proboscis, concealed nectar can be easily exploited. However, it also visits flowers with more or less freely presented nectar where a long proboscis is not required. Knoll (1921: p.105) was the first to mention pollen consumption by a *Bombylius* species (*B. medius*), and it has been shown recently that females, at least, are obligate pollen feeders since pollen is a necessary requirement for nourishing developing eggs (Boesi et al. 2009).

The proboscis can be used to suck up liquids, either fluid nectar or solid material like pollen grains suspended in salivary secretions. Knoll (1921) did not describe how *Bombylius* manages the uptake of pollen, and among zoologists there is discussion whether and how *Bombylius* can consume pollen directly from the anthers. On the one hand, it is described that the anthers are taken between the labella and that pollen is scraped off by rubbing and twisting movements (Schremmer 1961; Szucsich and Krenn 2002). On the other hand, pollen collection could be accomplished with the forelegs, which bear modified setae (bristles) playing a role in pollen removal; the forelegs then
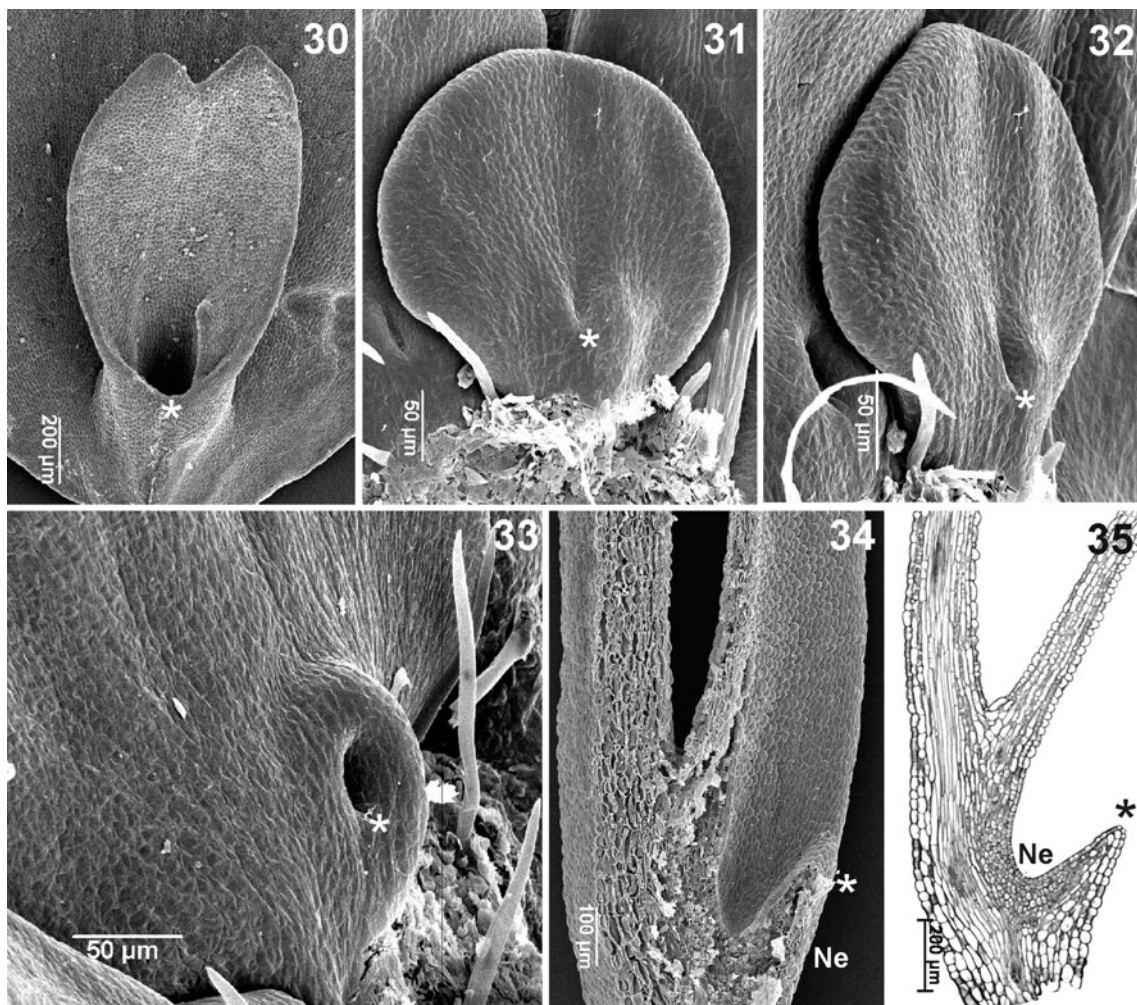
Figs. 30-35 Ontogeny of the nectary organ in *Ranunculus aconitifolius*. **Figs**. 30–34, SEM images. **Fig**. 30 Tubular scale of an adult nectary organ. **Figs**. 31–33 Early developmental stages of the tubular scale. **Fig**. 34 Tubular nectary scale cut in half. **Fig**. 35 Longitudinal section through the nectary. *Ne* Nectary. *Asterisk* Corresponding sites

transfer the pollen to the tip of the proboscis (Neff et al. 2003; see also Deyrup 1988, for another species of Bombyliidae).

It has been suggested that the pseudotracheal system in the labella serves to distribute saliva onto the labellar surface when feeding on solidified nectar (Krenn et al. 2005). Conversely, however, food seems not to be transported by the pseudotracheae as repeatedly suggested (e.g. Szucsich and Krenn 2002; Krenn et al. 2005). Instead, the food (liquid and solid) is conducted along furrows that are formed by interpseudotracheal folds (Schuhmacher and Hoffmann 1982; Gilbert and Jervis 1998), as shown by Dimmock in a simple experiment as early as 1881. He found that, after feeding the bee-fly with a mixture of sugar and gum arabic, coloured with carmine, and then plunging it suddenly into alcohol to fix the coloured solution in its mouthparts, the coloured solution of gum arabic had not entered the pseudotracheae.

Folding of the labellar surface has been denied in *Bombylius major* (see Szucsich and Krenn 2002; Krenn et al. 2005). However, using SEM, we could demonstrate such food furrows in two proboscises whose labella are spread: food furrows can be seen at the proximal part of a labellum, at the entrance to the epipharyngeal food canal (Figs. 13–15). In a feeding syrphid (*Eristalis*), Schuhmacher and Hoffmann (1982) demonstrated by instantaneous freezing that the interpseudotracheal folds bulged outwards (due to haemolymphal pressure) so that the pseudotracheae are at the bottom of the food furrow. In our material of *Bombylius major* (caught from the flower and not further treated by chemicals) the folding is just reverse, namely the pseudotracheae bulging outwards and presenting a zipper-like closure at the edge (Figs. 13–15).

Bombylius species that feed mainly on nectar from deep corollas exhibit only a few pseudotracheae (Gilbert and Jervis 1998). Since at least the females depend on pollen, saliva welling out of the pseudotracheae is necessary so that pollen mixed with a fluid can be sopped up. Our observation
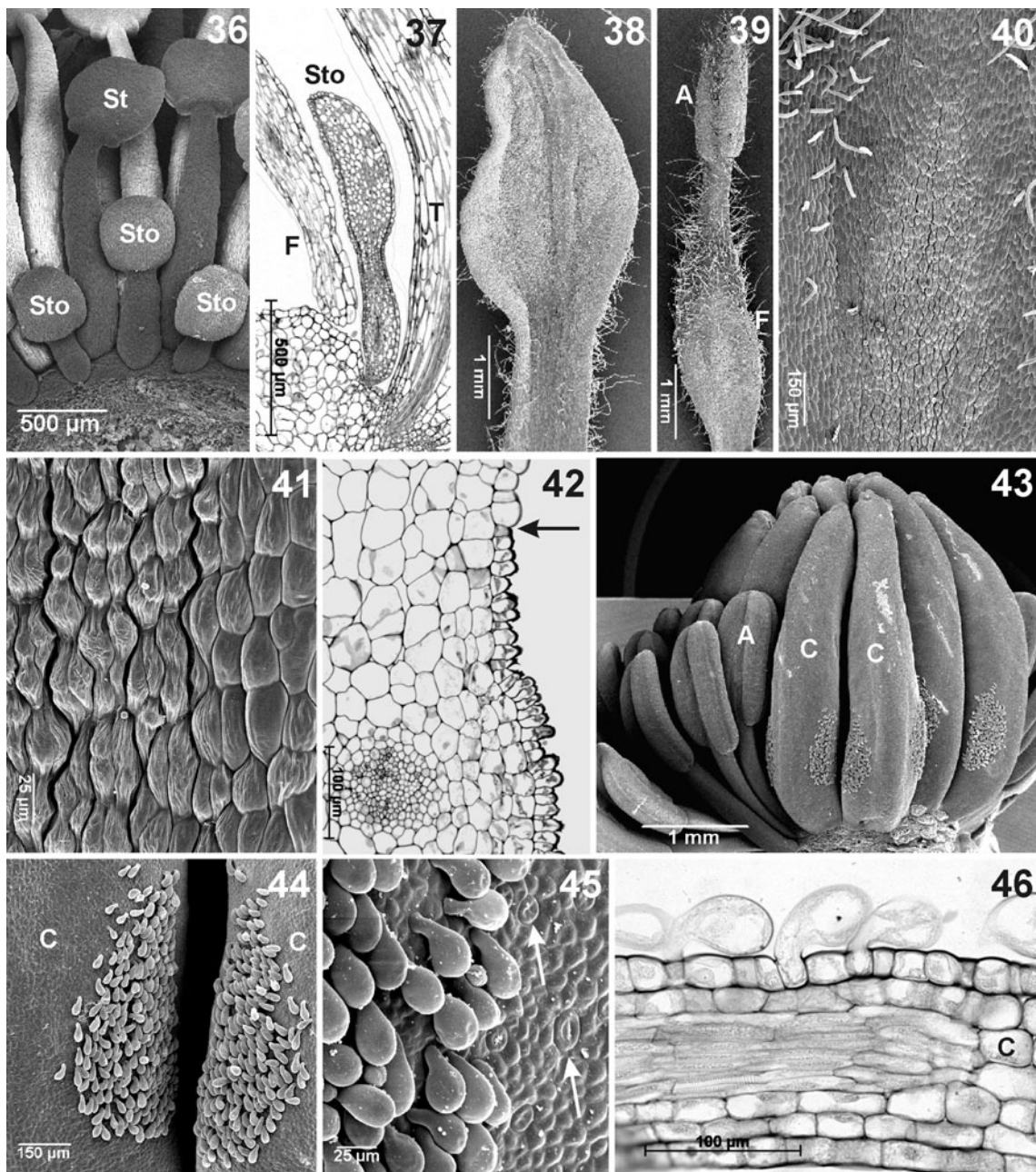
Figs. 36-46 Androecial and carpellary nectaries in Ranunculaceae. **Figs. 36–37** *Pulsatilla turczaninovii*. **Fig. 36** SEM image of part of the androecium with the outer staminodes retarded in development. **Fig. 37** Longitudinal section through a nectar-secreting staminode. **Figs. 38–42** *Clematis alpina*. **Fig. 38** Outer staminode. **Fig. 39** Fertile stamen with broadened filament. **Fig. 40** Magnification of the ventral oval nectar-secreting area of the filament. **Fig. 41** Transitional zone between the longitudinally furrowed nectar-secreting cells (*left*) and the surrounding cells (*right*). **Fig. 42** Longitudinal section through the nectar-secreting epidermis, upper end of nectar-secreting area indicated by an *arrow*. **Figs. 43–46** *Caltha palustris*. **Fig. 43** SEM image of the gynoecium. **Fig. 44** Group of nectar-secreting trichomes on either side of the carpels. **Fig. 45** Unicellular, clavate hairs at higher magnification, *arrows* indicate stomata for gas exchange. **Fig. 46** Cross section through the carpel wall with the nectar-secreting cells (carpellary trichome nectary). *A* Anther, *C* carpel, *F* filament, *St* stamen, *Sto* staminode, *T* tepal

of spreading labella at the bottom of the flowers (Figs. 1–2) allows the interpretation that solidified nectar is dissolved in saliva and is sucked along the food furrows to the opening of the epipharyngeal food canal.

Pollination of *Anemone nemorosa* by *Bombylius major*

More than 50 species of flowering plants are recorded to be pollinated by *Bombylius major* (e.g. Knuth 1898;
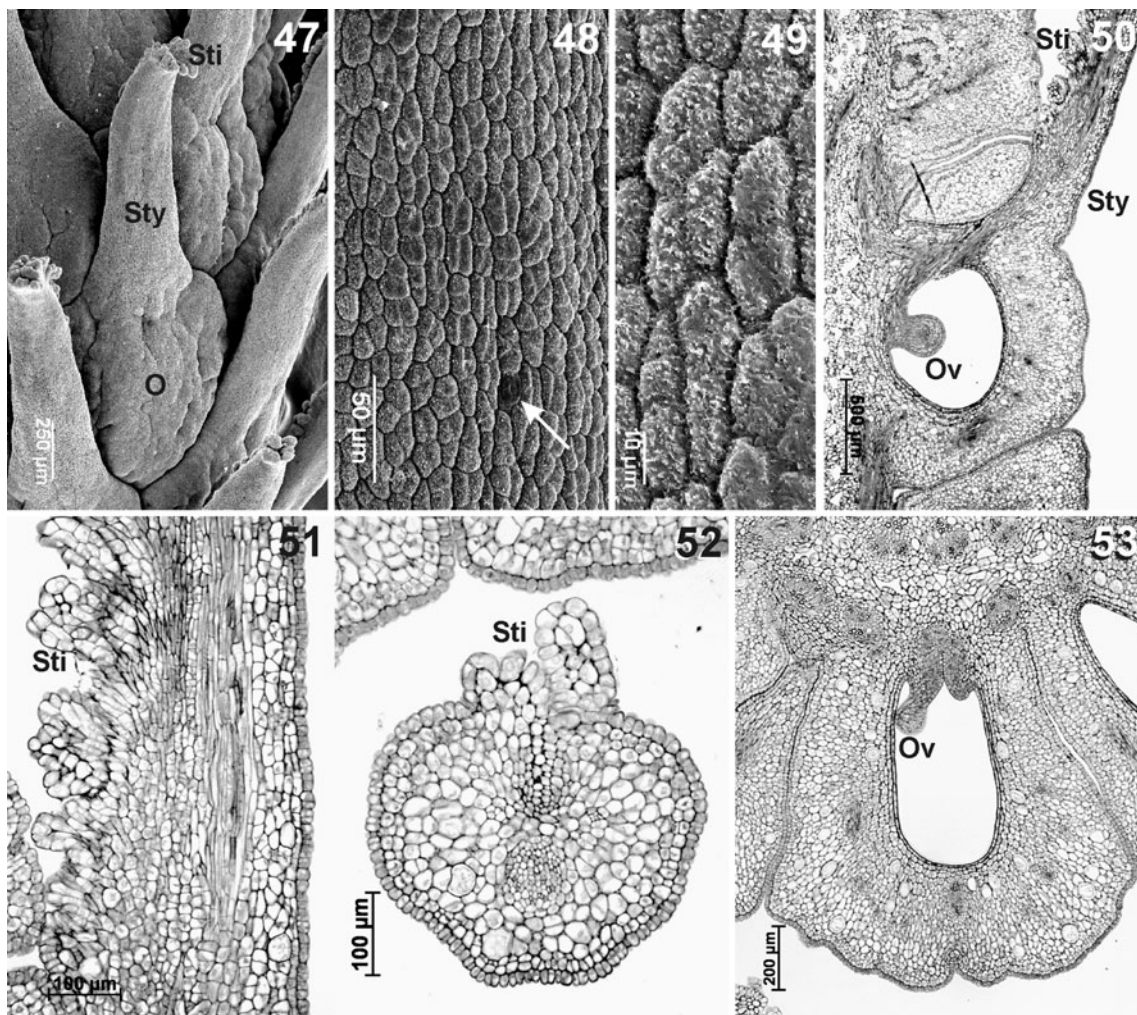
Figs. 47-53 *Magnolia stellata*. **Figs**. **47–49** SEM images. **Figs**. **50–53** Histological sections. **Figs**. **47–49** Surface of the carpels is covered with solidified nectar. **Figs**. **48–49** Stylar surface at higher magnification; *arrow* stoma for gas exchange. **Fig**. **50** Longitudinal section through a carpel. **Fig**. **51** Stylar region with the epithelial nectary (*right*). **Fig**. **52** Cross section through the style. **Fig**. **53** Cross section through the ovary. *O* Ovary, *Ov* ovule, *Sti* stigma, *Sty* style

Graenicher 1910; Hegi 1912; Kugler 1970; Proctor et al. 1996; Kastinger and Weber 2001; Panov 2007), most of them blooming in spring. The majority of flowers visited are violet, purple, blue, but also white and yellow, covering different blossom types (e.g. tubular, salverform, disc-shaped). *Anemone nemorosa* cannot be found among the flowers recorded for visits by *Bombylius major* (citations see above). Although Kastinger and Weber (2001) cite Fritsch (1927a, b), we could not find *Bombylius major* as pollinator of *Anemone nemorosa* in the compilations of Fritsch. But *Bombylius venosus* is mentioned as visitor of *Anemone nemorosa* (and two further *Anemone* species) by Barwisch (1938). Knight (1967), however, emphasises that *Anemone nemorosa*, no matter how abundant, were never seen by him to be visited by *Bombylius* species (study area in the midlands near Warwick, UK).

Since *Bombylius* individuals fly early in the year (from the end of March to June), they may be quite important as pollinators of spring flowers. However, the range of plants visited varies depending on the local flora (e.g. Grimaldi 1988; Panov 2007). Temperature and solar radiation determine the distribution and emergence of *Bombylius major*: It will not fly in temperatures less than 62 °F (16.5 °C) or on dull days (Knight 1967). In spring 2010, individuals of *Bombylius major* were busy pollinators of *Anemone nemorosa* at our observation site, visiting flowers at different phases of anthesis. Due to the need to dissolve the dried up nectar, they spend some time on the flowers (buzzing is briefly interrupted: Fig. 1). When spreading the labella, they move further down and thus come into contact with stigmas or anthers of the slightly proterogynous flowers (Figs. 1–4). Since, in addition to proterogyny, *Anemone nemorosa* is

self-incompatible (Shirreffs 1985; Müller et al. 2000), self-fertilisation is avoided.

Most authors emphasise the importance of vegetative reproduction by rhizome branching and fragmentation compared with sexual reproduction by seeds in *Anemone nemorosa* (e.g. Shirreffs 1985), and sexual reproduction has been considered to play only a marginal role in established populations (Tumidajowicz 1975). However, recent studies show that the amount of reproduction by seeds is higher than previously thought (Holderegger et al. 1998; Müller et al. 2000), and that there is high genetic variation in the population structure, confirming the importance of sexual reproduction (Stehlik and Holderegger 2000).

Nectar in other multistaminate Ranunculaceae and in *Magnolia*

Outside the Magnoliaceae and Ranunculaceae, nectar-secreting tissue is recorded in a number of multistaminate members of the lower organisational level of the angiosperms, e.g. Nymphaeaceae (Schneider et al. 2003), Illiciaceae (Thien et al. 1983), Calycanthaceae, Lauraceae, Monimiaceae (Endress 1992, 2010), and Annonaceae (Silberbauer-Gottsberger et al. 2003).

Ranunculaceae belong to the early-diverging eudicots and are a "transitional" group between basal angiosperms and core eudicots. This in-between group is diverse in its floral characters, including the site of nectar secretion. Most ranunculaceous flowers offer nectar in addition to pollen. The special nectary organs between perianth and androecium, which serve in nectar production and nectar presentation, are known widely and have been investigated developmentally in a number of studies (e.g. Hiepko 1965; Kosuge and Tamura 1989; Erbar et al. 1999; Ren et al. 2009, 2011; Zhao et al. 2012a). Their shapes can vary considerably: tubular, linear-oblong, flat (petaloid with the basal nectary pit mostly covered with a scale) or spurred. The nectariferous tissue is always mesophyllary. With *Ranunculus aconitifolius* we investigated for the first time the ontogeny of a tubular instead of a flat scale in a species outside the batrachian group. The results do not contradict the ontogenetic or structural resemblance to stamens (see Erbar et al. 1999; Leins and Erbar 2010). In *Ranunculus* (= *Batrachium*) *bungei* the ontogeny of the tubular scale is similar to *Ranunculus aconitifolius*: A horseshoe-shaped ridge develops into a circular rim and finally into an oblique cup-shaped scale (Zhao et al. 2012b; see also adult scale shapes in *Ranunculus* subgen. *Batrachium* in Dahlgren 1992). In *Ranunculus sceleratus* (Zhao et al. 2012b), early stages resemble those of *Ranunculus aconitifolius* and *Ranunculus bungei*, but formation of a circular rim does not occur, so that in adult stages the pocket-like nectary has a horseshoe-shaped rim.

It is well-known that some members of *Clematis* (Kratochwil 1988) and *Pulsatilla* (Daumann and Slavikova 1968) secrete nectar at the filaments; however, detailed studies are lacking. We could confirm that in *Pulsatilla* (*P. turczaninovii*) nectar is secreted by the outer short, club-shaped sterile stamens (staminodes), either from the filament or from the entire staminode (mesophyllary nectary). Within the genus *Clematis*, there are both pure pollen flowers such as *Clematis vitalba*, in which all stamens are fertile, and pollen-nectar flowers such as *Clematis alpina* with outer spatulate staminodes. However, it is not the staminodes, but the inner side of the fertile stamens that are the sites of epithelial nectary.

In addition to these cases of androecial nectaries (situated at staminodes or stamens), there is a well-known carpellary nectary, namely in the flower of *Caltha palustris*, which has been studied intensively by Petersen et al. (1979). Until this study, *Caltha* was the only case in Ranunculaceae in which a carpellary nectary was known. Its patches of hairs on both carpel flanks differ from the nectary of *Anemone nemorosa* presented here. There was a lost hint in an old textbook by Fritch and Salisbury from 1920 that, in *Anemone nemorosa*, nectar is secreted by papilla-like epidermal cells. Nevertheless, our investigations confirm this early observation, and add a new type of a carpellary nectary within the family Ranunculaceae. Fritch and Salisbury (1920, p. 148), however, do not specify which floral organ secretes nectar in *Anemone nemorosa*. Van Tieghem (1892, p. 432) assumed "un nectaire diffus" in the receptacle (in both *Anemone nemorosa* and *Caltha palustris*).

It is of interest that the site of nectar secretion differs in *Anemone nemorosa* and *Pulsatilla*. Recent molecular data suggest the inclusion of *Pulsatilla* (and further small genera) in a large genus *Anemone*. *Pulsatilla* (*Anemone* section *Pulsatilla*) appears to be sister to *Anemone* s.s. (*Anemone* section *Anemone*; Hoot et al. 2012).

Since an epithelial ovarian nectary, which we report for the first time in Ranunculaceae, was described by Daumann (1930) in *Magnolia* species, though without satisfactory evidence, we investigated *Magnolia stellata* in detail. Nectar secretion takes place only at the beginning of anthesis. As in *Anemone nemorosa*, there is no nectariferous tissue below the epidermis. We assume that nectar is secreted through the epidermal cell wall; we never found any nectary slits. There may be channels in the cuticula through which the nectar exits (as reported for the nectary of *Vicia* by Gunning and Steer 1975). In contrast to *Anemone nemorosa*, in *Magnolia* there is an epithelial nectary involving the epidermis of the entire carpel (and not only the epidermis of the ovary).

After preparation for SEM, the carpel surfaces of *Magnolia stellata* are evenly coated with a granular substance, presumably crystallized nectar (Figs. 47–49),

although distinct small nectar droplets are visible in the flowers (Fig. 29). After fixation, we consistently observed solidification or flocculation of substances in areas covered by nectar in the flowers in different groups of flowering plants. It is not known whether the granular material consists of sugar or more complex chemicals. Although the main ingredients of nectar are the three sugars sucrose, glucose, and fructose (and other carbohydrates like maltose and raffinose in small amounts), followed by amino acids and proteins (e.g. enzymes like invertase), nectar contains many other compounds, such as inorganic ions, organic acids, vitamins, antioxidants, phenolics, alkaloids, lipids, and terpenoids in minor concentrations (Lüttge 1961, 1962; Baker and Baker 1983; Nicolson and Thornburg 2007). Nectar chemistry may be altered by fixation with FAA (formalin, acetic acid, alcohol, water) as used in our preparation process. Pacini et al. (2003) emphasise that nectar is not removed during fixation, dehydration and staining with PAS (periodic acid Schiff).

The diverse structures, the rare occurrence, and scattered distribution of nectaries in the basal groups indicate convergent evolution. Disc nectaries, however, seem to occur only outside the ANITA (or ANA) grade, magnoliids, monocots, and also outside Ranunculales among basal eudicots (Endress 2008, 2010, 2011). Disc nectaries, or better receptacular disc nectaries, with the typical nectary slits are characteristic of many eudicots. It is worth mentioning that, in core eudicots, a transcription factor encoded by the *CRABS CLAW* gene is required for nectaries that may occur anywhere in the flower. First results in a limited number of taxa examined (e.g. *Arabidopsis*, *Cleome*, *Nicotiana*, *Petunia*) indicate that, irrespective of the position within the flower, the *CRABS CLAW* gene is essential for nectary development (Bowman and Smyth 1999; Lee et al. 2005a, b). Its expression is limited mostly to carpels (in that its ancestral function is involved in suppressing early radial growth of the gynoecium and in promoting its later elongation) and nectaries (Bowman and Smyth 1999; Fourquin et al. 2005). However, in basal eudicots, no evidence for expression of this gene in nectaries could be found (nectary spur of *Aquilegia* investigated; Lee et al. 2005a, b).

The evidence of nectar in *Anemone nemorosa* (fluid or solidified) may also resolve the puzzle that there are reports on insects tapping juicy tissues with the proboscis in what were thought to be nectar-less flowers: Müller (1873) reported a "piercing" bee-fly (*Bombylius canescens*) working on *Hypericum perforatum* and a "piercing" honey bee on *Anemone nemorosa*. It was assumed (see also Bonnier 1879 and Knuth 1898) that the insect bores with its proboscis into the base of the flower to obtain sap from floral tissue. However, Schremmer (1961) already pointed out that *Bombylius* is not able to penetrate floral tissue with its proboscis.

Why nectar secretion in pollen flowers?

In magnoliids, floral rewards are pollen, nectar, and food bodies (e.g. *Calycanthus*, Calycanthaceae) and, in some cases, pollination chambers (members of Annonaceae). If nectar, mostly in small amounts, is secreted, the nectaries are located on tepals, staminodes, stamens, or carpels (C.E., manuscript in preparation).

In *Anemone nemorosa*, *Caltha palustris*, and *Pulsatilla turczaninovii* as well as in *Magnolia stellata*, nectar production is limited mainly to the female phase of the (at least slightly) proterogynous flower. By this, the attractiveness of the flower is also assured in the non-pollen presenting phase of anthesis (or early male phase with only little pollen offered). Especially in *Magnolia*, with its numerous carpels (about 40 in *Magnolia stellata*) on the cone-like receptacle, the economic disadvantage of a choricarpous gynoecium compared to a coenocarpous one is compensated by nectar secretion of every carpel. In a coenocarpous gynoecium an uneven pollen deposition onto the stigmata can nevertheless result in the fertilization of all ovules due to the common inner gynoecial space, the so-called compitum (Endress 1982; Leins and Erbar 2010). However, when licking up the nectar droplets from all carpels in the choricarpous *Magnolia* flowers, contact of the insect's body with all stigmas might be achieved and thus, at best, fertilization of the ovules in all carpels.

References

Baker, H. G., & Baker, I. (1983). A brief historical review of the chemistry of floral nectar. In B. Bentley & T. Elias (Eds.), *The biology of nectaries* (pp. 126–152). New York: Columbia University Press.

Barwisch, H. (1938). Biologische Untersuchungen der blütenbesuchenden Insekten am Südwestabhang des Bisamberges. Dissertation, University of Vienna.

Boesi, R., Polidori, C., & Andrietti, F. (2009). Searching for the right target: oviposition and feeding behaviour in *Bombylius* bee flies (Diptera: Bombyliidae). *Zoological Studies, 48*, 141–150.

Bonnier, G. (1879). Les nectaires. Étude critique, anatomique et physiologique. *Annales des Sciences Naturelles*, *Botanique*, *Série 6*, *8*, 5–212.

Bowman, J. L., & Smyth, D. R. (1999). *CRABS CLAW*, a gene that regulates carpel and nectary development in *Arabidopsis*, encodes a novel protein with zinc finger and helix-loop-helix domains. *Development, 126*, 2387–2396.

Dahlgren, G. (1992). *Ranunculus* subgenus *Batrachium* on the Aegean islands and adjacent areas: nectary types and breeding system. *Nordic Journal of Botany, 12*, 299–310.

Daumann, E. (1930). Das Blütennektarium von *Magnolia* und die Futterkörper in der Blüte von *Calycanthus*. *Planta, 11*, 108–116.

Daumann, E., & Slavikova, Z. (1968). Zur Bluetenmorphologie der tschechoslowakischen *Clematis*-Arten. *Preslia, 40*, 225–244.

Deyrup, M. A. (1988). Pollen-feeding in *Poecilognathus punctipennis* (Diptera, Bombyliidae). *Florida Entomologist, 71*, 597–605.

Dimmock, G. (1881). *The anatomy of the mouthparts and of the sucking apparatus of some Diptera*. Boston: Williams.

Endress, P. K. (1982). Syncarpy and alternative modes of escaping disadvantages of apocarpy in primitive angiosperms. *Taxon, 31*, 48–52.

Endress, P. K. (1992). Protogynous flowers in Monimiaceae. *Plant Systematics and Evolution, 181*, 227–232.

Endress, P. K. (2008). Perianth biology in the basal grade of extant angiosperms. *International Journal of Plant Sciences, 169*, 844–862.

Endress, P. K. (2010). Flower structure and trends of evolution in eudicots and their major subclades. *Annals of the Missouri Botanical Garden, 97*, 541–583.

Endress, P. K. (2011). Evolutionary diversification of the flowers in angiosperms. *American Journal of Botany, 98*, 370–396.

Erbar, C., Kusma, S., & Leins, P. (1999). Development and interpretation of nectary organs in Ranunculaceae. *Flora, 194*, 317–332.

Fourquin, C., Vinauger, M., Fogliani, B., Dumas, C., & Scutt, C. P. (2005). Evidence that *CRABS CLAW* and *TOUSLED* have conserved their roles in carpel development since the ancestor of the extant angiosperms. *Proceedings of the National Academy of Sciences of the United States of America, 102*, 4649–4654.

Fritch, F. E., & Salisbury, E. J. (1920). *An introduction to the structure and reproduction of plants*. London: Bell.

Fritsch, K. (1927a). Beobachtungen über blütenbesuchende Insekten in Steiermark, 1905. *Feddes Repertorium, 46*, 41–70.

Fritsch, K. (1927b). Beobachtungen über blütenbesuchende Insekten in Steiermark 1906. *Sitzungsberichte der Akademie der Wissenschaften in Wien, Mathematisch-Naturwissenschaftliche Klasse Abt. I, 136*, 441–476.

Gilbert, F. S., & Jervis, M. (1998). Functional, evolutionary and ecological aspects of feeding-related mouthpart specializations in parasitoid flies. *Biological Journal of the Linnean Society, 63*, 495–535.

Graenicher, S. (1910). The bee-flies (Bombyliidae) in their relations to flowers. *Bulletin of the Wisconsin Natural History Society, 8*, 91–101.

Grimaldi, D. (1988). Bee flies and bluets, *Bombylius* (Diptera, Bombyliidae) flower constant on the distylous species, *Hedyotis caerula* (Rubiaceae), and the manner of foraging. *Journal of Natural History, 22*, 1–10.

Gunning, B. E. S., & Steer, M. W. (1975). *Plant cell biology, an ultrastructural approach*. London: Arnold.

Hegi, G. (1912). *Illustrierte Flora von Mitteleuropa III*. München: Lehmanns.

Hiepko, P. (1965). Vergleichend-morphologische und entwicklungsgeschichtliche Untersuchungen über das Perianth bei den Polycarpicae. *Botanische Jahrbücher für Systematik, 84* (359–426), 427–508.

Holderegger, R., Stehlik, I., & Schneller, J. J. (1998). Estimation of the relative importance of sexual and vegetative reproduction in the clonal woodland herb *Anemone nemorosa*. *Oecologia, 117*, 105–107.

Hoot, S. B., Meyer, K. M., & Manning, J. C. (2012). Phylogeny and reclassification of *Anemone* (Ranunculaceae), with an emphasis on austral species. *Systematic Botany, 37*, 139–152.

Kastinger, C., & Weber, A. (2001). Bee-flies (*Bombylius* spp., Bombyliidae, Diptera) and the pollination of flowers. *Flora, 196*, 3–25.

Knight, G. H. (1967). Observations of the behaviour of *Bombylius major* L. and *B. discolor* Mik. in the Midlands. *Entomologist's Monthly Magazine, 103*, 177–181.

Knoll, F. (1921). Insekten und Blumen. II. Bombylius fuliginosus und die Farbe der Blumen. *Abhandlungen der Zoologisch-Botanischen Gesellschaft in Wien, 12*, 17–119.

Knuth, P. (1898). *Handbuch der Blütenbiologie II*. Leipzig: Engelmann.

Kosuge, K., & Tamura, M. (1989). Ontogenetic studies on petals of the Ranunculaceae. *The Journal of Japanese Botany, 64*, 65–74.

Kratochwil, A. (1988). Zur Bestäubungsstrategie von *Pulsatilla vulgaris* Mill. *Flora, 181*, 261–324.

Krenn, H. W., Plant, J. D., & Szucsich, N. U. (2005). Mouthparts of flower-visiting insects. *Arthropod Structure & Development, 34*, 1–40.

Kugler, H. (1970). *Blütenökologie*. 2.Aufl. Stuttgart: Fischer.

Lee, J.-Y., Baum, S. F., Oh, S. H., Jiang, C.-Z., Chen, J.-C., & Bowman, J. L. (2005). Recruitment of *CRABS CLAW* to promote nectary development within the eudicot clade. *Development, 132*, 5021–5032.

Lee, J.-Y., Baum, S. F., Alvarez, J., Patel, A., Chitwood, D. H., & Bowman, J. L. (2005). Activation of *CRABS CLAW* in the nectaries and carpels of *Arabidopsis*. *The Plant Cell, 17*, 25–36.

Leins, P., & Erbar, C. (2010). *Flower and Fruit. Morphology, Ontogeny, Phylogeny, Function and Ecology*. Stuttgart: Schweizerbart.

Lüttge, U. (1961). Über die Zusammensetzung des Nektars und den Mechanismus seiner Sekretion. I. *Planta, 56*, 189–212.

Lüttge, U. (1962). Über die Zusammensetzung des Nektars und den Mechanismus seiner Sekretion. II. *Planta, 59*, 108–114.

Müller, H. (1873). *Die Befruchtung der Blumen durch Insekten und die gegenseitigen Anpassungen beider*. Leipzig: Engelmann.

Müller, A., Schneller, J. J., & Holderegger, R. (2000). Variation in breeding system among populations of the common woodland herb *Anemone nemorosa* (Ranunculaceae). *Plant Systematics and Evolution, 221*, 69–76.

Neff, J. L., Simpson, B. B., Evenhuis, N. L., & Dieringer, G. (2003). Character analysis of adaptations for tarsal pollen collection in the Bombyliidae (Insecta, Diptera), the benefits of putting your foot in your mouth. *Zootaxa, 157*, 1–14.

Nicolson, S. W., & Thornburg, R. W. (2007). Nectar chemistry. In S. W. Nicolson, M. Nepi, & E. Pacini (Eds.), *Nectaries and nectar* (pp. 215–264). Dordrecht: Springer.

Pacini, E., Nepi, M., & Vesprini, J. L. (2003). Nectar biodiversity: a short review. *Plant Systematics and Evolution, 238*, 7–21.

Panov, A. A. (2007). Sex-related diet specificity in *Bombylius major* and some other Bombyliidae (Diptera). *Entomological Review, 87*, 812–821.

Petersen, R. L., Scott, M. G., & Miller, S. L. (1979). Some aspects of carpel structure in *Caltha palustris* L. (Ranunculaceae). *American Journal of Botany, 66*, 334–342.

Proctor, M., Yeo, P., & Lack, A. (1996). *The natural history of pollination*. Portland: Timber.

Ren, Y., Chang, H., Tian, X., Song, P., & Endress, P. K. (2009). Floral development in Adonideae (Ranunculaceae). *Flora, 204*, 506–517.

Ren, Y., Gu, T., & Chang, H. (2011). Floral development of *Dichocarpum*, *Thalictrum*, and *Aquilegia* (Thalictroideae, Ranunculaceae). *Plant Systematics and Evolution, 292*, 203–213.

Schneider, E. L., Tucker, S. C., & Williamson, P. S. (2003). Floral development in the Nymphaeales. *International Journal of Plant Sciences, 164*, S279–S292.

Schremmer, F. (1961). Morphologische Anpassungen von Tieren - insbesondere Insekten - an die Gewinnung von Blumennahrung. *Verhandlungen der Deutschen Zoologischen Gesellschaft, 25*, 375–401.

Schuhmacher, H., & Hoffmann, H. (1982). Zur Funktion der Mundwerkzeuge von Schwebfliegen bei der Nahrungsaufnahme (Diptera, Syrphidae). *Entomologia Generalis, 7*, 327–342.

Shirreffs, D. A. (1985). Biological Flora of the British Isles. *Anemone nemorosa* L. *Journal of Ecology, 73*, 1005–1020.

Silberbauer-Gottsberger, I., Gottsberger, G., & Webber, A. C. (2003). Morphological and functional flower characteristics of New and

Old World Annonaceae with respect to their mode of pollination. *Taxon, 52*, 701–718.

Sprengel, C. K. (1793). *Das entdeckte Geheimnis der Natur im Bau und in der Befruchtung der Blumen*. Berlin: Vieweg.

Stehlik, I., & Holderegger, R. (2000). Spatial genetic structure and clonal diversity of *Anemone nemorosa* in late successional deciduous woodlands of Central Europe. *Journal of Ecology, 88*, 424–435.

Szucsich, N. U., & Krenn, H. W. (2002). Flies and concealed nectar sources, morphological innovations in the proboscis of Bombyliidae (Diptera). *Acta Zoologica, 83*, 183–192.

Thien, L. B., White, D. A., & Yatsu, L. Y. (1983). The reproductive biology of a relict *Illicium floridanum* Ellis. *American Journal of Botany, 70*, 719–727.

Tumidajowicz, D. (1975). Population dynamics of *Anemone nemorosa* in the Niepolomice forest. *Bulletin de l'Académie Polonaise des Sciences. Série des Sciences Biologiques, 23*, 101–108.

Van Tieghem, P. (1892). *Traité de botanique*. BHL. http://www.biodiversitylibrary.org/page/24076964

Zhao, L., Wang, W., Ren, Y., & Bachelier, J. B. (2012). Floral development in *Asteropyrum* (Ranunculaceae), implications for its systematic position. *Annales Botanici Fennici, 49*, 31–42.

Zhao, L., Bachelier, J. B., Chang, H.-L., Tian, X.-H., & Ren, Y. (2012). Inflorescence and floral development in *Ranunculus* and three allied genera in Ranunculeae (Ranunculoideae, Ranunculaceae). *Plant Systematics and Evolution, 298*, 1057–1071.