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ORIGINAL RESEARCH PAPER

Ecological aspects of the floral structure and flowering in *Pulsatilla* species

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Abstract

In terms of flowering ecology, *Pulsatilla* flowers are classified as "pollen flowers" producing inconsiderable amounts of nectar. The aim of this study was to assess the length of the flowering period in *Pulsatilla slavica* and *P. vulgaris* and to investigate the structure of the epidermis of the perianth and generative elements of their flowers. Special focus was placed on the structure of hairs and the distribution of stomata. The weight of nectar released by the flowers of the two *Pulsatilla* species and the content of sugars in the nectar was also evaluated. In SE Poland, both species flowered for similar periods between the second half of April and the first half of May. The flower life-span of both was determined to be 9–14 days. The lower part of each sepal was observed to be covered by long hairs having cellulose-pectin cell walls of varying thickness. Hairs present on the pistil style are thinner; they may provide some protection against cold and can play a role of a secondary pollen presenter for insects. The bowl-shaped structure of the perianth and the nature of the adaxial surface of the sepal epidermis may facilitate reflection of sunlight into the inner parts of the flower, which may contribute to an elevation of the intraflower temperature. This is particularly important for the functionality of the ovary. The surface of the hairs was seen to be covered by a cuticle ensuring water impermeability. Flowers are visited by honeybees, bumblebees, butterflies, and ants, for which nectar and pollen are the main attractants. Ants, which are regarded as illegitimate flower visitors, were found to cause damage to the androecium. The number of fruits produced in the flowers of both *Pulsatilla* species was lower than 50% of the number of pistils.

Keywords

pasque flower; structure; flowers; hairs; stomata; attractiveness to insects; nectar; pollen

Introduction

The genus *Pulsatilla* (Ranunculaceae) comprises more than 30 species growing in the Northern Hemisphere, primarily in Europe and temperate Asia, with an eastern occurrence range in Japan. Two species occur in North America [1,2]. Species growing in Europe are perennials, characteristic for members of xerothermic communities. Since they have large, decorative flowers, many species are cultivated as early-spring ornamental plants with *P. vulgaris* subsp. *grandis* as one of the most attractive [2]. Different cultivars of this species have flowers varying in color from dark purple through blue-violet and red to white [3]. In Poland, there are six native *Pulsatilla* species growing in different habitats: *P. alba*, *P. patens*, *P. pratensis*, *P. slavica*, *P. vernalis*, and *P. vulgaris*. All these species are strictly protected but *P. vulgaris* probably no longer grows in natural sites in

Poland [4,5]. Recent investigations of the ecological structure of *P. patens* populations in northeast Poland (Białowieża Primeval Forest) have demonstrated low numbers of individuals of this species [6].

From the point of view of flowering ecology, there are many important traits in the flower structure and function determining reproductive success. These include the size, shape, symmetry, color, odor, and texture of the perianth, the presence of color indicators, exposure of stamens and pistils, and floral rewards for pollinators, i.e., pollen, nectar, heat, resin, and oil [7–10]. The large *Pulsatilla* flowers with variously colored perianths formed by petaloid sepals are attractive to many insect groups such as small bees and beetles [11], bumblebees [12–15], solitary bees [15], honeybees [8,16], and butterflies [7,17]. Pollen is the main floral reward offered to pollinators [7,10,18]. It has been shown that in *P .vulgaris* and *P. rubra* the stamens and pollen emit a scent and the chemical composition of these anther volatiles has been determined [19]. Some species of *Pulsatilla* develop staminodial nectaries and produce nectar [7,14,17,18]. In several species of this genus, for example in *P. alpina*, no floral nectaries have been found [19,20]. *Pulsatilla* flowers have been classified as either bell-shaped [7] or bowl-shaped [19] on the basis of the perianth shape, which determines the accessibility of nectar and pollen to pollinators,

Studies conducted by several researchers have indicated that for some *Pulsatilla* species the temperature in the interior of flowers is higher than the ambient temperature. Knutson [11] found that the intraflower temperature in *P. patens* was 8.9–10.0°C higher than that of the surrounding environment in bright sunshine. Luzar and Gottsberger [21] showed that in *P. alpina* the temperature exceeded ambient values by a maximum of 6.2°C. This species was found to be the most effective in terms of floral heating among five alpine species studied. It is assumed that the increased intraflower temperature in plants flowering in early spring accelerates the development of pistils and pollen tubes as well as offers an additional reward for pollinators in the form of intrafloral warming and heated nectar [9].

The aim of the present study was to compare the length of the flowering period in two species from the genus *Pulsatilla* and to document ecologically-significant features in the structure of the perianth, gynoecium, and androecium with special emphasis on the structure of hairs and the distribution of stomata. An assessment of the attractiveness of *Pulsatilla* flowers to insects was also performed, complemented by an evaluation of nectar production in the two species.

Material and methods

The *Pulsatilla* species selected for study were *P. vulgaris* Mill., *P. slavica* G. Reuss., and *P. pratensis* (L.) Mill. growing in the collection of the Botanical Garden of Maria Curie-Skłodowska University in Lublin, and three cultivars of *P. vulgaris* Mill., 'Roede Klokke', 'Blue Shadow', and 'Alba' collected from a private garden in Świdnik near Lublin.

Nectar production by flowers of *P. vulgaris* and *P. slavica* was investigated using the method developed by Jabłoński [22]. The flowering dates and flowering duration in 2009–2011 were recorded in both species and in the three ornamental cultivars of *P. vulgaris*. The life-span of a single flower was recorded and the percentage of fruits set calculated. Sepal structure, generative elements, and staminodia were investigated using both light and scanning electron microscopy.

Light microscopy (LM) and histochemical tests

The staminodia, stamens, and pistils were counted (n = 30 for each structure) under a stereoscopic microscope STM 800 (Microlab). The lengths of stamens, pistils, staminodia, and fruits (n = 30 for each structure) were measured. Glycerine preparations were made from fresh sepals (marginal portions of the central area of the sepal), stamens, pistils, staminodia, and pollen grains. The lengths of the hairs located on the surface of the sepals and pistils and the thickness of the hair cell walls were measured (n = 30). Additionally, the polar (P) and equatorial (E) axes of the pollen grains (n = 50) were measured and the shape indices (P/E) calculated. The hair preparations were subjected to histochemical tests to assess the presence of lipids using Sudan IV (POCH) [23], pectins – ruthenium red (POCH) [23], polysaccharides – periodic acid–Schiff reagent (PAS) (Sigma-Aldrich) [24], and lignin – toluidine blue (Sigma-Aldrich) [25], phloroglucinol-HCl (POCH) [26], and safranin (Sigma-Aldrich) [27]. In order to detect the presence of pollenkitt on the surface of the pollen grains and to visualize exine ornamentation, various reagents were used for staining: basic fuchsin (POCH) [28], Sudan IV, toluidine blue, malachite green (POCH) [27], ruthenium red, phloroglucinol-HCl, and safranin. All stains were matched with non-stained plant material. Photographic documentation was prepared with a Coolpix 4500 (Nikon) camera coupled to an Eclipse 400 (Nikon) light microscope.

Scanning electron microscopy (SEM)

Fragments of sepals, staminodia, and stamens were fixed at a temperature of 4°C for 12 h in a 2% solution of glutaraldehyde with 2.5% paraformaldehyde in 0.75 M phosphate buffer (pH 6.8). Subsequently, the samples were rinsed with the same buffer and then dehydrated in an ethanol series and dried at the critical point in liquid CO_2 using a K850 (Emitech) dryer. They were then coated with gold using a sputter coater K550X (Emitech). The preparations were examined under a Tescan Vega II LMU (Tescan) scanning electron microscope at an accelerating voltage of 30 kV.

Nectar production

Nectar was sampled from the flowers of *P. pratensis* and *P. slavica* in 2010, on the fifth day of flower life in the initial pollen release phase (first anthers opened, the others closed). These flowers had been isolated from insects with a tulle net for three sunny days. Nectar collection was carried out on April 28, 2010 at 6 p.m. It was placed in tared glass Jabłoński micropipettes [29], the pipettes weighed on a WPS 36 electronic balance (Radwag), and the weight of nectar calculated. Subsequently, a drop of nectar was placed in an Abbe refractometer (Carl Zeiss AG) and the concentration of sugars recorded. The total sugar (mg) secreted in nectar per 10 flowers was then calculated.

Results

Flowering time and span

Pulsatilla slavica and *P. vulgaris* plants flowered from the second half of April to the first week of May in 2010. However, the duration of *P. slavica* flowering was 5 days longer than that of the other species recorded (Tab. 1). The flowers of both species had similar periods of anthesis persisting for 9–10 days (Tab. 1). Depending on the year, the ornamental cultivars of *P. vulgaris* flowered between the first half of April and the first 10 days of May (Tab. 2, Fig. 1). Cultivars 'Roede Klokke' and 'Blue Shadow' entered anthesis at the same date in both study years. Cultivar 'Alba' commenced flowering a week later than the other two in 2011 (Tab. 1, Fig. 1). The longest flowering period (nearly 18 days) in both study years was noted for cv. 'Blue Shadow', and the other two cultivars had a shorter flowering period (ca. 2 weeks). The longest, i.e., almost 2-week, persistence of flowers was noted in cultivars 'Blue Shadow' and 'Roede Klokke', whereas the life-span of the white flowered cultivar was shorter, on average by 5 days (Tab. 2).

Flower and fruit morphology

Plants of *P. slavica* and *P. vulgaris* flowered abundantly (Fig. 2a). The mean flower life-span was 9.1 and 10.3 days, respectively. The flowers were visited by honeybees (Fig. 2e), bumblebees, butterflies, and flies (Fig. 2d) opening around noon and most

Tab. 1 Morphological features of flowers and the flowering period of two *Pulsatilla* species in 2010.

Features	*Pulsatilla slavica*			*Pulsatilla vulgaris*		
	range	mean	*SD*	range	mean	*SD*
Flowering period (days)	April 11 – May 7 (27)			April 15 – May 5 (22)		
Flower life-span (days)	5–12	9.1	±2.2	8–11	10.3	±1.0
Number of stamens	192–305	258.0	±31.5	197–278	233.0	±26.1
Number of staminodia	20–30	25.0	±2.8	16–31	25.0	±4.8
Number of pistils	135–236	179.0	±33.5	139–207	172.0	±16.4

Tab. 2 Flower life-span and the flowering period of three *Pulsatilla vulgaris* ornamental cultivars in 2010–2011.

Cultivar	Year	Flowering period (days)	Flower life-span (days)		
			range	mean	*SD*
'Alba' 	2010	18 April – 7 May (19)	7–12	9.0	±2.4
	2011	16 April – 25 April (9)	7–10	8.3	±1.2
Mean		(14)		8.7	
'Blue Shadow' 	2010	18 April – 7 May (19)	7–13	11.4	±2.3
	2011	9 April – 25 April (16)	14–19	15.7	±2.4
Mean		(17.5)		13.6	
'Roede Klokke' 	2010	19 April – 3 May (14)	7–11	9.7	±1.9
	2011	9 April – 25 April (16)	14–19	15.7	±2.4
Mean		(15)		12.7	

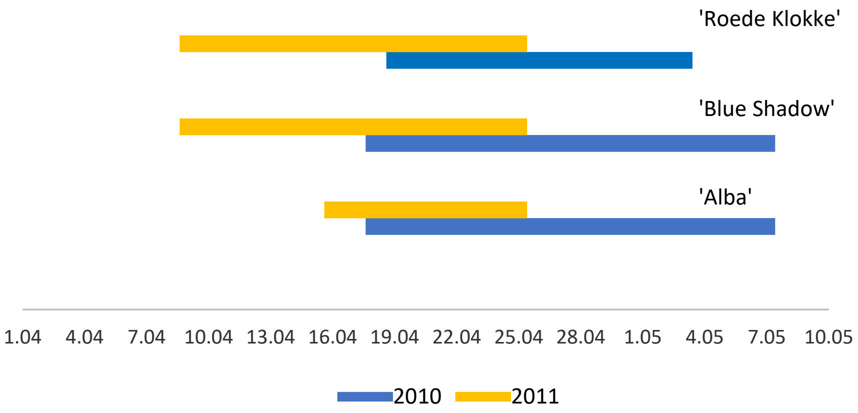

Fig. 1 Comparison of the flowering period of three *P. vulgaris* ornamental cultivars in 2010–2011.

Fig. 2 General habit of flowers and elements of the perianth in *P. slavica* and *P. vulgaris*. **a** General habit of *P. slavica*. **b** Bowl-shaped flower of *P. slavica*; the arrow indicates hairs on the abaxial surface of sepals. **c** Mechanical hairs on the abaxial surface of a *P. vulgaris* sepal (stained with the periodic acid–Schiff reagent; insert – ruthenium red reaction). **d** Ants – nectar "robbers" (arrows) in a *P. slavica* flower; the arrowheads indicate bitten off anthers. **e** *Apis mellifera* worker in a *P. vulgaris* flower; the arrow indicates hairs on the abaxial surface of sepals. **f,g** Fragments of mechanical hairs with irregularly thickened cell walls on the abaxial surface of *P. vulgaris* sepals (**f** Sudan IV staining; insert – non-stained control). **h** Fragment of the marginal area of a *P. slavica* sepal (adaxial surface) (SEM). **i** Adaxial epidermis cells in a *P. slavica* petaloid sepal (SEM). **j,k** Stomata on the abaxial surface of *P. vulgaris* sepals [periodic acid–Schiff reagent (**j**) or no staining (**k**)]; the arrow indicates chloroplasts. **l** Fragment of the abaxial surface of a *P. slavica* sepal with visible hairs; the arrows indicate massive hair base cells (SEM).

often assuming an erect position (Fig. 2a). The blue-purple perianth in *P. slavica* was composed of six to eight petaloid sepals and always six in *P. vulgaris* (Fig. 2b). The abaxial surface of the sepals in both species was covered by numerous silky hairs (Fig. 2b,c,e,f,k), with a length in the range of 0.89–2.35 (mean 1.37) mm in *P. vulgaris*. The diameter of the hairs in this species was in the range of 11.9–14.3 µm. These hairs were dead, cylindrical, pointed, and unicellular (Fig. 2c). Substantial differences were noted in the thickness of opposite cell walls of some hairs (Fig. 2f) with wall thickness ranging from 1.93 to 6.43 µm (mean 4.0 µm). Hair cell walls were non-lignified (negative result of the acid phloroglucinol assay) and contained pectin (positive result of the ruthenium red assay) (a magnified fragment in Fig. 2c). The hairs in both *Pulsatilla* species had a massive base formed of five to six cells (Fig. 2k). Numerous, evenly distributed stomata were found in the sepal abaxial epidermis (Fig. 2i,l). In both species, the upper surface of the sepals was slightly glossy (Fig. 2b). Depressions were visible along the vascular bundles (Fig. 2g). The cells of the adaxial epidermis were slightly convex but do not form papillae (Fig. 2h) and were arranged in regular rows along the axis of the sepal.

The androecium of *P. slavica* had on average 258 stamens, but only 233 stamens were noted in *P. vulgaris* (Tab. 1) and a lower number (186–204) in the ornamental cultivars. The length of the stamens in *P. vulgaris* flowers was in the range of 5.6–10.1 mm. Stamens that were located higher on the receptacle had longer filaments (Fig. 3a,b). Mean anther length in *P. vulgaris* was 1.3 µm. The concave, widened connectivum (Fig. 3d) was covered by smaller epidermal cells than those on the anthers (Fig. 3e,f). There were stomata in the epidermis of this connectivum in both species (Fig. 3c,e,f).

Pollen grains of *P. vulgaris* were tricolpate with exine covered by microspines at a high density within the furrows (Fig. 3g). The surface of the pollen grains was shown to be abundantly covered by pollenkitt, by means of Sudan IV staining (Fig. 3j). There were visible columellae in the relatively thick exine (Fig. 3l,r). Mean length of the P axis of the pollen grains was 35.3 µm and the E axis 36.8 µm; the shape index (P/E) was 0.96, which indicates an oblate-spheroidal shape of the pollen grains. Toluidine blue and basic fuchsin staining of the pollen grains allowed better visualization of the exine sculpture both in the intercolpium and within the furrows (Fig. 3h,i,k). Malachite green stained the cell nuclei and exine fragments (Fig. 3p,r). Safranin produced red staining of the inner part of the pollen grain cell wall (Fig. 3m). After treatment with ruthenium red, the stain reaction indicating the presence of pectins was only visible within the furrows, in particular at their edges (Fig. 3n,o).

There were small, sterile stamens, i.e., staminodia, at the base of the androecium in the flowers of both *Pulsatilla* species, (Fig. 3b). These had reduced anthers and a strongly expanded connectivum and served as staminodial nectaries. There were stomata through which nectar exudation occurs on the abaxial part of the modified head of the staminodium. Drops of secreted nectar were observed on the surface of staminodia and at the base of the petaloid sepals. The structure of both the upper parts and filaments of the staminodium were polymorphic in their length, width, and shape (Fig. 5a–h).

The color of the gynoecium matched with that of the petaloid sepals and created an attractive contrast to the stamens (Fig. 2a,b, Fig. 4a). The mean number of pistils in the flower of *P. slavica* (Fig. 5k) and *P. vulgaris* was 179 and 172, respectively. The mean length of the ovary in *P. vulgaris* was 1.3 mm and the length of the style 14.7 mm. Both the ovary and pistil style were covered by dead hairs (Fig. 4f,h–k), which were similar to those observed on the sepals but were shorter, with a mean length of ca. 1.08 mm. The hair surface was covered by a thin cuticle (Fig. 4j). In contrast to the hairs present on the sepals, those located on the pistil were characterized by evenly thickened walls along its length (Fig. 4j,k). They were thinner (mean width 1.63 µm) than the sepal hairs and had numerous pollen grains attached (Fig. 4f,i). Stomata were visible in the style epidermis (Fig. 4h).

A vascular bundle containing tracheary elements with visible spiral secondarily thickened walls was present in the style (Fig. 4g). The asymmetric stigma of the pistil in *P. vulgaris* was equipped with many apically rounded papillae (Fig. 4b,c,e). Vacuoles of cells forming the papillae were stained with anthocyanins in the fresh material preparations (Fig. 4d). During fruit formation, the hairy style was strongly elongated (constituting 88% of the length of the entire fruit), forming a flight apparatus adapted to anemochory (Fig. 5i,j,l,m). This apparatus was nearly fourfold longer than the style (Fig. 5i). It was noted that the peduncles nearly doubled their length; 43% of *P. vulgaris*

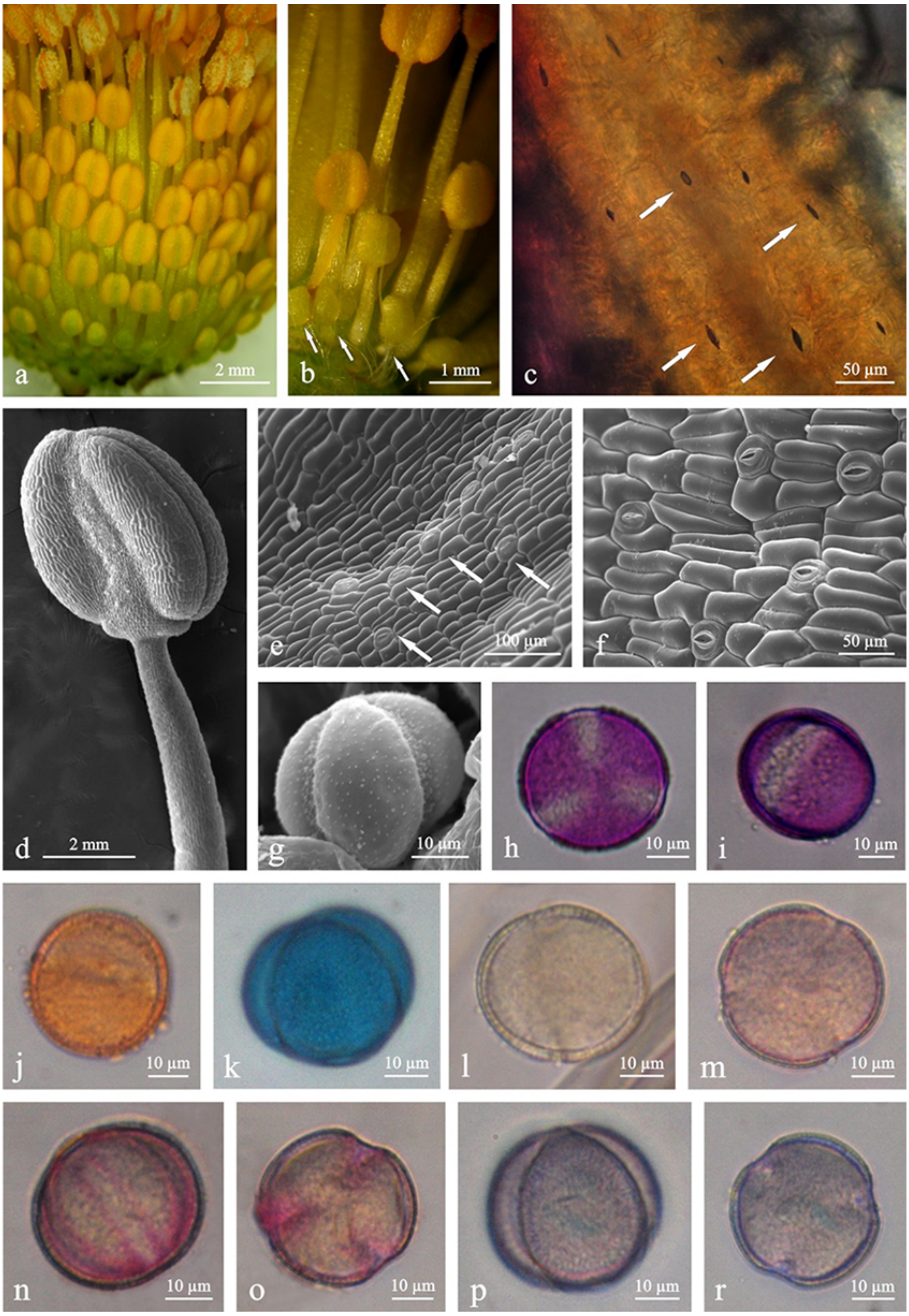

Fig. 3 Androecium and pollen grains in *P. slavica* and *P. vulgaris*. **a** Numerous spirally arranged stamens in *P. slavica*. **b** Differentially long stamens and staminodia (arrows) in a *P. slavica* flower. **c** Fragment of the adaxial surface of the connectivum in *P. vulgaris* with numerous stomata (arrows) – ruthenium red staining. **d** Fragment of a *P. slavica* stamen before pollen release. **e** Fragment of the adaxial surface of the connectivum in *P. slavica* with visible stomata (arrows; SEM). **f** Stomata on the adaxial surface of the connectivum in *P. slavica* (SEM). **g** Pollen grain of *P. slavica* in equatorial view under SEM. **h** Pollen grain of *P. slavica* in polar view under LM (fuchsin staining). **i** Pollen grain of *P. slavica* in equatorial view under LM (fuchsin staining). **j** Pollen grain of *P. vulgaris* in equatorial view (Sudan IV staining). **k** Pollen grain of *P. vulgaris* in equatorial view (toluidine blue staining). **l** Pollen grain of *P. vulgaris* in equatorial view (phloroglucinol-HCl staining). **m** Pollen grain of *P. vulgaris* in polar view (safranin staining). **n,o** Pollen grains of *P. vulgaris* in equatorial view (**n**), in polar view (**o**) after ruthenium red staining. **p,r** Pollen grains of *P. vulgaris* in equatorial view (**p**), in polar view (**r**), malachite green reaction.

Fig. 4 Stigmata and styles of *P. slavica* and *P. vulgaris* pistils. **a** Upper part of pistils and upper androecium whorl in a *P. slavica* flower. **b,c** Stigmata of *P. vulgaris* pistils with numerous surface papillae and pollen grains (arrows) (phloroglucinol-HCl staining). **d,e** Papillae on the stigma in *P. vulgaris*; Sudan IV (**d**) and phloroglucinol-HCl staining (**e**). **f** Fragment of the style in *P. vulgaris* with numerous hairs (toluidine blue staining). **g** Spiral vessels in the *P. vulgaris* style (phloroglucinol-HCl staining). **h** Fragment of *P. vulgaris* style with numerous stomata (arrows) and surface hairs playing the role of pollen presenters (phloroglucinol-HCl staining); the magnified fragment of the photograph shows a stoma. **i** Mechanical hairs on the surface of the *P. vulgaris* style and pollen grains (no staining). **j** Fragment of the *P. vulgaris* style with mechanical hairs (Sudan IV staining). **k** Fragment of a non-glandular hair from the surface of the *P. vulgaris* style (toluidine blue staining).

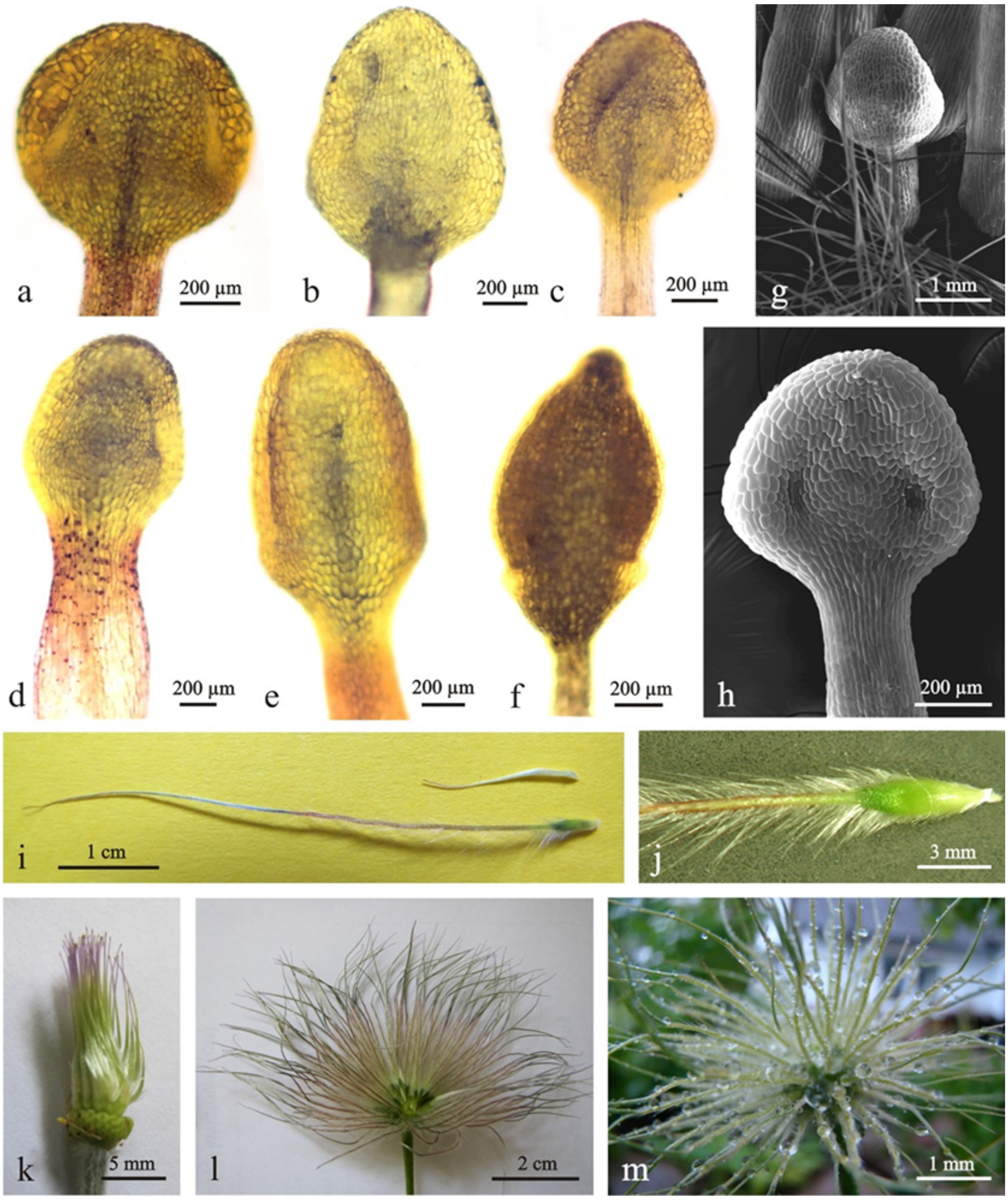

Fig. 5 Staminodia, pistils, and aggregate achenes in two pasque flower species. **a–f** Staminodia with various shapes in *P. vulgaris* flowers. **g,h** Staminodia in *P. slavica* viewed under SEM. **i** Morphology of the pistil and fruit in *P. slavica*. **j** Fragment of a *P. slavica* achene with the basal part of the style equipped with mechanical hairs facilitating dispersal. **k** *Pulsatilla slavica* gynoecium. **l** *P. slavica* aggregate achene. **m** *Pulsatilla vulgaris* aggregate fruit with rain drops retained on the hair surface.

pistils and 45.5% of those in *P. slavica* developed into achene fruits. Aggregate achenes were formed, on average, by 74 (*P. vulgaris*) and 81 (*P. slavica*) single fruits reaching nearly 4 cm in length. Due to the presence of the cuticle, the hairs covering the pistil were impermeable to water, thus offering protection for the fruit; water droplets were retained on the hair surface (Fig. 5m).

Nectar

The weight of the nectar sampled from 10 flowers was 11 mg in *P. slavica* and 19.75 mg in *P. pratensis* (Tab. 3). Sugar content in *P. pratensis* and *P. slavica* nectar were found to be 66.1% and 72.5%, respectively. The weight of sugars in the nectar from the *P. pratensis* flowers was therefore almost twofold greater than that in *P. slavica* nectar (Tab. 3).

Tab. 3 Nectar production (mg), sugar concentration (% w/w), and sugar mass (mg) in 10 flowers of *P. pratensis* and *P. slavica*.

Features	*Pulsatilla pratensis*			*Pulsatilla slavica*		
	range	mean	*SD*	range	mean	*SD*
Nectar production per 10 flowers (mg)	16.3–25.1	19.7	±3.3	9.0–13.7	11.0	±2.2
Sugar concentration (% w/w)	57.0–75.0	66.1	±6.8	68.6–76.5	72.5	±3.3
Mass of sugars in nectar of 10 flowers (mg)	11.5–14.3	13.0	±1.2	6.7–9.5	7.9	±1.2

Discussion

Flowering span

Plants flowering in early spring provide insects with pollen and nectar, including honeybees, which begin intensive development at this time [30]. In 2010, the *P. slavica* and *P. vulgaris* plants and the three *P. vulgaris* cultivars all flowered between the second part of April and the first half of May. The flowering dates only differed by 3–4 days. In 2011, we only investigated the flowering of the ornamental *P. vulgaris* cultivars, which took place in April. The differences in the onset of flowering of these ornamental cultivars observed for both years ranged from 2 to 10 days; they were probably caused by the differences in the prevailing weather conditions. Slight variations (3–5 days) in the flowering onset dates between the three *Pulsatilla* species in 2012, 2013, and 2016 were also observed by Strzałkowska-Abramek et al. [18]. Delayed flowering has been recorded in certain years for other early spring species from the family Ranunculaceae, e.g., *Helleborus* (2–6 days) [31], *Eranthis* (7–20 days) [32], and *Anemone* (16 days) [33]. The life-span of a single *Pulsatilla* flower recorded in this study in 2010 was similar, 9.0–11.4 days. In 2011, the flowering time span of two *P. vulgaris* cultivars was 4–6 days longer than in the previous year.

Ecological characteristics associated with pollen production in *Pulsatilla* flowers

Pollen flowers are characterized by a rich androecium; here pollen is usually the only food attractant for pollinators as the flowers do not produce nectar. Examples are in the genera *Anemone*, *Hypericum*, *Papaver*, and *Rosa* [7,10,34,35]. Kugler [7] found that in terms of pollination ecology, *Pulsatilla* flowers represent a group of "nectar-producing pollen flowers". It has been calculated that a single *P. vulgaris* flower containing on average 186 stamens produced 13 mg of pollen [18]. The mean number of stamens in the *P. vulgaris* flowers analyzed in this study was 233; it can therefore be assumed that the flowers of these plants produce greater amounts of pollen, proportionally to the number of stamens. The large number and size of stamens determine the abundance of pollen production by flowers [7,36]. The multistaminate flowers of members of the Ranunculaceae produce substantial quantities of pollen [30,37–40]. The amounts of pollen produced by *P. vulgaris* flowers are in the range of mean values observed for other species from this family.

Odor of *Pulsatilla* flowers

We found that the *P. vulgaris* pollen grains were surrounded by substantial quantities of pollenkitt. It was shown previously that stamens and pollen of *P. vulgaris* and *P. rubra* produce volatile compounds [19]. The pollen scent is determined by pollenkitt, which contains various compounds such as neutral lipids, carotenoids, flavonoids, izoprenoids (terpenoids), and benzenoids [41–43]. The last-mentioned two groups of organics are floral volatile compounds [44,45]. One of the functions of pollen volatiles is to attract pollinators. Pollen scent is often determined by chemicals with documented defense activity primarily against non-pollinator, pollen-feeding pathogens [42,46]. Phytochemical analyses of volatile in *P. vulgaris* stamens and pollen have demonstrated a dominance of protoanemonin, which can act as a defense against phytophagous insects [19]. This may have been the reason why the insects observed in *P. vulgaris* flowers collected mainly nectar.

Heating of flowers

Flowers of some plants with a bowl-shaped corolla have an ability to raise the intrafloral temperature, since they function as parabolic reflectors of solar radiation into the flower interior, thus warming up the ovaries. The microtexture of the upper surface of the perianth plays an important role in this phenomenon as it facilitates maximum reflection of the incoming radiation onto the center of the flower [10,47,48]. In this study, we observed that the upper surface of the petaloid sepals in *P. slavica* and *P. vulgaris* flowers exhibited a slightly glossy texture, likely determined by the structure of epidermal cells with slightly convex outer walls. The structure and location of sepals in the bowl-shaped *P. vulgaris* flowers, which have an internal parabolic shape, indicate that they can reflect solar radiation and direct it towards the gynoecium and androecium, as in other plant species. To date, heat-retaining flowers have only been identified in *P. patens* and *P. alpina*, with a temperature of the interior several degrees higher than the ambient temperature [11,21].

Hairs

The abaxial surface of *P. vulgaris* sepals is covered by long, dead hairs. We believe that the differential thickness of the cellulose cell walls in different parts of the hairs ensures their high flexibility. The hairs are characterized by extremely thick cell walls (up to 6.5 μm). The presence of such hairs on the surface of sepals in flowers blooming in early spring may be associated with their role as an insulating barrier providing thermal protection for the internal parts of the flower. The presence of thick hairs on the surface of sepals is regarded as one of the mechanisms of floral temperature elevation, as in the classic example of inflorescences of the mountain *Leontopodium* species [49]. It has been shown that many alpine or arctic plant species possess woolly sepals or petals. Another role for the unwettable hairs covering the petaloid sepals in *Pulsatilla* is probably to protect the pollen inside the flower against precipitation [10]. Hairs present on the surface of *P. vulgaris* petaloid sepals can also cover the numerous stomata present in the abaxial epidermis, thus protecting them from excess transpiration. The style and ovary in *P. vulgaris* are also covered by hairs. These can serve a variety of functions including the provision of thermal protection for ovules, whereas the style hairs can be a pollen presenter retaining pollen released from anthers and offering it to pollinators, as confirmed by the presence of numerous pollen grains between the hairs. The presence of pollen presenters on the pistil style of flowers of plants from various families (e.g., Asteraceae, Campanulaceae) has been reported in earlier studies [50–53].

Stomata on stamens and pistils

We observed numerous stomata on the style in the *P. vulgaris* and *P. slavica* flowers. The presence of stomata in this part of the flower was reported in *Lasquerella fendleri* (Brassicaceae) by Kehl and Erickson [54] and at the base of the style in some Apiaceae and Asteraceae by Fahn [55] and Davis et al. [56]. Kehl and Erickson [54] associate the presence of gynoecial stomata with secretion of products that can attract pollinators, which may represent an adaptation to ensure cross-pollination. The presence of numerous stomata on both surfaces of the connectiva in the stamens of *P. vulgaris* and *P. slavica* is notable. Previously, we reported the presence of stomata in the connectivum epidermis in the stamens of *Ruta graveolens* [57]. The presence of many stomata provides effective gas exchange facilitating intense respiration in stylar tissues and stamen connectiva. An efficient process of respiration combined with an alternative metabolic pathway is characteristic of thermogenic flowers [58]. A possible assumption that can be proposed is the involvement of the stomata present on *P. vulgaris* and *P. slavica* stamens and styles in the elevation of the intrafloral temperature. However, this suggestion requires further detailed research for confirmation.

Staminodia, nectar

Several types of nectaries can be distinguished in flowers of the Ranunculaceae. They derive from different parts of the flower and vary in shape, location, and number [59]. The majority of *Pulsatilla* species contain nectar-producing staminodia in their flowers [7,17,18,35,60]. In our previous study, we showed that staminodia accounted for 12–15% of the androecium in *P. vulgaris* and *P. slavica* [17]. The staminodia in these species are characterized by highly diverse shapes and sizes. Nectar is secreted through stomata present on modified anthers [17]. It is an important reward for insects visiting *Pulsatilla* flowers, which has been confirmed by many authors in studies on nectar-collecting insects [14,17,18]. In the present investigation carried out during one growth season, we found that the mean weight of nectar produced by 10 *P. pratensis* and *P. slavica* flowers was 19.75 mg and 11.0 mg, respectively. This is a minimal amount in comparison to the nectar weight produced by 10 flowers of, e.g., *Aconitum lycoctonum* (86 mg) or *Aconitum carmichaelii* (217 mg) [61]. This confirms that pasque flowers represent an ecological type of "pollen flowers" producing only small quantities of nectar.

Insect visitors to *Pulsatilla* flowers

We noted honeybees, bumblebees, and ants visiting *P. slavica* and *P. vulgaris* flowers. The flowers of *P. vulgaris* were additionally visited by butterflies, which mainly foraged for nectar, whereas bees and bumblebees additionally fed on pollen. Kugler [7] classified *Pulsatilla* flowers as flowers pollinated by bees and butterflies. Szklanowska [16] observed *Apis mellifera* foraging on flowers of three species of the genus *Pulsatilla*. In our research, we found that the nectar of the two *Pulsatilla* species contained over 60% of sugars. Nectar with such a high concentration of sugars is very eagerly collected by *Apis mellifera* foragers, as they lose little energy for evaporation in order to achieve the final 80% condensation of sugars [29]. Ruszkowski [12] and Ruszkowski and Żak [13] reported seven species from the genus *Bombus* feeding on nectar or pollen from *P. vulgaris* and *P. pratensis* flowers. Torvik et al. [14] showed that a majority of visitors in two populations of *P. pratensis* were representatives of the genus *Bombus*, which mainly foraged for nectar and behaved like nectar robbers in some cases. Since *Pulsatilla* flowers are visited by insects representing many Hymenoptera species, Huang et al. [15] suggested that plants from this genus have no legitimate pollinators. The ants observed in this study in *P. slavica* and *P. vulgaris* flowers foraged for nectar. Moreover, they damaged flowers by biting off stamens, whose anthers dropped onto the sepals. Ants are often classified as non-specialist or illegitimate flower visitors and nectar robbers, as they are usually too small to pollinate, but highly attracted to any sugary fluids [10].

Floral attractants in *Pulsatilla slavica* and *P. vulgaris*

The results presented above and data in the existing literature [2,3,11,17] indicate that *P. slavica* and *P. vulgaris* flowers produce a number of visual and volatile attractants, which are attractive to pollinators. These include a large, colorful, bowl-shaped perianth, glossy, petaloid sepals reflecting the sunrays, fragrant stamens and pollen, and a well-exposed colorful and massive androecium and gynoecium enabling insects to stay inside the flower. The floral rewards offered by the flowers to pollinators include a large amount of pollen, a small quantity of nectar, and possibly a higher intraflower temperature than that of the ambient environment, as shown previously in two other pasque flower species.

Conclusions

The long, dead, silky hairs on the lower surface of *P. slavica* and *P. vulgaris* petaloid sepals and on pistils may ensure protection against cold. These hairs exhibit a high degree of flexibility probably due to the varied thickness of the cellulose-pectin cell walls. The small amounts of nectar produced by the staminodial nectaries of both *Pulsatilla* species are characterized by a high concentration of sugars (over 60%), which ensures a high attractiveness to insects. This attractiveness in the *Pulsatilla* taxa investigated recommends them for growing in gardens, not only for the perceived beauty of their flowers but also the long flowering period of the plants (over 14 days), and single flowers (9–14 days), which is important for both humans and pollinators.

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Ekologiczne cechy struktur kwiatowych i kwitnienie gatunków z rodzaju *Pulsatilla*

Streszczenie

Z punktu widzenia ekologii kwitnienia kwiaty *Pulsatilla* zaliczane są do kwiatów pyłkowych, które wytwarzają niewielkie ilości nektaru. Celem badań było określenie długości kwitnienia *Pulsatilla slavica* i *Pulsatilla vulgaris* oraz analiza budowy okwiatu i elementów generatywnych w aspekcie ekologii zapylania. Szczególną uwagę zwróciliśmy na budowę włosków i występowanie aparatów szparkowych. Określiliśmy również masę nektaru wydzielanego przez kwiaty dwu gatunków *Pulsatilla* oraz zawartość cukrów w nektarze.

Badane gatunki kwitną w zbliżonych terminach od drugiej dekady kwietnia do pierwszej dekady maja. Długość życia ich kwiatów wynosi 9–14 dni. Występujące na dolnej powierzchni listków okwiatu długie włoski mają celulozowo-pektynowe ściany komórkowe o zróżnicowanej grubości. Włoski zlokalizowane na szyjce słupka są cieńsze i służą prawdopodobnie jako ochrona przed chłodem oraz mogą stanowić wtórny prezenter pyłkowy. Miseczkowaty kształt okwiatu i budowa powierzchni doosiowej epidermy listków okwiatu mogą umożliwiać odbicie promieni słonecznych do środkowej części kwiatu i skutkować podwyższeniem temperatury w jego wnętrzu. Jest to szczególnie ważne dla funkcjonowania zalążni. Powierzchnię włosków pokrywa kutykula, co czyni je nieprzepuszczalnymi dla wody. Kwiaty odwiedzane są przez pszczołę miodną, trzmiele, motyle i mrówki, dla których istotnym atraktantem jest nektar i pyłek. Mrówki zaliczane do nieuprawnionych owadów odwiedzających kwiaty powodowały uszkodzenia pręcikowia. Stwierdziliśmy, że liczba powstających owoców w kwiatach dwóch gatunków *Pulsatilla* wynosi mniej niż 50% w stosunku do liczby słupków w kwiecie.