Morphology of *Anemone sylvestris* L. flower (Ranunculaceae)

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Abstract – During the monitoring of populations of *Anemone sylvestris* L. (Ranunculaceae), a protected species in Poland, we found that the seed set is impaired. The flower is considered an adaptation that has co-evolved to achieve effective pollination and successful fertilization. Therefore we have focused on the morphological and anatomical characteristics of the flowers of *A. sylvestris* L. as a prelude to the study of the species' pollination biology and plant breeding system. The large size of the flower (50.6 ± 16.4 mm in dimensions) and its bowl shape fulfil both the biotic pollination syndrome and the aerodynamic requirements for pollen dispersal and capture. The opening and closing of the perianth provide a shelter for beetles. The odourless perianth, absence of nectar, scarcity of pollen (approximately 200 000 pollen grains per flower) and its traits – small size (axis $P = 18.52 \pm 1.0$ μm; $E = 16.59 \pm 0.9$ μm), lack of balsam on the exine surface, starch accumulation in more than 95% of pollen grains correspond to the specialization in anemophily. The stigma is papillous, the dense hairs are situated between single carpels indicating adaptation to capturing dry pollen and specialization in the wind pollination syndrome. The flower of *A. sylvestris* is an example for an intermediate form between entomophily and anemophily, i.e. a secondary and more advanced feature among Ranunculaceae.

Keywords: androecium, *Anemone sylvestris*, anther, gynoecium, papille, pollen, Ranunculaceae, trichomes

Introduction

Different interdependent factors may influence the efficiency of pollen dispersal between individuals, i.e. density of population or pollen vectors (Nishikawa and Kudo 1995, Aizen and Harder 2007). The most significant adaptation that co-evolved in order to maximize pollination is that based on floral morphology (Faegri and Van Der Pijl 1979, Konarska 2014, Sulborska et al. 2014). Typically, colour, shape, scent, display size, and symmetry start the reaction chain of direct or indirect action that leads to pollination (Willmer 2011). However, some floral structures, e.g. nectaries, characteristics of the style and stamens, or pollen traits have been recognized as fundamental traits of specialization in the pollination syndrome (e.g. anemophily vs. entomophily vs. self-pollination), although numerous exceptions from typical adaptation exist (Friedman and Barrett 2009).

The Ranunculaceae is a family with 59 genera and is considered the most primitive of angiosperms (Tamura 1995), as well as one of the basic groups of eudicots in APG III (Chase and Reveal 2009). Within this botanical family, diverse evolutionary tendencies in the flower morphology and pollination syndrome occur (Endress 1995, Tamura 1995, Willmer 2011). Some genera, i.e. *Ranunculus, Hepatica*, *Adonis*, or *Ficaria* possess flowers with morphological traits fulfilling the requirements of generalist insect pollinators (Denisow et al. 2014). The species that develop spurs or pockets from the perianth and share nectaries contribute to the specialized insect pollination syndrome, e.g. *Aconitum* or *Aquilegia* (Endress 1995, Denisow and Antoń 2012, Antoń and Denisow 2014), whereas others are adapted to wind pollination, e.g. species from the genus *Thalictrum* (Friedman and Barrett 2009). A combination of insect and wind pollination has been reported for some species from the genus *Clematis* (Tamura 1995).

The genus *Anemone* L. s. str. (Ranunculaceae) is closely related to *Hepatica, Clematis, Anemonella,* and *Ranunculus* (Chase and Reveal 2009). The *Anemone* species are distributed primarily in the northern temperate zones (Hegi 1974, Tamura 1995). In Europe, approximately 17 species from four different subgenera may be found in natural populations (Müller 2002, Ziman et al. 2011). Four species are native to Polish flora; they occur in different habitats, i.e. de-

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ciduous forests, xerothermic grasslands, or in mountain flora (Zając and Zając 2009).

Flowers from the genus *Anemone* were recognized as nectarless and exclusively polleniferous (Horovitz 1991, Nishikawa and Kudo 1995, Szklanowska 1995, Denisow and Bożek 2006); however, nectar production has been documented in *Anemone nemorosa* (Erbar and Leins 2013). Generally, flowers devoid of nectaries are considered less specialized with regard to the pollination system, than flowers that do develop nectaries (Erbar et al. 1999).

Anemone sylvestris L. is a perennial herb native to Eurasia and distributed from Central Europe through the Caucasus, South Siberia to the northwest regions of China (Hegi 1974). It is a common species in the Balkans (Albania, Bosnia and Herzegovina, Bulgaria, Croatia, Greece, Macedonia, Montenegro, Serbia, and Slovenia), occurring in semi-shady steppe-forest communities (Zimman et al. 2011). In Northern Europe, *A. sylvestris* is found in xerothermic grasslands of the *Festuco-Brometea* class and is a characteristic species for the *Geranio-Anemonetum sylvestris* association or grows in thermophilous thickets of *Corylus avellana* (Piękoś-Mirkowa and Mirek 2006, Maciejewska-Rutkowska and Antkowiak 2013). In Poland, it is an endangered plant, protected by law (Piękoś-Mirkowa and Mirek 2006, Kwiatkowska-Falińska and Faliński 2007). Recovery programs for *A. sylvestris* primarily aim at conservation and management of the habitat (Wrzesień and Denisow 2006, Chmura et al. 2013). It is a long-lived, rhizomatous clonal species. Individuals may produce several flowers each year. The species also has a capability of sexual reproduction (Ehrendorfer et al. 2009). A number of studies have pointed out that sexual reproduction is a prerequisite for maintenance and development of sustainable population, even for clonal species, since it counteracts the inbreeding depression (Müller 2002).

The monitoring of three wild populations of *A. sylvestris* located in SE Poland conducted in short- and long-term perspective revealed their decrease in size (Denisow and Wrzesień 2015, *in press*). Vegetation changes, e.g. shrub encroachment, are possible explanations (Kwiatkowska-Falińska and Faliński 2007). In addition, there are problems with seed set, as we observed only 9–12% of seeds developed in relation to one-ovuled pistils.

In the present paper, we focused on the morphological characteristics of the flowers of *A. sylvestris* L. as a prelude to the study of the species' pollination biology and breeding system. In particular, we focused on (i) primary and secondary floral attractants, (ii) pollen production and pollen traits.

Materials and methods

Flowering of *Anemone sylvestris* L. (Ranunculaceae) was observed in 2013 and 2014 in a population located in Stawska Góra (51°13'N, 23°25'E; 224.8 m a.s.l.) in the Lublin Upland, SE Poland. The *A. sylvestris* was a component of a loose grassland patch of *Brachypodio-Teucrietum* from the Festuco-Brometea class. Additionally, for microscopic examinations performed in 2014, we used flowers from in-

dividuals cultivated in the Botanical Garden of Maria Curie-Skłodowska University, Lublin, SE Poland (51°15' 44''N, 22°30'48''E). The collection was established on the basis of individuals derived from the Stawska Góra population.

Flower biology

The duration of the flower life span was recorded in 2013 (n = 11 flowers) and in 2014 (n = 15 flowers). We defined the total flower lifetime as the period from flower opening to corolla shedding. To determine the temporal separation of stigma receptivity and anther dehiscence, the flower development was monitored from the bud stage until the end of pollen presentation. The number of anthers and the number of pistils per flower (n = 23 in 2013 and n = 27 in 2014) were established. Every day we recorded the degree to which stigmas were exerted as well as the number of dehiscing anthers.

Stigma receptivity

The timing of stigma receptivity was determined; 30% hydrogen peroxide was applied for detection of peroxidase activity (SPA) (Dafni 1992). The entire gynoecium from the flowers was extracted and was subdivided for three parts (low, medial, apical). Each part was placed on a glass slide separately and coated with a drop of H_2O_2. Stigmas that produced bubbles within 2–3 min were considered receptive. The number of receptive stigmas was counted for each individual flower (n = 9 flowers per consecutive day of anthesis) under a binocular microscope (NIKON SMZ-2B).

Flower size and micromorphology

The flower size was established by means of flower diameter measurements (n = 20 flowers) at the full flowering stage. The length between the external points of the petaloid sepals was measured. These measurements were performed using a digital calliper with an accuracy of 0.02 mm.

The morphological and anatomical details were examined by means of light microscopy (LM) and scanning electron microscopy (SEM). The photographic documentation was made on freshly cut material using an Olympus SZX12 stereomicroscope equipped with a Canon EOS 550D digital camera. The material used for SEM was fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4; 0.1 M) at a temperature of 4 °C for 12 hours. Next, the material was washed in phosphate buffer and dehydrated in graded acetone series, respectively. Afterwards, the plant material was critical-point dried using liquid CO_2, sputter coated with gold, and examined at an accelerating voltage of 30 kV with a TESCAN/VEGA LMU scanning electron microscope.

Pollen production and pollen characteristics

The number of pollen grains per anther and flower and pollen grain size were determined. The anthers (n = 16 in 2013 and n = 20 in 2014) were dissected from closed flow-

ers (n = 11 in 2013 and n = 14 in 2014). Next, the anthers were placed on a microscopic glass, the pollen sacs were squashed, and the anther walls were carefully removed. Afterwards, we put on a drop of aniline blue with glycerine and the number of pollen grains was counted. Pollen grain dimensions were determined in glycerol-gelatine slides (Erdtman 1954). The lengths of the polar axis (P) and the equatorial axis (E) were determined (n = 4 × 50 per year). These observations were conducted using a Nikon Eclipse 200 light microscope. The protein content was detected in dry samples collected during the study period. The Kjeldahl method was used for nitrogen content determination and crude protein was estimated using factor 6.25 (Roulston and Cane 2000). Starch accumulation was detected with the Lugol's iodine solution in 200 pollen grains per year.

Data analysis

Standard ANOVA was applied to assess inter-year differences in the mean values of the analyzed criteria. In order to detect differences between the means, *post hoc* comparison was made by means of the Tukey test. Data are presented as mean values ± standard deviation (SD). The level of statistical significance required to measure differences between the means for all the analyses was P = 0.05. All data analyses were performed using STATISTICA 10.0 (Statsoft Inc.) software.

Results

The flowering of *A. sylvestris* lasted 4–6 weeks, with peak flowering periods differing up to 4 weeks between the years (Tab. 1). The peak of the flowering time of the species was recorded in April (2013) or in May (2014). The reproductive shoot is unbranched, 10–15 cm high. The flower is perfect, actinomorphic, and odour-less. The pentamerous sepals are white and obovate; the flower lacks petals (Figs. 1A–C). The mean dimension of the flower is 50.6 ± 16.4 mm (n = 20). Anthesis of a single flower typically lasted 4–6 days (n = 11–15). For the first 2–3 days, the flower opened between 9.00 and 11.00 and closed at ca. 17.00. A temperature decrease (< 10 °C) kept the flowers closed, or their closure was observed before 17.00. Beetles, e.g.

Tab. 1. Phenology and floral characteristics (mean ± standard deviation, with range in parentheses) of *Anemone sylvestris* from natural population in Stawska Góra, SE Poland, during a 2 year study. Means with the same small letter do not differ significantly between years at P < 0.05, based on HSD Tukey test.

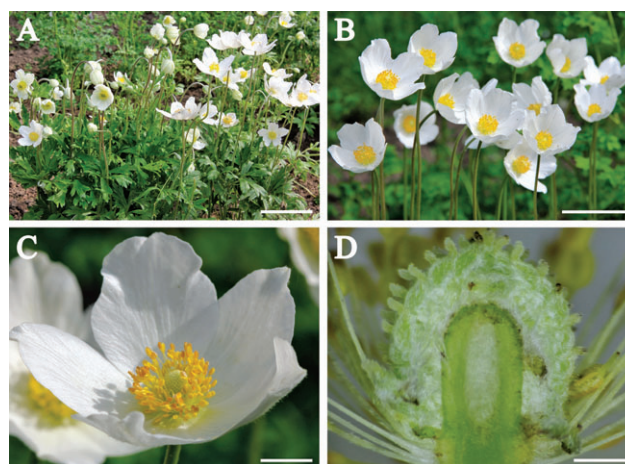| Feature	2013	2014
Flowering period	10 April – 10 May	26 April – 5 June
Duration of flowering (days)	31	55
No. of anthers per flower	103.5[a] ± 36.2 (87 – 131) (n = 23)	87.9[b] ± 23.1 (57 – 146) (n = 27)
No. of pistils per flower	112.3[a] ± 26.8 (94 – 126) (n = 23)	157.6[b] ± 53.1 (108 – 184) (n = 27)

Fig. 1. Macrophotographs of the of *Anemone sylvestris*: A) plants in the experimental population (bar = 10 cm); B) solitary, white flowers (bar = 5 cm); C) spirally arranged, multi-staminate androecium (bar = 1 cm); D) longitudinal section through gynoecium showing numerous carpels (bar = 0.5 cm).

Mordellistena sp. were observed in the flowers. During the course of flowering, sepal turgor gradually decreased, and from the third day of anthesis, the sepals in many flowers were not able to open fully. When the flower opened the first time, multiple stigmas were inserted over the top of the immature anthers. The first anthers dehisced on day 3–4 after the first flower opening, and the remaining anthers dehisced progressively during the successive days.

The flower development of *A. sylvestris* allowed us to distinguish 5 relatively distinct stages: stage 1 – tight white-greenish bud, no stigma receptivity and no pollen release; stage 2 – petals closed, beginning of stigma receptivity; stage 3 – petals opened, stigma receptivity in > 30% of pistils, stage 4 – intermezzo – early male stage, dehiscence of the first anthers, stigma receptivity; stage 5 – > 30% of anthers had dehisced, aborted ovules, or developed achenes.

Androecium and gynoecium characteristics

The floral organs are free and arranged in a spiral sequence on a convex receptacle (Figs. 1C–D). Nectariferous tissue is absent. The androecium consists of numerous stamens, ranging from 57 to 146 per flower (Tab. 1). The number of stamens per flower varied significantly from year to year ($F_{1,11}$ = 19.7, P = 0.044). The anthers are ovoid-elongated. The anthers within the androecium dehisce gradually, starting at the middle. Opening of the ripe anther is longitudinal. Three-fold disparities in the number of pollen grains produced per anther were found to occur between the study seasons ($F_{1,16}$ = 9.6, P = 0.034; Tab. 2).

The gynoecium possesses numerous apocarpic carpels (Figs. 1C–D), whose number ranged from 94 to 184 per flower. The number of pistils per flower varied significantly from year to year ($F_{1,11}$ = 12.1, P = 0.032).

Flower micromorphology

The adaxial epidermis of the petaloid sepals is smooth (Fig. 2A), whereas the abaxial epidermis bears numerous,

Tab. 2. Pollen production and pollen grain characteristics (mean ± standard deviation, with range in parentheses) of *Anemone sylvestris* from natural population in Stawska Góra, SE Poland, during a 2 year study. Means with the same small letter do not differ significantly between years at P < 0.05, based on Tukey test.

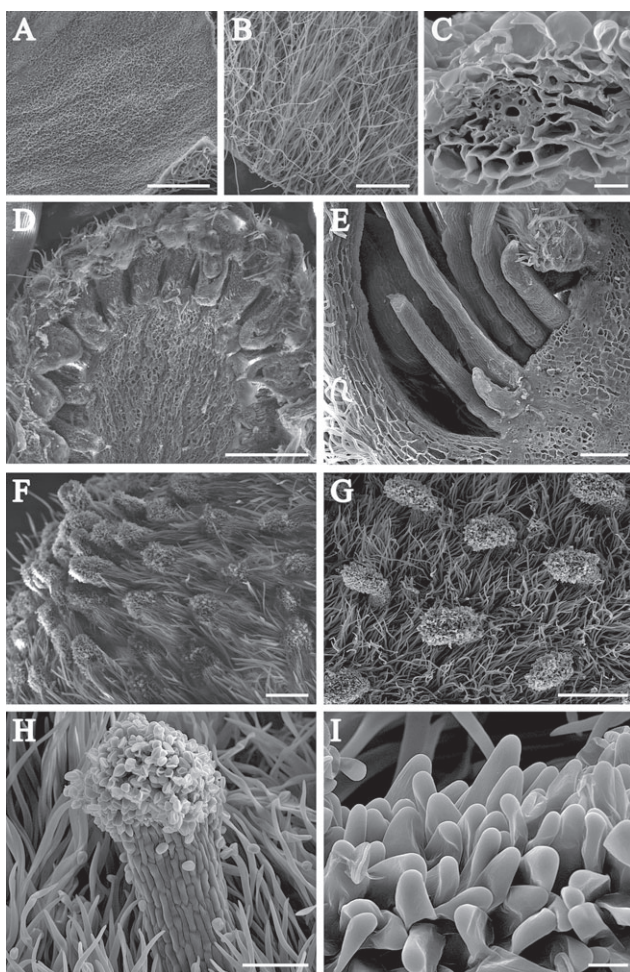| Year	No. of pollen grains		Length of axis (µm)		Shape index (P/E)
	per anther	per flower	polar (n =200)	equatorial (n = 200)	
2013	930.2[a] ± 484.0 (110 – 1790) (n = 16)	96275 (80927 – 121856)	18.31[a] ± 1.02 (16.56 – 19.68)	16.71[a] ± 0.61 (14.02 – 17.23)	1.09
2014	3361.0[b] ±825.3 (70 – 4480) (n = 20)	295432 (191577 – 490706)	18.73[a] ± 0.91 (17.34 – 20.88)	16.47[a] ± 0.85 (14.67 – 17.96)	1.13
Mean for years	2144.7	195853	18.52 ± 1.01	16.59 ± 0.92	1.11

Fig. 2. Scanning electron micrographs of the floral organs of *A. sylvestris*: A) smooth adaxial surface of the petaloid-sepal (bar = 500 µm); B) abaxial surface of the petaloid-sepal with numerous hairs (bar = 500 µm);C) longitudinal section through petaloid-sepal, note reduced vascular bundle (bar = 20 µm); D) section through gynoecium showing numerous pistils (bar = 500 µm); E) longitudinal section through flower, from the external to internal part: petaloid-sepals, stamens, pistil (bar = 200 µm); F, G) general aspect of the multiple gynoecium with distinct apocarpic carpels (bars = 200 µm and 500 µm, respectively); H) details of a single carpel with easily distinguishable stigmatic area (bar = 100 µm); I) stigmatic area of pistils with a number of unicellular and conical papillae (bar = 20 µm).

long, unicellular hairs with the greatest density observed at the base (Figs. 2B, E). In cross sections, the petaloid sepals are relatively thin with 4–6 mesophyll layers and have reduced vascular bundles (Fig. 2C).

Numerous hairs between distinct apocarpic carpels are present (Fig. 1D; Figs. 2D–H). Each carpel contains a single ovule, which develops into a one-seeded achene. The receptacle enlarges after anthesis and continues to enlarge during achene formation in a fruit cluster of fruitlets. Stigmatic tissue is visible at the apex of the style (Figs. 2F–H). The stigmatic area is easily distinguishable from the style, bearing a number of unicellular and conical papillae, whereas the surface of the style is covered by smooth epidermal cells (Figs. 2H–I). The presented stigma is dry.

The anther walls consist of the epidermis, endothecium, middle layer, and tapetum (Figs. 3A–D). The endothecium has thickenings that are differentiated in the lateral walls of the cells (Figs. 3C–D). The pollen grains are dry, tricolpate with a reticulate surface (Fig. 3E), and prolato-spheroid (shape index 1.09–1.13). The diameter of the polar axis (P)
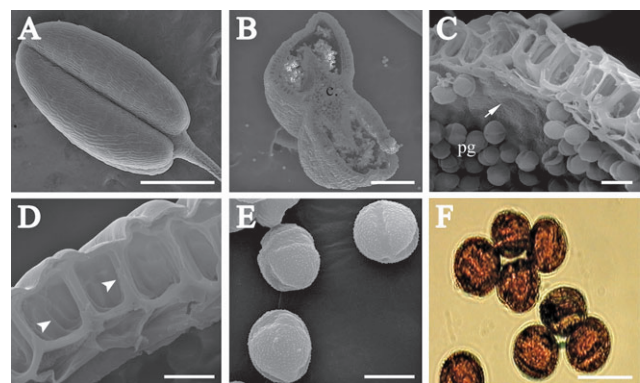

Fig. 3. Scanning electron (A–E) and light (F) micrographs of the anther and pollen grains of *A. sylvestris*: A**)** general aspect of the stamen (bar = 500 µm); B) longitudinal section through anther with visible connective (c) (bar = 200 µm); C–D) section through anther showing tapetum (arrow), endothecium cells with wall thicknesses (arrowhead) and numerous pollen grains (pg) (bars = 20 µm); E) tricolpate pollen grains (bar = 20 µm); f) pollen grains stained with Lugol's iodine (bar = 10 µm).

ranges from 16.56 to 20.88 μm and the equatorial axis (E) is in the range of 14.02–17.96 μm. No year effect on the mean values of the diameter of pollen axis was found ($F_{1,6}$ = 2.3, P = 0.124 for polar and $F_{1,6}$ = 1.2, P = 0.085 for equatorial). Starch was present in more than 95% of pollen grains (Fig. 3F). The mean protein content in the pollen is 24.6% of dry matter ± 0.75.

Discussion

The morphological architecture and extrafloral attributes of *A. sylvestris* flowers imply that two pollination modes may interact to affect pollen dispersal. Secondary attractants, *sensu* Faegri and Van Der Pijl (1979), i.e. visual and/or temperature attractants, indicate that the flowers of *A. sylvestris* may lure insects as potential pollen vectors. On the other hand, the specificity of floral reward, the traits of pollen grains, as well as some gynoecium traits indicate involvement of wind in the dispersal of pollen.

A. sylvestris flowers are large and bowl-shaped, according to the classification of Faegri and Van Der Pijl (1979). In general, this flower type seems to function as an advertising organ for luring insect visitors (Willmer 2011); it also fulfils the aerodynamic requirements for pollen dispersal and capture (Friedman and Barrett 2009). The perianth is scentless and the feature corresponds to that in anemophilous taxa whose perianth is still visually attractive (Willmer 2011).

From the third day of anthesis, the turgor of the sepals decreased, which apparently impaired sepal opening. We also observed closing of the flower for the night and during the day when temperature dropped below 10 °C. Closed flowers prevent the wind from serving as a pollen dispersal agent. However, the anthers of *A. sylvestris* inserted on thin filaments are likely to contact stigmas when the flower is blown by the wind; therefore, self-pollination is highly possible. Closed flowers are also temporarily unavailable for insects. We found beetles both in closed flowers (presumably warming up or avoiding the wind) and in opened flowers on sunny days (presumably maximizing exposure to solar radiation in order to build up heat before take-off). The syndrome of the opening and closure of the flower, which provides a shelter for ectotherms, is referred to as a temperature attractant (Faegri and Van Der Pijl 1979) and has already been documented for several species, e.g. *Adonis vernalis* (Denisow et al. 2014), *Anemone patens* (Ordway 1986), *A. canadensis* (Douglas and Cruden 1994), or *A. nemorosa* (Van Doorn and Van Meeteren 2003). However, the significance of insects that take shelter inside a flower is not obvious, and requires further experimental investigations to establish their efficiency in the pollination process, e.g. the number of pollen grains they actually transfer and deposit on the stigmas.

In most species, perianth movements of a thermonastic type are associated with a difference in the growth rate of mesophyll cells (Van Doorn and Van Meeteren 2003). Presumably, the relatively thin mesophyll layer of the petaloid sepals revealed in *A. sylvestris* optimizes the movement at a minimal metabolic cost.

Among the noteworthy results of the current study is the finding that *A. sylvestris* flowers lack nectar-secreting structures. The attribute can be considered as promotion of *anemophily* (Friedman and Barrett 2009) and is interpreted as a co-evolutionary adaptation to attraction of specific type of insects (Waldbauer and Friedman 1991). Absence of nectar was confirmed for other *Anemone* species, i.e. *A. coronaria*, *A. canadensis*, *A. flaccida,* or *A. japonica*, the flowers of which were visited mainly by beetles; however, flies or primitive Apoidea (Anthophoridae, Halictidae) were also observed (Horovitz 1975, Horowitz 1991, Douglas and Cruden 1994, Nishikawa and Kudo 1995, Denisow and Bożek 2006). Conversely, nectar-producing flowers of *A. nemorosa* were reported to be visited by *Bombylius* spp.*,* honeybees, or bumblebees (Szklanowska 1995, Erbar and Leins 2013). Probably, the nectar deficiency in the flowers of *A. sylvestris* restricts the range of the insect spectrum, resulting in the scarcity of dipterans and bee visitors in our experimental population (manuscript in preparation).

Not only was nectar absent, but a relatively small amount of pollen was produced in a single flower of *A. sylvestris*. Altogether, the primary attractant characteristics possibly explain the low interest of insect visitors. Although the flowers are multi-staminate, they released only approx. 200 000 pollen grains per flower. The value is ten-fold lower than that established for *Anemone coronaria* (approx. two million). Generally, taxa from the family Ranunculaceae are reported to produce copious amounts of pollen (Szklanowska 1995, Denisow and Bożek 2006, Denisow and Antoń 2012). The low pollen production in *A. sylvestris* may be related to the genetic potential of the species; pollen production is reported to be highly genetically dependent (Szklanowska 1995, Denisow 2011). However, the year-to-year disparities in the number of pollen grains produced indicate that pollen production in *A. sylvestris* is sensitive to external factors. For example, weather conditions (shortage of precipitation or air temperature anomalies) are known to limit pollen production in a number of taxa (Denisow and Bożek 2006, Aizen and Harder 2007, Denisow et al. 2014).

The pollen grains of *A. sylvestris* are small, within a range of 10–25 μm (Erdtman 1954). The protein content of pollen is approximately 25% of the dry mass. The protein concentration in pollen grains is a highly conserved trait within plant genera and families; however, the correlation between the protein content and the type of pollination is not always obvious (Roulston and Cane 2000, Denisow 2011). Nonetheless, small pollen grains, lack of balsam on the exine surface, and starch accumulation correspond well to specialization in anemophily (Friedman and Barrett 2009). Similar pollen traits were described for *Anemone coronaria* by Horowitz (1991), who proved that pollen was dispersed by air currents up to the distance of < 1.5 m from the flower.

We found papillous stigma and dense hairs situated between single carpels. It is possible that these properties enhance the capture of dry pollen and may indicate specialization in the wind pollination syndrome, as suggested by Horowitz (1991). However, the pistils placed on the convex receptacle are well exposed and provide an unobstructed

path for pollen transported by both abiotic and biotic agents (Friedman and Barrett 2009). The other ecologically significant adjustment of these dense hairs seems to be protection of pistils and ovules from overheating and/or heat loss. Low temperatures are particularly frequent during early spring, and they are often hazardous for carpels, which are considered the most sensitive plant structures (Hedhly 2011).

The anthers of *A. sylvestris* dehisce longitudinally. We found lignified wall thickenings in the endothecium cells. Many investigation results are consistent in showing that cell wall thickenings control the dynamics of anther opening by regulating the dehydration and shrinkage of cells, which may indirectly support pollen dispersal (Keijzer 1987).

The abaxial surface of the petaloid sepals is velvety. It is associated with the presence of numerous hairs on the abaxial epidermis. The same characteristic of abaxial epidermis was evidenced in the flowers of *Adonis vernalis* (Gostin 2009). Epidermal hairs are reported to protect epidermal and mesophyllous cells against excessive heating or water loss, which is especially important in xerothermic habitats (Karabourniotis et al. 1992). The adaptive significance of epidermal hairs in the defence against phytophagous insects has also been confirmed (Hanley et al. 2007).

A. sylvestris flowers are protogynous, which has been confirmed by the findings for other *Anemone* species (Horowitz 1991, Denisow and Bożek 2006). Protogyny is

commonly described in cantharophilous flowers (Faegri and Van Der Pijl 1979) as well as in anemophilous ones (Friedman and Barrett 2009). In the protogynous flowers of *A. sylvestris*, the absence of nectar may affect the function of the female phase by decreasing the attractiveness of the phase to potential pollinators and influence the mating opportunity of the flower.

Presumably, the flower of *A. sylvestris* is an example of an intermediate form between entomophily and anemophily, i.e. a secondary and more advanced feature among Ranunculaceae (Endress 1995). The floral morphology and the type of the floral attractant indicate the existence of alternative modes of pollination (partial wind, self-pollination, or biotic pollination).

Acknowledgements

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