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## ORIGINAL RESEARCH PAPER

# Nectar secretion and pollen production in protandrous flowers of *Campanula patula* L. (Campanulaceae)

Bożena Denisow<sup>1\*</sup>, Monika Strzałkowska-Abramek<sup>1</sup>, Małgorzata Wrzesień<sup>2</sup><sup>1</sup> Department of Botany, University of Life Sciences in Lublin, Akademicka 15, 20-950 Lublin, Poland<sup>2</sup> Department of Geobotany, Institute of Biology and Biochemistry, Maria Curie-Skłodowska University in Lublin, Akademicka 19, 20-033 Lublin, Poland\* Corresponding author. Email: [bozena.denisow@up.lublin.pl](mailto:bozena.denisow@up.lublin.pl)**Abstract**

Nectar secretion was noted both in the male and female floral phases of the protandrous flowers of *Campanula patula* (Campanulaceae). Female-biased sugar accumulation was evidenced and plasticity in the duration of sexual phases observed. Flowers in the male phase produced twofold less nectar with lower sugar concentrations compared to female-phase flowers. The sugar mass content averaged  $0.6 \text{ mg} \pm 0.45 \text{ SD}$  per flower in the male phase and  $1.4 \pm 0.5 \text{ SD}$  per flower in the female phase. The pollen mass averaged  $0.16 \text{ mg} \pm 0.10 \text{ SD}$  per flower. An understanding of the evolution of functional relationships between floral sexes requires consideration of the compensation of the reproductive costs, including the plastic response to interdependent factors, i.e., photosynthesis and growth, the effect of pollinators, pollen robbers, and external environmental forces.

**Keywords**

floral longevity; dichogamy; male phase; female phase; nectar production

**Introduction**

Both nectar and pollen represent a considerable investment for plants in terms of assimilate deployment [1–3]. It has been proved that these secondary sexual traits were evolved to increase plant reproductive success via the impact on the behavior of insect visitors [4]. Nectar varies in its sugar concentration from 10–75% [5–7], therefore as a primary energy source for insect visitors, it is subject to selection pressure. Quite notably, the quantity of nectar production is positively correlated with the frequency and abundance of pollinator visits [8–10], the number of flowers visited per plant, as well as impact the duration of the visit [11]. Consequently, nectar production influences plant sexual reproduction by indirect involvement in the dissemination of pollen to conspecific stigmas [12,13]. Pollen has a double function as a provider of male gametes and a protein source for insects [10,14,15].

Many biotic and abiotic factors (e.g., temperature, air humidity) determine the amount of nectar available in a flower and therefore the nectar secretion rate can differ between species [11], during the flower life-span [16], or even throughout the day [6]. Nectar production usually differs between growing seasons [17]. Pollen production is also affected by genetic and environmental factors [9,10]. Besides floral reward, flower longevity is involved in the allocation of resources [18] and reproductive success [19]. Trade-offs between floral longevity and nectar sugar production as well as

between nectar sugar production and seed production have been documented [3,20]. In dichogamous plants, effective reproduction depends on pollinator visits to both male and female flowers [15,21]. Models of sex allocation assume that total reproductive effort is resource-limited and the costs have to be partitioned between male and female functions [22]. The proportion of resource allocation to each sexual phase reflects the plants' emphasis on each sexual function [23]. Most experiments have examined the involvement of resources in sexual reproduction by estimation of the dry weight or amounts of inorganic nutrients utilized for the floral structures [13,24], disregarding floral features essential for plant–pollinator interactions such as nectar and pollen production.

The genus *Campanula* (Campanulaceae) includes about 400 species distributed mainly in the temperate zone (mainly in Europe and Asia), with nine species recorded in Poland [25–27]. Flowers of *Campanula* are protandrous with a secondary pollen presentation mechanism [5,28–33]. Dichogamous plants with functional separation of gender phases are ideal for use in studies that analyze the male and female impact on different flower functions. In dichogamous species, nectar secretion may be greater in one of the sexual phases or the nectar production rate may be unbiased [6,34]. *Campanula patula* L. is a biennial herb distributed throughout Central and Western Europe. The species occurs mainly on lowland and mountain meadows and is also common on field margins and other anthropogenically transformed sites. By preference, it grows on dry, well-drained, sunny sites on fairly infertile soils. *Campanula patula* bears lateral branches of pale blue or white perfect flowers that are upright and funnel-shaped [27,28].

In the current study, we examined the nectar and pollen reward available in flowers of *Campanula patula* and considered how sexual floral phases may potentially affect nectar secretion. Therefore, we determined (i) the nectar production in the male and female phases of flower development and (ii) estimated the total output of sugar and pollen resources available. Accordingly, details of floral biology, i.e., floral longevity and duration of sexual phases have also been monitored.

## Material and methods

The observations and measurements of *Campanula patula* L. were conducted in 2008 and 2010 in a meadow from the vegetation alliance *Arrhenatherion*, within the Dąbrowica area, (51°18' N and 21°32' E), Lublin Upland, Poland. All observations of flowering biology were made during the full bloom of the population and the procedures described by Dafni [35] and Denisow [9] were applied. To determine the flower life-span, we randomly chose and marked buds ( $n = 11–15$  per year) from different plants ( $n = 5$ ). Simultaneously, we observed changes and determined the duration of the male and female phases, the position and dehiscence of anthers, and stigma receptivity ( $n = 8–12$ ). Receptive stigmas were detected by a characteristic bubbling observed on their surface when placed in 30% H<sub>2</sub>O<sub>2</sub> [35]. The beginning of anthesis was recognized when the petals opened wide enough to allow pollinators to enter the flower. The end of anthesis was the time when the petals wilted. The male phase was the period from the beginning of anther dehiscence through to presentation of pollen on the presenter trichomes and the beginning of stigma lobe opening. The functional female phase was the time between stigma opening and anther wilting.

We examined nectar secretion using the pipette method [36]. To determine the amount of nectar, we prevented insect visitors. We therefore bagged inflorescences in the bud stage with tulle isolators (mesh size 1 mm) on different individuals ( $n = 6–8$ ). The isolators remained until nectar sampling. Nectar collection was conducted in six replications during the blooming period. For each replication, five–six samples were collected and a composite sample contained nectar from three–six flowers. The nectar was collected from flowers at two different phases of flower development, i.e., at the end of the male phase with pollen presented and stigmas closed and at the end of the female phase, separately. Sugar concentration in nectar was measured using an Abbe refractometer. The amounts of nectar and sugar concentration were then used to calculate the total sugar mass in each sample, and the amount of sugars accumulated per flower (in mg) in both floral phases could thus be determined.

Pollen production was rated by the ether-ethanol method described by Denisow [9,10]. Well-developed flower buds were collected from different plants ( $n = 8-10$ ), and anthers ( $n = 50$ ) were dissected and placed in tarred vessels prepared for the purpose. Measurements were performed in four replications. Anthers were assayed for dry matter and for the mass of pollen delivered. Pollen was washed out of the anthers with ether (2–3 mL) and then with 70% ethyl alcohol (4–6 mL) four–five times.

Data are presented as means with *SD* (standard deviation). The analysis of the number of flowers, floral phases duration, nectar secretion, and pollen production was conducted by means of analysis of variance [37]. Post hoc comparisons of means were tested by the HSD Tukey test. The level of statistical significance for all analyses was set at  $\alpha = 0.05$ . All analyses were performed using Statistica ver. 10.0 (StatSoft, Poland).

## Results

The bell-shaped flowers of *C. patula* are bisexual, actinomorphic with a pentamerous perianth. Flowers are arranged in a compound dichasium. Both functionally female and male flowers were usually present on individuals (Tab. 1). During the day, flower opening continued until 6:00 p.m. (GMT + 2 h), however, they were mostly open between 10:00 a.m. and 2:00 p.m. A significant year effect was found for the duration of flower life-span (Tab. 2). The anthers were rigid and formed a narrow tube in the bud. The style with closed lobes was centrally located; the anthers firmly touched the style. Flowers were distinctly protandrous. Usually, anthers dehisced in well-developed buds. The male phase started before corolla opening (Tab. 1, Tab. 2). The release of pollen began in the apical parts of anthers. The pollen was sticky and was presented on the style trichomes. Most anthers started to release pollen between 10:00–16.00 h, and pollen presentation took less time (mean =  $1.6 \pm 0.9$  *SD* days,  $n = 20$ ) than stigma presentation (mean =  $3.0 \pm 0.6$  *SD* day,  $n = 20$ ) (Tab. 2). After flower opening, the style was elongated and

**Tab. 1** Morphological criteria of floral phases during flower development in *Campanula patula*.

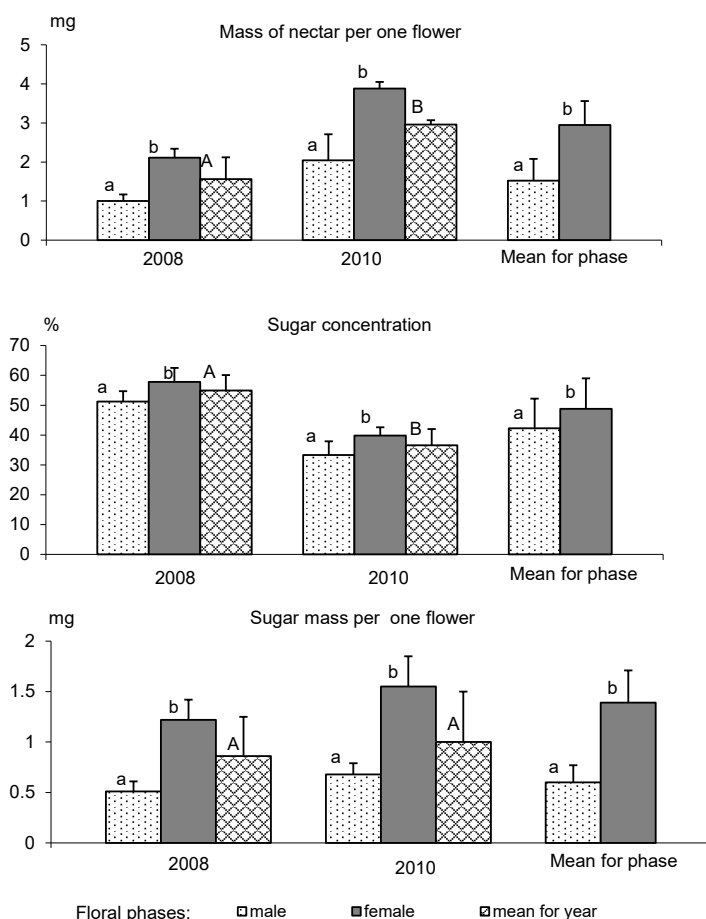
Phase	Anthers dehiscence	Pollen exposition	Stigmas	Corolla condition
Bud	Yes	Not presented	Not visible	Turgid
Male	All	Presented on the style trichomes and visible	Not visible	Turgid
Neuter	All	Presented on the style trichomes and visible	Visible, but closed	Turgid
Female	Anthers dropped out	Remnants of pollen visible under microscope	Three-curved fully exposed, receptive	Turgid, color lightening

**Tab. 2** The number of flowers per stem, the duration of total life-span, the male and female phases of *Campanula patula* flowers in the years 2008 ( $n = 9$ ) and 2010 ( $n = 11$ ). Definitions of sexual phases are given in the text.

Variable	2008		2010		Mean
	Mean	$\pm$ SD	Mean	$\pm$ SD	
Number of flowers per stem	8.2 <sup>a</sup>	3.6	9.4 <sup>a</sup>	7.1	8.8
Life-span (days)	3.6 <sup>a</sup>	1.0	5.4 <sup>b</sup>	2.4	4.5
Male phase (days)	1.2 <sup>a</sup>	0.7	1.9 <sup>b</sup>	1.7	1.6 <sup>A</sup>
Female phase (days)	2.4 <sup>a</sup>	1.5	3.6 <sup>b</sup>	2.3	3.0 <sup>B</sup>

Means within the row indicated with the same small letters are not significantly different between seasons; means indicated with capital letters show differences between floral sexual phases at  $\alpha = 0.05$  according to HSD Tukey test.

the female phase was correlated with unfolding of stigma lobes. The second day of floral life-span appeared to be a neuter phase; pollen was presented but stigmas remained closed, thereby it had been receiving little if any pollen. The centrally exerted style exhibited a three-curved stigma during the female phase. Simultaneously with flower opening, the anthers started the process of wilting. At the time of lobe unfolding, the style was 2–4 times longer than at the beginning of the male stage. Stigma lobes coiled up like a spiral to touch the style towards the end of anthesis. After the corolla opened, pollen was



**Fig. 1** Nectar secretion, sugar concentration, and sugar mass in the male and female phases of dichogamous flowers of *Campanula patula* in the years 2008 and 2010. Means indicated with the same small letters are not significantly different between floral phases and with the same capital letters are not significantly different between years of study, at  $\alpha = 0.05$ , according to HSD Tukey test.

**Tab. 3** The dry mass of anthers and the pollen production in the flowers of *Campanula patula* in the years 2008 and 2010.

Year	Dry mass of 100 anthers		Pollen mass per flower	
	Mean	$\pm SD$	Mean	$\pm SD$
2008	24.8 <sup>a</sup>	$\pm 3.4$	0.15 <sup>a</sup>	$\pm 0.07$
2010	24.4 <sup>a</sup>	$\pm 9.3$	0.16 <sup>a</sup>	$\pm 0.06$
Mean	24.6	$\pm 6.9$	0.16	$\pm 0.10$

Means indicated by the same small letter are not significantly different between seasons at  $\alpha = 0.05$ , according to HSD Tukey test.

offered to insects for 1–2 days. The number of flowers formed per plant ranged from six to 18 (mean =  $8.8 \pm 3.5$  SD) and a year effect was found for the abundance of blooming.

Flowers produced both nectar and pollen. The amount of nectar secreted differed significantly between the male and female phases (Fig. 1). Flowers in the male phase produced two-fold less nectar [ $F(1, 28) = 20.2, p = 0.0001$ ] with a lower sugar nectar concentration [ $F(1, 28) = 4.5, p = 0.0420$ ] compared to female-phase flowers. Consequently, the amount of accumulated sugar had higher values in female-phase flowers [ $F(1, 28) = 80.24, p = 0.000$ ] than in male-phase flowers. The sugar mass content averaged  $0.6 \text{ mg} \pm 0.45$  SD per flower in the male phase and  $1.4 \pm 0.5$  SD per flower in the female phase. The nectar produced in 2008 was more concentrated than that in 2010 [mean = 54.9% and 36.6%, respectively,  $F(1, 28) = 95.1, p = 0.0002$ ]. However, the amount of nectar was almost 1.5-fold lower [ $F(1, 28) = 17.04, p = 0.0002$ ], therefore there were no statistically significant differences in the total mass of sugars obtained from the flowers between the years of study [ $F(1, 28) = 1.4, p = 0.247$ ].

No year effect was found for the size of anthers, measured as dry mass of anthers [ $F(1, 5) = 0.005, p = 0.918$ ], and for the amount of the pollen produced in anthers [ $F(1, 6) = 0.28, p = 0.871$ ]. The pollen mass averaged 0.16 mg per flower (Tab. 3).

## Discussion

The flowers of *C. patula* are protandrous. Protandry is considered characteristic for the genus *Campanula* [29,38]. The second day of floral life in *C. patula* appears to be a neuter phase; pollen removal is almost completed but stigmas remain closed. Neuter phases have been recorded in other dichogamous taxa, although it is not clear whether this is a common phenomenon [39,40]. Although dichogamy has been almost universally interpreted as an outcrossing mechanism [41], we observed that stigma lobes can coil back and touch the remnants of pollen from the surface of style trichomes in some flowers. Dichogamous species are equipped with different properties to complete reproduction through autogamy during unfavorable conditions, i.e., in the case of disappointing activity of pollinators or the absence of visits [21,22,28,29,42]. It seems reasonable that the movement of stigma lobes at the end of anthesis may ensure self-pollination in *C. patula*, even though at the termination of anthesis remaining pollen accounts for less than 1% of total pollen produced [43]. Considering the seasonal development (= phenology) of the gender in protandrous *C. patula*, the mechanism seems to be particularly important at the end of the season. Then, the number of male-phase flowers decrease in a population and female-phase flowers are less likely to receive pollen. In the protandrous flowers of *C.*

*patula*, regardless of the year of the study, the female phase dominated over the male phase and nectar production was female-biased. This indicates that both features are determined genetically and corresponds to the interpretation involving sexual selection in the evolution of dichogamy [34,41].

Our findings support Primack's [18] opinion that the male phase in dichogamous flowers is generally shorter than the female phase. A longer female phase than the male phase has been reported in protandrous species from different plant families, e.g., in *Carum carvi* (Apiaceae) [44] or *Polemonium caeruleum* (Polemoniaceae) [16]. Overall, the predominance of female vs. male phase duration is combined with higher costs of maintenance of the male phase (concurrent nectar and pollen production). Higher allocation of resources to the male phase was reported by Konuma and Yahara [45] and Obeso [24]. Both nectar and pollen production require much energy, e.g., the costs of nectar can be up to 37% of daily photosynthesis [2], while 7% of photosynthetic carbon might be used for pollen production [46]. In the flowers of *C. patula*, the peak of nectar and sugar accumulation was associated with the female phase, which is in agreement with the findings of Carlson and Harms [34], who classified protandrous Campanulaceae species as female-biased nectar producers. Concurrently, about one-third of the entire sugar production was offered already in the male phase, i.e., in flowers with nonreceptive stigmas, when ovules cannot be fertilized. This indicates the high costs of male function in protandrous *C. patula* and the male phase shortening seems to be an evolutionary compromise to reduce the costs of the male phase. The pattern of nectar secretion observed in *C. patula* was slightly different to that found in *C. rotundifolia* [42] or *C. lingulata* [47]. In our study, nectar was present in the flowers at the beginning of anthesis, whilst in *C. rotundifolia* nectar secretion was shifted towards the termination of pollen presentation. The direct costs for maintenance of flowers functionally prepared for reproduction include, in addition to nectar and pollen costs, resources invested in respiration and transpiration by floral structures (calyx, corolla, androecium, gynoecium) [2,48]. The shift of nectar production between the sexual phases among *Campanula* species may reflect various trade-off relationships, e.g., the corolla size vs. derivative costs of respiration. *Campanula patula* flowers are smaller than those of *C. rotundifolia*, therefore their production costs are expected to be lower, and for that reason, precise control of nectar secretion was probably not subjected to as strong selection as in *C. rotundifolia*.

Completely different habitats and ecological conditions between the lowland *C. patula* and alpine *C. lingulata* may also impose different strategies for encouragement of reproductive cost reduction. The environmental perspective for optimization of the resource allocation strategy has been underlined by different authors [3,18,48].

In our observations, the total flower life-span and the duration of sexual phases differed between the years of study. These results confirmed the potential for the plastic response in floral life-span to environmental factors, which was described in many species [9,10,42]. The length of the gender phases may also be modified by adjunctive factors, e.g., activity of flower visitors, pollen deposition to the stigma, or pollen tube growth [20].

Nectar production also differed between the growing seasons, unlike the pollen yield. Generally, the potential for nectar and pollen yield are multigene traits [6,14], although they are modified by environment. For example, air temperature and humidity, vapor pressure, and soil moisture are known to affect nectar secretion and concentration of sugars [14,17]. Pollen production is also impacted by external factors such as prolonged drought, which limits the quantity of pollen in some taxa [10,12,32]. Our data indicate that nectar secretion and pollen production are related to different abiotic factors in *C. patula*.

Floral life-span, duration of the sexual phases, and nectar secretion require to be considered in relation to pollination. Firstly, the appearance of nectar at the beginning of anthesis raises the costs of maintenance of the male phase in *C. patula* but may be cost-effective in terms of pollination. Even minor amounts of nectar in the male phase increase flower attractiveness, likewise the restricted nectar production may accelerate pollen limitation due to low flower attractiveness [8]. Secondly, the dominance of the female phase over the male phase may be an effect of the selective pressure of changes in the pollinator visitation rate during the flowering season [23,42]. An increase in the duration of the female phase in *C. patula* increases the possibility for pollen deposition

onto the stigma when the proportion of female flowers increases during the flowering season. The female-biased sex ratio late in the season is characteristic for populations of protandrous species [49] and creates strong competition for pollinators. The prolongation of the female phase may be an evolutionary compromise for increasing the chance of pollination and mating assurance. However, according to Richardson and Stephenson [50], in protandrous plants the male phase that affects the opportunity for pollen donation should be lengthened. Generally, the duration of pollen release and exposure is due to the androecium type and the more numerous stamens, the longer pollen presentation [10,14]. In flowers with a five-staminate androecium, i.e., the type observed in *C. patula*, pollen presentation usually lasts 2–8 hours [9,10]. We found that pollen presentation on the style trichomes (PCHs-pollen collecting hairs) lasted 1.6 days, on average. The secondary pollen exposition to pollinators occurs in 16 angiosperm families [30]. In *C. patula* flowers, the stylar pollen presentation (SPP-system) allows pollen removal away from the beginning of anthesis, i.e., during style elongation, while the lobes are joined together and the stigma is not receptive. Immediately after flower opening, pollen presented on the style was actively collected by insect visitors. We also observed insects whose bodies became dusted with pollen presented on the style presenter while they were attempting to collect nectar. Insect visitor activity and frequency may play an important role in the duration of the male phase in dichogamous flowers as pollen-thieving insects may limit the pollen availability for pollination [15]. Furthermore, the dominance of the female phase (1.5–2-fold) over the male phase together with the greater amount of nectar secreted during stigma presentation may indicate self-incompatibility (SI) of the species. According to Inoue [23], sex allocation is related to the breeding system and the higher the costs of the female phase is (e.g., a longer phase), the higher the rate of outcrossing and SI.

## Conclusion

*Campanula patula* nectar secretion both in the male and female floral phases, female-biased sugar accumulation, and plasticity in the duration of the sexual phases have been evidenced. An understanding of the evolution of functional relationships between floral sexes evidently requires consideration of the compensation of reproductive costs, including the plastic response to interdependent factors, i.e., photosynthesis and growth, the effect of pollinators, the pollen robbers, and external environmental forces.

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**Nektarowanie i pylenie protandrycznych kwiatów *Campanula patula* L.  
(Campanulaceae)**

**Streszczenie**

W protandrycznych kwiatach *Campanula patula* (Campanulaceae) sekrecja nektaru odbywa się w fazie męskiej i żeńskiej kwiatu. Kwiaty w fazie męskiej produkowały 2-krotnie mniej nektaru, o niższej koncentracji cukrów, niż kwiaty w fazie żeńskiej. Masa wydzielanych cukrów wynosiła średnio  $0,6 \text{ mg} \pm 0,45 \text{ SD}$  w jednym kwiecie w fazie męskiej oraz  $1,4 \pm 0,5 \text{ SD}$  w jednym kwiecie w fazie żeńskiej. Zrozumienie relacji pomiędzy fazami płciowymi kwiatów wymaga określenia korzyści i kosztów funkcjonowania fazy męskiej i żeńskiej, np. wpływu potencjału fotosyntetycznego, zachowania zapylaczy, strat pyłku, wpływu czynników abiotycznych.