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## The sexual reproductive strategy in clonal *Scopolia carniolica* is based on obligatory cross-pollination and self-incompatibility

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Understanding the reproductive biology and breeding system of rare species is crucial for effective conservation. We examined floral biology, assessed the true pollinators, and investigated the reproductive effect of six pollination treatments (spontaneous and induced autogamy, geitonogamy, spontaneous and supplemental cross-pollination, control - flowers exposed to natural pollinators) on the fruit and seed set and their quality (size, viability) in two populations (artificial and natural) of clonal *Scopolia carniolica* (Solanaceae), a rare species distributed across Central and South-Eastern Europe. Hermaphroditic flowers of *S. carniolica* represent movement herkogamy and incomplete protogyny. Pollinators are necessary for transferring pollen onto stigmas (there was no fruit and seed set after a test for spontaneous self-pollination), and the species shows the system of obligatorily xenogamy (only cross-pollination guarantees the production of seeds with fertile embryos ensuring viable offspring) and self-incompatibility (no seeds are produced after within-flower self-pollination). Although fruits can be developed through geitonogamous self-pollination, this is associated with late-acting self-incompatibility because the seeds produced are non-viable (contain no embryos and endosperm). Bumble bees are the most effective pollinators; honeybees also contribute to pollen deposition. Conserving pollinator diversity is essential for rare clonal *S. carniolica*, sustaining reproduction, preventing inbreeding depression, and supporting long-term population viability.

**Keywords** Incomplete protogyny, Geitonogamy, Fruit set, Seed set, Seed viability, Bumble bees, P/O ratio

Studies of reproductive biology and pollination ecology are essential for predicting plant population survival<sup>1</sup>. The results can help to identify factors disturbing the reproduction of individuals and may be crucial for the implementation of conservation programs aimed at maintaining or restoring rare, narrowly distributed, or endangered populations<sup>2,3</sup>.

Flowering plants exhibit a wide array of floral adaptations and reproductive strategies that have an impact on their fitness, genetic makeup, and population dynamics<sup>4,5</sup>. Most angiosperm species (ca. 78% in the temperate-zone and 94% in the tropical zone) rely on insect vectors for pollen transfer to mediate the pollination process<sup>6</sup>. However, their mating systems can vary widely - from autonomous selfing (self-pollination without external assistance) via mixed mating systems (combining sexual and asexual reproduction or involving selfing and outcrossing) to obligate outcrossing (relying entirely on cross-pollination)<sup>7-9</sup>. In general, cross-pollination is considered more advantageous than self-pollination due to a higher proportion of heterozygous individuals and increased genetic diversity of offspring<sup>8,10</sup>. Nevertheless, reproductive assurance is an important selective force that can strengthen self-fertilization in order to secure offspring production in environmental conditions non-conducive for cross-fertilization, e.g. a lack of compatible mates or pollinator limitation<sup>9,10</sup>.

A large proportion of angiosperm flowers are hermaphroditic, with the potential for self-pollination<sup>11</sup>. Self-pollination may take place within a flower (autogamy - autonomous or vector-mediated) or between flowers (geitonogamy) of the same individual<sup>12</sup> and references therein). Despite the possibility for self-pollination, a large number of species have evolved an extraordinary variety of adaptive mechanisms, e.g. spatial and temporal

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separation of male and female function or separation of male and female sex organs onto different individuals in order to force transfer of cross-pollen and ensure cross-fertilization<sup>13–16</sup>.

The only strategy that guarantees full protection against self-fertilization is the self-incompatibility (SI) system<sup>17,18</sup>. The self-incompatibility phenomenon is regarded as an effective mechanism for preventing inbreeding depression<sup>9</sup>. There are two main types of self-incompatibility, i.e. gametophytic (GSI) and sporophytic (SSI)<sup>19,20</sup>. In the Solanaceae family, the self-incompatibility mechanism is primary gametophytic (GSI), which is based on the interaction between proteins present in the pollen and in the pistil<sup>21–23</sup>. Less commonly, the late-acting self-incompatibility phenomenon occurs in other Solanaceae plants, in which the incompatibility reaction is delayed and arises after fertilization<sup>24,25</sup>. The degree of self-incompatibility can vary not only between species but also within a single species (among populations) or even individual plants<sup>12,26</sup>. This variability includes species being polymorphic for SI, with some populations being self-incompatible (SI) and others self-compatible (SC), and individual plants potentially transitioning from SI to SC over their lifespan<sup>27,28</sup>.

Presently, there is evidence that a majority of small angiosperm populations occurring in an altered environment (e.g. ecological conditions and pollinator assemblages have been modified by anthropogenic disturbance or climate change) are subjected to pollen limitation resulting from a lack of pollinators<sup>29,30</sup>. Therefore, well-organized conservation strategies require information on plant-pollinator interactions, which are fundamental for evaluation of the reproductive capacity of individuals and the capability of a population for maintenance and regeneration in both natural and human-changed ecosystems<sup>6,31</sup>.

The genus *Scopolia* Jacq. includes three species; two species are distributed across Central and South-Eastern Europe and the Caucasus region and one occurs in Japan and the Korean Peninsula<sup>32</sup>. Among them, *Scopolia carniolica* is a rare species and has been entered in the IUCN Red List of Threatened Species and classified as LC (Least Concern)<sup>33</sup>. In Poland, after the latest regulation, *S. carniolica* is under partial protection<sup>34</sup>.

Our previous research evidenced that *S. carniolica* flowers exhibit several traits that support entomophily: a large-size corolla, a visual guide that can serve as a signal pattern of floral rewards, abundant nectar and pollen attractants, the nectar composition (sucrose-dominant nectar), and a papillate wet stigma<sup>35</sup>.

Despite the importance of conservation of the rare *S. carniolica* species, its reproductive biology is unknown. Therefore, the aim of the present study was to examine the aspects of floral morphology and biology as well as the breeding system of *S. carniolica* in two populations. Specifically, we (i) studied the period of stigma receptivity and timing of pollen release during the course of anthesis and determined pollen quantity and quality, (ii) compared the fruit and seed set in diverse pollination experiments, (iii) examined the pollination treatment effects on seed viability, (iv) tried to assess whether the species is pollen-limited, i.e. if its reproduction is restricted by insufficient pollination service, and (v) attempted to identify the effective pollinator(s) of the species by means of the effectiveness of pollen transfer and deposition onto the stigma.

## Materials and methods

### Study species

*Scopolia carniolica* Jacq. (Solanaceae Juss.) is a perennial plant that develops vertical green above-ground stems (ca. 40–60 cm high) and underground rhizomes<sup>33,36</sup>. It prefers moist, shaded habitats in beech forests, especially on slopes and in valleys. Flowering occurs in early spring (from early April to early May). Its hermaphroditic flowers are set in leaf axils on relatively long pedicels<sup>36</sup>. The flowers are large (size = 20–25 mm in length; 7–10 mm in diameter) with campanulate brownish-violet corolla and the presence of nectar and pollen attractants<sup>37,38</sup>. Our previous study revealed that the flower life-span is ca. 3.3 days; sucrose-dominant nectar is secreted throughout the flower life-span, with a peak coinciding with the beginning of pollen release from the anthers. One flower produces ca.  $1.67 \pm 0.47$  mg of nectar,  $0.54 \pm 0.22$  mg of sugars, and  $1.95 \pm 0.56$  mg of pollen<sup>35</sup>. The species was described as protogynous and insect-pollinated<sup>39</sup>. Bumble bee queens are reported as the main floral visitors<sup>35</sup>. Fertilization occurs one day after pollination<sup>40</sup>. The fruit is a capsule that matures at the end of May and beginning of June (unpublished own observations).

### Study locations and populations

The study was carried out in two *S. carniolica* populations (artificial and natural) located at a distance of ca. 130 km away from each other (Fig. 1S a - b). The research in the artificial population was conducted in 2018 and 2019. The artificial population was located in the city of Lublin (L-population), SE Poland ( $51^\circ 16' N$ ,  $22^\circ 30' E$ , 200 m a.s.l.), within an area belonging to the Botanical Garden of Maria Curie-Skłodowska University. This population was established in 1975 and originated from natural sites in the Bieszczady Mountains (SE Poland): inventory number 877 from Zasan ( $49^\circ 23' N$   $22^\circ 25' E$ ) and 2166 from Widelki ( $49^\circ 07' N$   $22^\circ 41' E$ ). Each population covered an area of ca. 20 m<sup>2</sup>. The plants were grown on loess-origin soil. The surrounding vegetation consisted of species associated with the communities of the Fagellalia sylvaticae order (e.g. *Fagus sylvatica* L., *Ranunculus cassubicus* L., *Sympyton cordatum* L., *Scilla bifolia* L., *Corydalis solida* (L.) Clairv., *Corydalis cava* (L.) Schweigg. and Körte, *Allium ursinum* L., *Galium odoratum* (L.) Scop.).

A similar experiment was performed in 2023 in a natural population located in Potoki Forestry Division (P-population), Tomaszow Lubelski Forest District, SE Poland ( $50^\circ 18' N$   $23^\circ 30' E$ ). This population covered an area of ca. 60 m<sup>2</sup> and occurred in the ground flora of typical Fagellum carpaticum forest (= Carpathian beech forest) composed predominantly of *Pulmonaria obscura* L., *Anemone nemorosa* L., *Galium odoratum* (L.) Scop., *Ranunculus ficaria* L., and *Lathraea squamaria* L. The soil was fertile brown typical for beech wood<sup>41</sup>.

According to the Köppen classification<sup>42</sup>, both populations occur in the temperate climate zone (Dfb - warm-summer humid continental climate).

## Reproductive biology observations and pollen transfer effectiveness

### Stigma receptivity

The receptivity of the stigma was assessed in stigmas dissected from buds and 1-, 2-, 3, and 4-day-old flowers. Entire gynoecia were excised from randomly selected flowers, placed on a microscope slide, and covered with a drop of 3% hydrogen peroxide ( $H_2O_2$ ) to detect peroxidase activity. Stigmas that produced bubbles within 2–4 min were considered receptive<sup>43</sup>. The proportion of ‘bubbling stigmas’ in relation to all that were checked was determined ( $n = 20$  stigmas for every point of flower life-span per year, i.e. 300 stigmas in total were checked, with 8–10 plants sampled from each population per year). These observations were conducted under a binocular stereomicroscope (NIKON SMZ-2B).

### Pollen quantity and quality

Flowers were selected randomly from at least six individuals and collected before pollen presentation at the bud stage ( $n = 20$  flowers per population). All anthers from individual flowers were placed separately in Eppendorf tubes (2.0 ml) and, after pollen grains were released, 2 ml of distilled water was added. Then, anther tissues were removed thoroughly, and the pollen grains were counted in 20- $\mu$ l samples using a hemocytometer (Bürker chamber) under a light microscope at 40 $\times$  magnification. The results were expressed as the average number of pollen grains per 2 ml of suspension using the formula described by<sup>44</sup>, giving the number of pollen grains per flower.

Pollen grain viability was determined in pollen collected during pollen presentation, i.e. in 2-, 3-, and 4-day-old flowers. At each time point, pollen was collected from  $n = 5$  flowers originating from 2 to 3 plants. Pollen was stained with 2% acetocarmine and fixed in glycerogelatin). Pollen grains which stained red were considered viable, while deformed or unstained grains were regarded as non-viable. For each time point, 4 repetitions  $\times$  100 pollen grains were examined across different fields of view<sup>45</sup>.

### Number of ovules

A total of 40 flowers ( $n = 20$  flowers per population) randomly selected from different individuals were examined for the number of ovules formed per ovary. The ovaries were cut lengthwise manually, and the ovules were counted under a stereoscopic microscope (Nikon SMZ-2B). The ovule number and pollen quantity data were used to assess the P/O – the pollen-to-ovule ratio was estimated according to Cruden<sup>7</sup>.

## Reproductive and breeding system study

### Pollination treatments

The flowers were marked at the bud stage (ca. 2–3 days prior to anthesis). Five flowers were labeled on three different days, i.e. in total 20 flowers were examined for each pollination treatment in each population. The flowers were selected randomly from at least six different plants.

The following treatments were performed: (1) spontaneous self-pollination (= flowers enclosed in a fine mesh bag to prevent access by insect visitors) to evaluate the possibility of autogamy, (2) induced self-pollination (= obligate autogamy; flowers isolated and hand-pollinated within the same flower with self-pollen), (3) geitonogamy (flowers isolated and hand-pollinated with pollen collected from a different flower in the same individual plant); (4) open out-crossed pollination (non-manipulated flowers available to insects (= control) to demonstrate insect importance in pollination, (5) induced cross-pollination (xenogamy; buds were emasculated before supplemental manual pollination with fresh cross-pollen collected from different individual plants and then isolated from further insect visits), (6) supplemental cross-pollination (flowers hand-pollinated with pollen collected from different individual plants, uncovered for open-pollination) to check pollen limitation. The flowers were also tested for apomixis, (flowers were emasculated and then bagged with isolators;  $n = 10$  flowers per year per population). Since *S. carniolica* is capable of vegetative propagation via rhizomes, pollen for cross-pollination originated from individual plants occurring  $> 15$  m from pollen receptor individuals to ensure that it was derived from different genets.

### Fruit and seed collection and analysis of phenotypic features

Fruits obtained from the pollination treatments were harvested in the beginning of July prior to opening and the number of fruits in relation to pollinated flowers was established. In the laboratory, we determined (i) the fruit size based on their length measured with an electronic caliper (accuracy of 0.1 mm) – the measurements were made along the longitudinal axis from the capsule base to the top point, (ii) the number of seeds per each capsule, and (iii) the mass of 1,000 seeds.

Seed viability was checked using a TTC test (2,3,5-triphenyl tetrazolium chloride test)<sup>46,47</sup>, and performed on seeds that were at the same stage of development. The main indicator of seed suitability for testing was a change in the capsule color from green to greenish-light brown and the presence of dark brown, hard, and dry seeds inside the capsule. The seeds ( $n = 40$  per pollination treatment per year) were soaked in distilled water in a thermal chamber at a temperature of 20–25 °C for 24 h. Then, they were filtered and scarified, i.e. cut in such a way that the two halves (cotyledons) remained connected. Next, the seeds were flooded with a 0.1% solution of 2,3,5-triphenyltetrazolium chloride with distilled water and placed in a thermal chamber at 35 °C for 24 h. The seeds were filtered and then cut longitudinally. They were classified as either (i) viable – with the embryo evenly stained red or (ii) non-viable – with the embryo completely unstained or light pink or with the absence of an embryo and endosperm. Seeds were viewed under a stereoscopic microscope.

### Pollen transfer and deposition effectiveness

Pollen transfer and pollen deposition experiments were conducted in 2018 in the L-population and in 2023 in the P-population. To determine the ability of insect pollinators to transfer *S. carniolica* pollen, the method

described in detail by Zych<sup>48</sup> was applied. Briefly, we captured flower-visiting insects using an entomological net and carefully removed the pollen from their bodies and transferred it onto a microscope slide (stained with a 2% acetocarmine, fixed in glycerogelatin). The number of pollen grains of *S. carniolica* and 'other' species was counted. The visiting insects were assigned to the following groups: *Bombus* spp., *Apis mellifera*, and solitary bees ( $n = 3\text{--}12$  individuals were examined for each group of insects).

To establish the effectiveness of a pollinator in depositing pollen onto the stigma, we determined the quantity and purity of the pollen load deposited during a single visit. First, we randomly selected flowers at the bud stage ( $n = 24$  in the L-population and  $n = 9$  in P-population) from different plants. The flowers were emasculated and enclosed in tulle isolators to exclude stigma contamination. Then, after the flower opened, the isolator was carefully removed and the insect was allowed to visit the flower once. After this single visit, the flower was re-isolated and labeled, indicating the type of the visiting insect. On the next day, the stigmas were removed from the experimental flowers with clean forceps and microscopic slides were made (stained with 2% acetocarmine, fixed in glycerogelatin)<sup>49</sup>. The number of *S. carniolica* pollen grains and pollen of 'other' species was counted in each sample. Control flowers were subjected to identical experimental conditions but were excluded from insect visitation ( $n = 10$  per population). These observations were made using a light microscope (Nikon Eclipse E200; Tokyo, Japan).

### Statistical analyses

Analysis of variance (ANOVA) was used to test the significance of differences between mean values of the analyzed floral features (pollen viability, stigma receptivity between floral phases) and pollen production between the populations; prior to performing any tests, the data were checked for normality (Shapiro-Wilk test). If the data were not normally distributed, the log transformation ( $\ln$ ) was applied.

A generalized linear model (GLM) was used to assess the effects of population and pollination treatment on fruit set and number of seeds produced per capsule. For percentage of set fruits, a beta regression model was used to analyze continuous response variables bounded within the unit interval (0,1). The model was fitted using the betareg package in R, employing a logit link function to map the percentage data onto the real line. For the seed number (response variable), a negative binomial (NB) regression model was used to account for overdispersion in the count data. The explanatory variables included year, population, and treatment. A log link function was applied to relate the expected seed count to the linear predictor. The model was fitted using the glm.nb() function from the MASS package in R (v4.3.1<sup>50</sup>).

A generalized linear model (GLM) with a Gaussian distribution and a log link function was applied to assess the effects of explanatory variables (pollination treatment and population) on fruit length and seed weight. Seed viability (proportion of viable seeds per fruit) was analyzed using a beta regression with a logit link (betareg package, R), which is appropriate for modeling proportional data bounded between 0 and 1.

The generalized linear models were built using the R programming language (R Core Team, 2023) in R Studio, version 1.3.1093 (PBC).

The Kruskal-Wallis test was used to analyze the number of pollen grains transported on insect bodies and deposited on the stigma. Statistica 13.3 software (Statsoft, Kraków, Poland) was applied for these calculations.

## Results

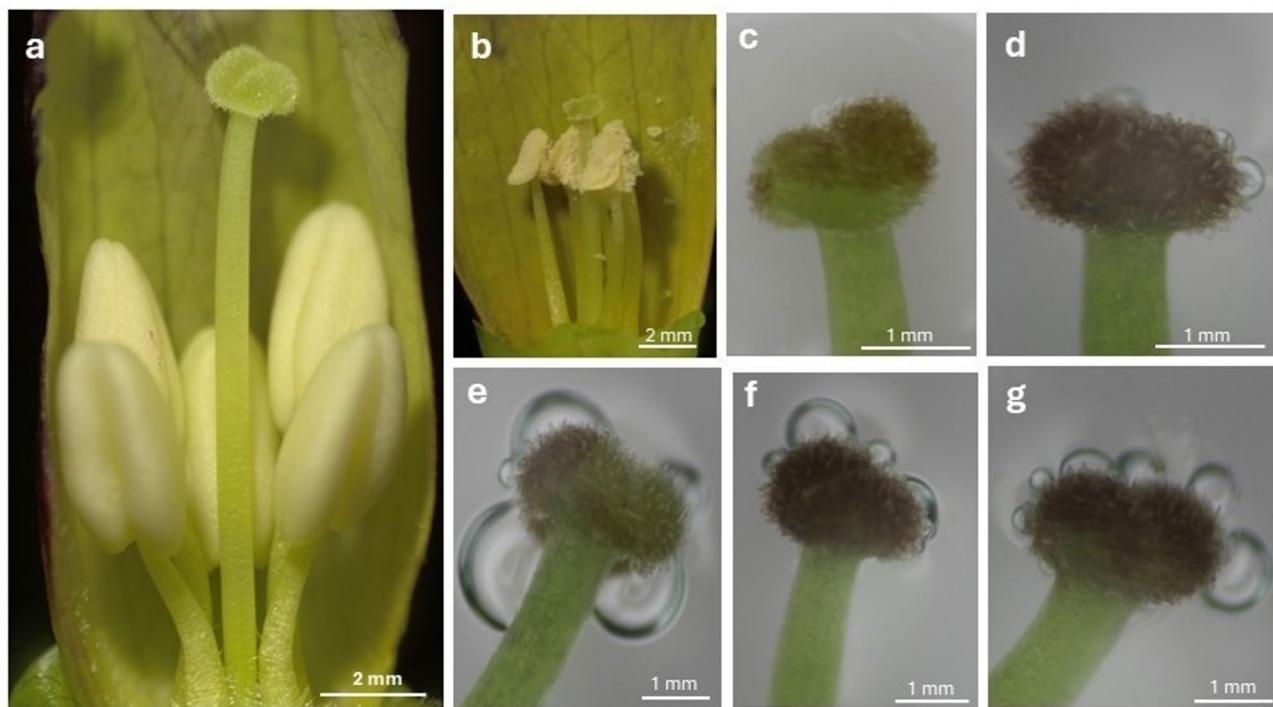
### Reproductive biology: flower features

Flowering of *Scopolia carniolica* occurs in early spring, with the timing varying between years. Depending on the year, flowering begins either in early April and extends to the first days of May, or from mid-April to the beginning of May (Fig. 2S). The species has hermaphroditic flowers. Initially, in the buds and 1-day-old flowers, the pistil is positioned above the anthers, but later, the anther-stigma distance shortened during anthesis and the stigma was placed almost at the level of the pollen-bearing anthers or slightly above the anthers (Fig. 1a-b). Stigma receptivity differed significantly over the flower life-span ( $F_{4,25} = 576.85, P < 0.001$ ) (Fig. 1c-g). It started in the floral bud stage ( $8.5 \pm 2.0\%$  of receptive stigmas) and gradually increased, reaching a maximum on the 3rd day of anthesis ( $97.0 \pm 2.0\%$ ), and lasted until the end of the flower life-span. Anther dehiscence began on the 2nd day of anthesis. The average pollen viability was high ( $79.9 \pm 5.0\%$ ) and differed significantly across the pollen presentation time ( $F_{2,21} = 181.48, P < 0.001$ ); the highest viability was recorded on the 3rd day of anthesis ( $91.9 \pm 3.0\%$ ), whereas the lowest value was obtained in the 4th day ( $72.8 \pm 3.0\%$ ). From the second day until the end of the flower life-span, an overlapping period of both female and male functions was observed (Fig. 2).

The pollen productivity varied between the populations ( $F_{1,38} = 16.04, p = 0.0002$ ). It amounted to  $46,149.0 \pm 16,334.0$  pollen grains per flower in the L-population and was ca. 55% higher in the P-population (mean =  $72,423.9 \pm 23,468.2$  pollen grains per flower). The number of ovules per ovary was similar in both populations ( $p > 0.05$ ;  $43.2 \pm 13.4$  in the L-population and  $45.5 \pm 12.6$  in the P-population). The average calculated P/O ratio was  $1102.1 \pm 303.1$  in the L-population and  $1692.5 \pm 677.8$  in the P-population.

### Fruit and seed set

Apomixis was not identified in *S. carniolica*. Pollination treatment had a strong effect on the reproductive performance of the species (beta regression, logit link; Fig. 3a). Neither population nor year significantly influenced fruit set. No fruits developed when insect vectors were excluded (spontaneous self-pollination, treatment 1), indicating the absence of autonomous within-flower self-pollination (Table 1). The highest fruits set were recorded in the natural and induced cross-pollination treatments (treatments 4, 5; estimate =  $-0.1845$ , odds ratio [OR] =  $0.83, p > 0.05$ ). Relative to open cross-pollination (GLM reference treatment 4), induced self-pollination (treatment 2) resulted in a 90% reduction (OR =  $0.10, p < 0.001$ ) while geitonogamous pollination (treatment 3) showed a 64% reduction (OR =  $0.363, p < 0.001$ ) in fruit set. Supplemental cross-pollination (treatment 6) reduced fruit set by approximately 61% (OR =  $0.39, p < 0.001$ ) (Tabel 1 S).



**Fig. 1.** Macrophotographs of *S. carniolica* showing: (a) flower with stamens before pollen presentation and anthers clearly separated in a 1-day-old flower; (b) stigma placed slightly above the pollen presenting anthers in a 3-day-old flower, (c-g) stigma receptivity evaluated using the peroxidase test at various flower stages: (c) bud; (d) 1- day-old flower; (e) 2-day-old flower; (f) 3-day-old flower; (g) 4-day-old flower.

No seeds were produced after within-flower self-pollination and induced self-pollination, indicating self-incompatibility (Fig. 3b). Seed production was similar between open and induced cross-pollination (multiplicative effect = 0.84–0.90,  $p > 0.05$ ; Table 2). Geitonogamy (treatment 3) resulted in a 37–46% reduction in seed number compared with open cross-pollination (multiplicative effect = 0.54–0.63,  $p < 0.01$ ). However, the seeds formed were non-functional. Supplemental cross-pollination (treatment 6) reduced strongly seed number by 39–49% (multiplicative effect = 0.51–0.61,  $p < 0.001$ ) compared to open cross-pollination. Population origin also influenced seed set, with plants from P-population producing approximately 31% fewer seeds than those from L-population (multiplicative effect = 0.69,  $p < 0.001$ ). Year had no detectable effect on seed production in L-population (Table S2).

#### Fruit and seed quality

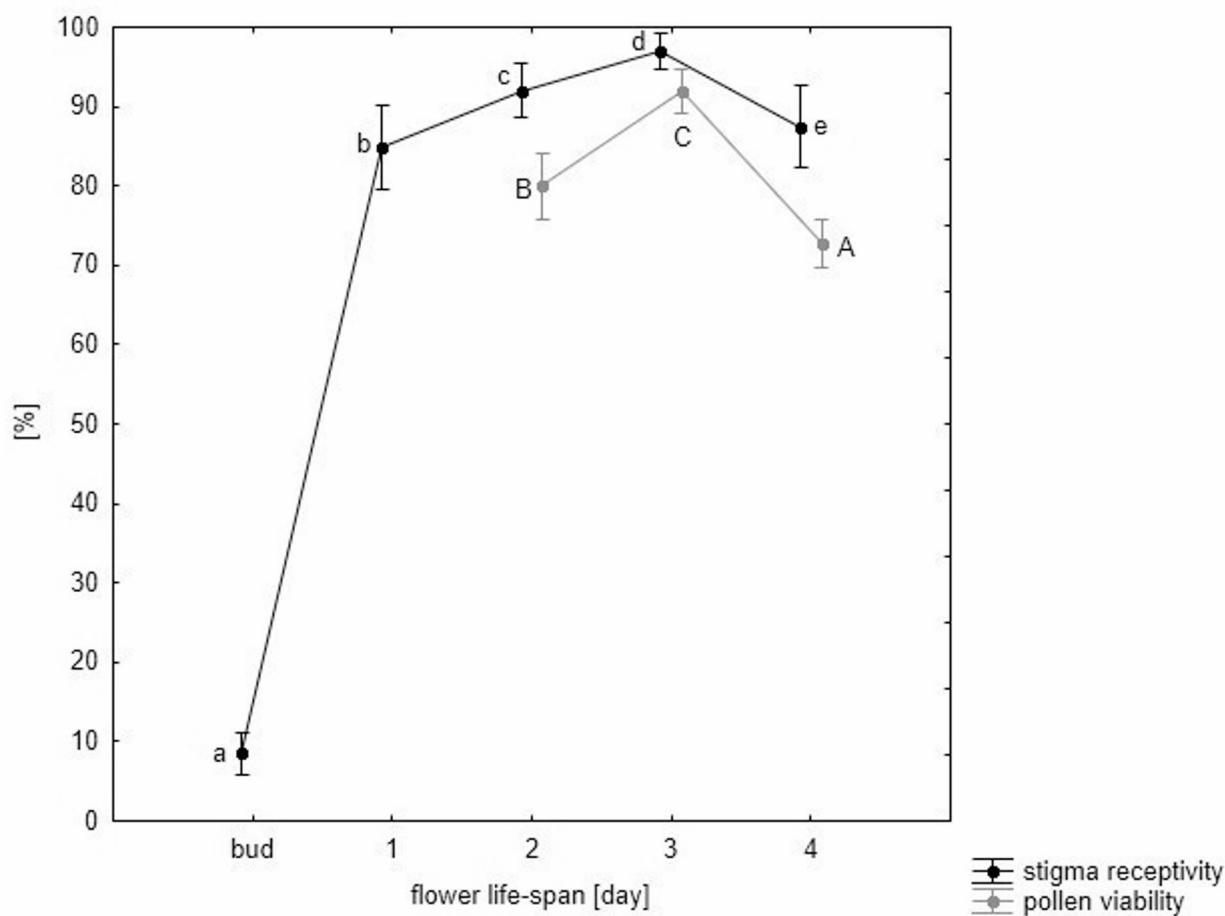
Population had no significant effect on any of the measured fruit and seed quality traits (fruit length:  $p = 0.086$ ; seed weight:  $p = 0.612$ ; seed viability:  $p = 0.518$ ; full model outputs are provided in Supplementary materials; Tables S3–S5). Therefore, subsequent summaries focused on the effects of pollination treatments, which strongly influenced fruit and seed traits (Table 3). Overall, open cross-pollination maintained the highest fruit and seed quality. The largest fruits were produced in the natural and induced cross-pollination groups (treatments 4, 5, Fig. 4). In contrast, induced self-pollination reduced fruit length by 38.3% (multiplicative effect = 0.62,  $p < 0.001$ ), geitonogamous pollination by 18.5% (0.82,  $p < 0.001$ ), and supplemental cross-pollination by 15.0% (0.85,  $p < 0.01$ ).

For seed weight, geitonogamous fruits produced seeds about 62% lighter than those from open cross-pollination (estimate = 0.38,  $p < 0.001$ ), whereas induced and supplemental cross-pollination treatments did not differ significantly from open cross-pollination ( $p > 0.05$ ).

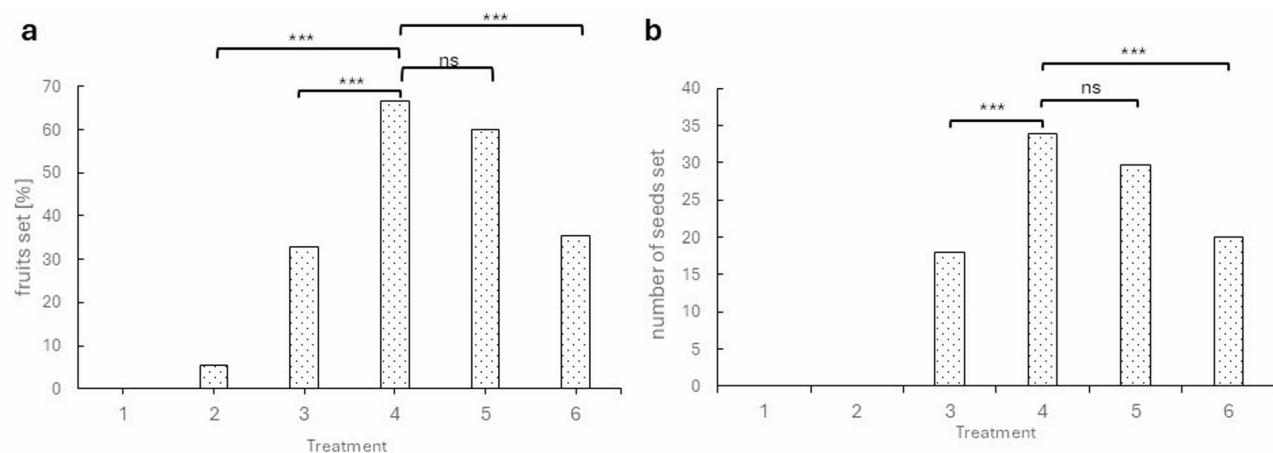
The seeds developed in the cross-pollination treatment (treatments 4–6) were fully formed, i.e. they had well-developed embryos and endosperm (Fig. 4). Beta regression analysis revealed that the seed viability did not differ significantly between induced and open cross-pollination ( $p < 0.05$ ), whereas supplemental cross-pollination significantly reduced seed viability, compared to cross-pollination ( $p < 0.001$ ; Tables 3 and 5 S). The seeds produced in the geitonogamous pollination treatment were non-viable, as they had no embryo and no endosperm.

#### Pollen transfer and deposition effectiveness

All investigated insect visitors carried the pollen of *S. carniolica* and ‘other’ plant species. We found differences in the number of pollen grains of both *Scopolia* (Kruskal–Wallis test:  $H_{(2, N=33)} = 7.28$ ,  $P = 0.026$ ) and ‘other’ species ( $H_{(2, N=33)} = 8.02$ ,  $P = 0.018$ ) carried by the various insect groups. The number of conspecific pollen grains carried by *Bombus* species (mean =  $551.06 \pm 379.79$ , range 8.0–904.0,  $n = 15$ ) was statistically similar to



**Fig. 2.** Changes in stigma receptivity and pollen viability in *S. carniolica*. Values are means calculated across populations and study years. Whiskers represent standard errors. Different letters indicate statistically significant differences between flower ages (a-d for stigma receptivity; A-C for pollen viability) at  $p < 0.05$ , based on HSD Tukey's test.



**Fig. 3.** Average fruit set (a) and seed number per fruit (b) in *S. carniolica* subjected to different pollination treatments: 1 - spontaneous self-pollination, 2 - induced self-pollination, 3 - geitonogamous pollination, 4 - open out-crossed pollination, 5 - induced cross-pollination (xenogamy), 6 - supplemental cross-pollination. Significance codes: \*\*\*  $p < 0.001$ , \*\*  $p < 0.01$ , ns = not significant, based on generalized linear models (GLMs). Note: no fruit or seeds were produced in spontaneous self-pollination (treatment 1) or induced self-pollination (treatment 2); therefore, values for these treatments are not available.

Predictor	Estimate	Std. Error	z-value	p-value	Significance
(Intercept)	0.455	0.165	2.754	<0.01	**
Population: P-population	0.127	0.130	0.973	0.331	ns
Treatment 2: induced self-pollination	-2.306	0.468	-4.926	<0.001	***
Treatment 3: geitonogamous pollination	-1.015	0.256	-3.972	<0.001	***
Treatment 5: induced cross-pollination	-0.185	0.184	-1.001	0.317	ns
Treatment 6: supplemental cross-pollination	-0.936	0.230	-4.070	<0.001	***

**Table 1.** Effects of population and pollination treatment on fruit set in *S. carniolica* analyzed using a beta regression GLM with a logit link function. Estimates are expressed relative to the cross-pollination (treatment 4), which served as the reference category. Significance codes: \*\*\*  $p < 0.001$ , \*\*  $p < 0.01$ , ns = not significant. Note: estimates for spontaneous self-pollination (treatment 1) are not available as no fruit set was observed after this treatment.

Predictor	Estimate	Std. Error	z-value	p-value	Significance
(Intercept)	3.641	0.065	55.878	<0.001	***
Population: P-population	-0.372	0.078	-4.785	<0.001	***
Treatment 3: geitonogamous pollination	-0.615	0.108	-5.691	<0.001	***
Treatment 5: induced cross-pollination	-0.165	0.089	-1.858	0.063	ns
Treatment 6: supplemental cross-pollination	-0.495	0.109	-4.594	<0.001	***

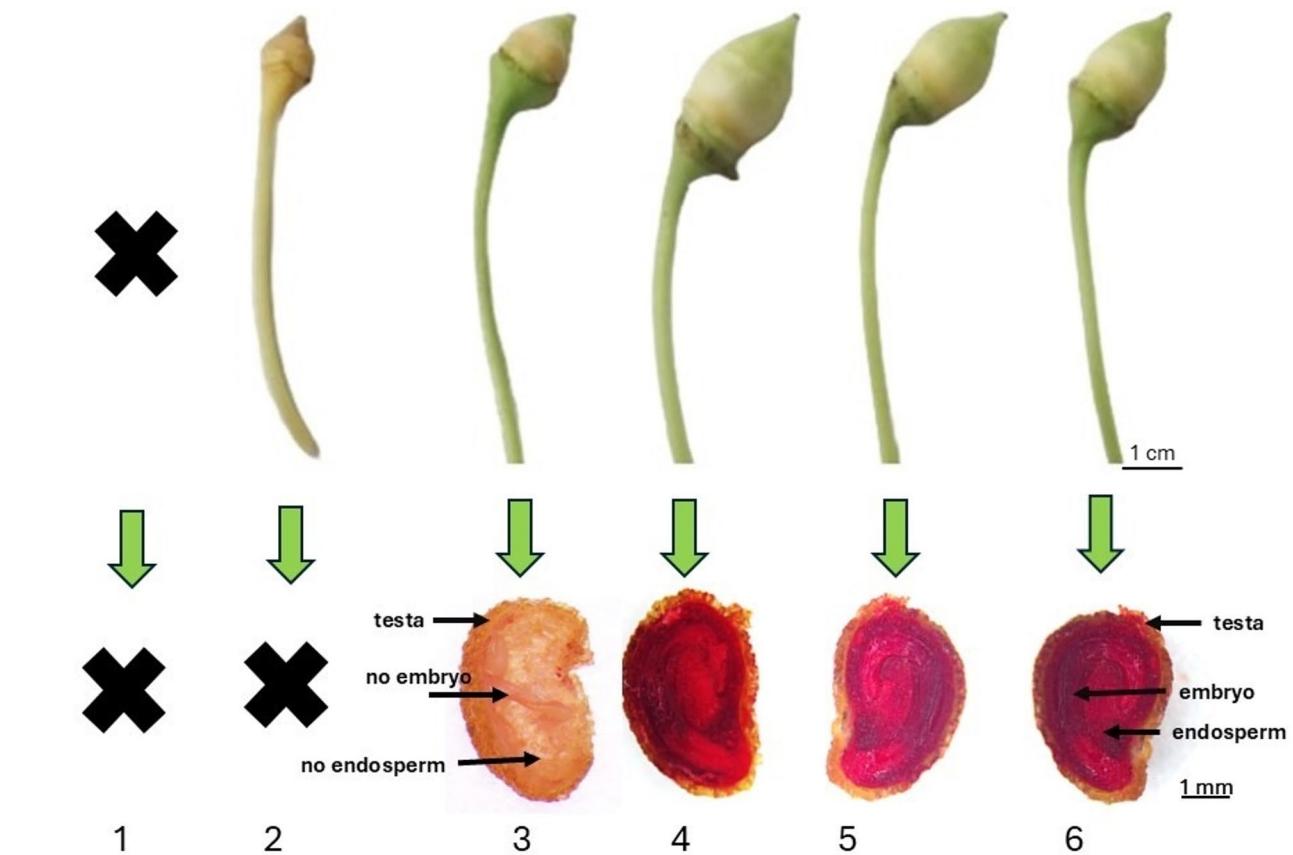
**Table 2.** Effects of population and pollination treatment on seed number in *S. carniolica* analyzed using a negative binomial generalized linear model (GLM). The model used a log link function. Significant negative coefficients indicate reduced seed set to the cross-pollination (treatment 4), which served as the reference category. Significance codes: \*\*\*  $p < 0.001$ , \*\*  $p < 0.01$ , \*  $p < 0.05$ , ns = not significant. Note: estimates for spontaneous self-pollination (treatment 1) and induced self-pollination (treatment 2) are not available because no fruit set was observed following these treatments.

Trait	Treatment	Estimate	Multiplicative effect (exp[Estimate])	Approx. % change vs. open pollination	Significance
Fruit length [mm]	Treatment 2: induced self-pollination	-0.483	0.617	-38.3%	***
	Treatment 3: geitonogamous pollination	-0.205	0.815	-18.5%	***
	Treatment 5: induced cross-pollination	-0.071	0.931	-6.9%	ns
	Treatment 6: supplemental cross-pollination	-0.162	0.850	-15.0%	**
1000 seed weight [g]	Treatment 3: geitonogamous pollination	-0.965	0.381	-61.9%	***
	Treatment 5: induced cross-pollination	0.001	1.001	+0.1%	ns
	Treatment 6: supplemental cross-pollination	0.022	1.022	+2.2%	ns
Seed viability [%]	Treatment 5: induced cross-pollination	-0.233	-	-	ns
	Treatment 6: supplemental cross-pollination	-0.539	-	-	***

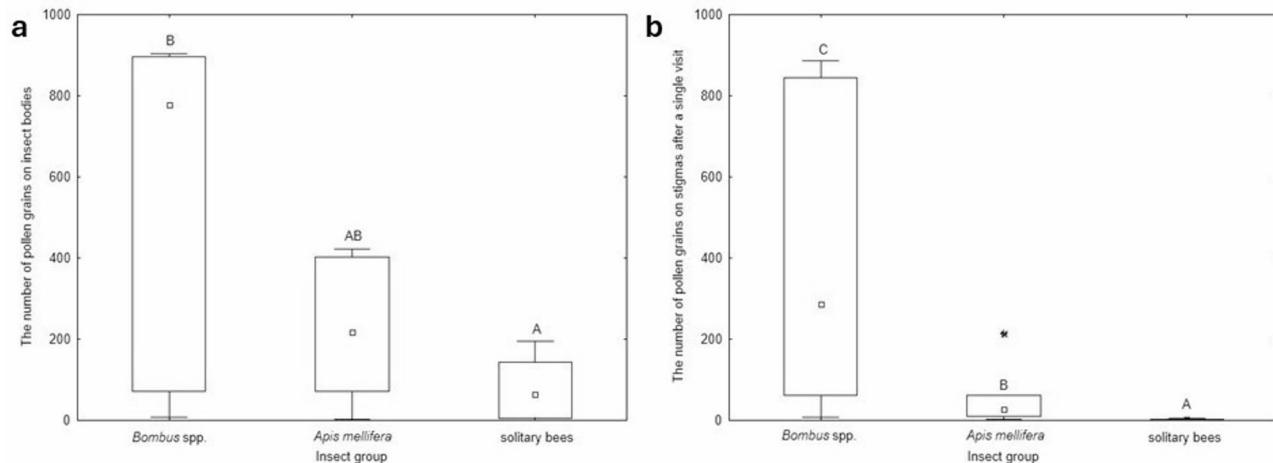
**Table 3.** Effects of different pollination treatments on fruit length, 1000-seed weight, and seed viability in *S. carniolica*, relative to open cross-pollination. Estimates from generalized linear models (Gaussian family; log link) and beta regression models (logit link) are shown. Multiplicative effects (exp[Estimate]) and approximate percent changes relative to open pollination are provided for fruit length and seed weight. Seed viability estimates are presented on the logit scale of the beta regression model. Significance codes: \*\*\*  $p < 0.001$ , \*\*  $p < 0.01$ , ns = not significant. Note: seed weight estimates for treatments 1–2 and seed viability estimates for treatments 1–3 are not available because no seeds were produced following these treatments.

that carried by *Apis mellifera* (mean =  $323.8 \pm 367.7$ , range 3.0–1405.0,  $n = 11$ ; Fig. 5a). Solitary bees transferred the smallest amount of *S. carniolica* pollen grains on their bodies (mean =  $82.8 \pm 70.9$ , range 4.0–194.0,  $n = 7$ ). The pollen loads carried by bumble bees were the most diverse with a high share of pollen of ‘other’ plants. No ‘other’ pollen grains were noted on the *S. carniolica* stigmas.

The number of pollen grains deposited on the stigma per single visit also differed between the insect groups ( $H(2, N = 33) = 20.09, P = 0.0001$ ). The highest amount of cognate pollen on the stigma during a single visit (mean =  $413.9 \pm 354.4$ , range 7.0–886.0,  $n = 15$ ; Fig. 5b) was deposited by bumble bees, revealing their greatest effectiveness. Significantly less pollen was left by *Apis mellifera* (mean =  $60.9 \pm 73.9$ , range 2.0–215.0,  $n = 11$ ), whereas only  $2.1 \pm 1.4$  pollen grains (range 1.0–5.0,  $n = 7$ ) were found to be loaded by solitary bees.



**Fig. 4.** Appearance of fruits and seeds developed after different pollination treatments in *S. carniolica*: 1 - spontaneous self-pollination, 2 - induced self-pollination, 3 - geitonogamous pollination, 4 – open out- crossed pollination, 5 - induced cross-pollination (xenogamy), 6 supplemental cross-pollination. Seed viability was assessed using the TTC test. The symbol X indicates absence of fruit or seed set.



**Fig. 5.** The number of *S. carniolica* pollen grains carried by three insect groups (*Bombus* spp., *Apis mellifera*, and solitary bees): (a) on insects' body, (b) deposited on stigma. The mark within each box indicates the median; box boundaries represent the 25th and 75th percentiles; whiskers indicate the range of non-outlier values; asterisks (\*) mark extreme values. Values represent means across observations. Different letters indicate significant differences between groups according to the Kruskal-Wallis test ( $p < 0.05$ ).

## Discussion

Our study provides the first description of the floral traits related to reproductive biology and the breeding system in *Scopolia carniolica*. The floral morphology and reproductive biology of *S. carniolica* indicate that the species has evolved mechanisms promoting cross-pollination, while maintaining the potential for self-pollination. The species is strongly self-incompatible and strictly dependent on insect-mediated cross-pollination, as self-pollination and pollinator exclusion produced no fruits or viable seeds. Natural and induced cross-pollination yielded the highest fruit and seed set, whereas within-flower self-pollination and geitonogamous pollination produced few or non-functional seeds, highlighting the critical role of pollinators in reproductive success. Population-level differences in seed production, suggesting that reproductive success across populations may be subject to limitations (local pollinator activity, compatible pollen availability, pollen quality, timing of pollination). However, for this rare species, conserving pollinator communities (mainly bumblebees), maintaining population connectivity, and preventing habitat fragmentation are critical to ensure effective cross-pollination and long-term survival.

### Floral traits: support of both cross- and self-pollination

*Scopolia carniolica* flowers have evolved morphological and biological characteristics that encourage cross-pollination but still carry the risk of self-pollination. We observed the changes in the position of the stigma in relation to the anthers, i.e. a phenomenon described by Boucher et al.<sup>51</sup> as movement herkogamy. This phenomenon was reported in Solanaceae species, e.g. in *Solanum lycopersicum*<sup>52</sup>. Herkogamy has long been regarded as an important floral trait that plays an advantageous role in increasing outcrossing rates and reducing self-pollination<sup>53,54</sup>. In *S. carniolica*, the shortening of the anther-pistil distance was observed during anthesis - the stigma reached the level of pollen-presenting anthers or was located slightly above the anthers in the 2-day-old flowers, which might increase the likelihood of automatic self-pollination.

In particular, we documented incomplete protogyny at the intrafloral level based on the criteria of Lloyd and Webb<sup>54</sup>. Knuth<sup>59</sup> described *S. carniolica* flowers as protogynous, but the study did not consider the receptivity of stigmas. We found that the stigma became receptive before anther dehiscence and remained functional throughout pollen presentation up to the end of anthesis. In general, such an overlapping of sexual functions might allow within-flower self-pollination<sup>8,54,55</sup>. However, even if self-pollen was deposited on the stigma (due to the anther-stigma position and overlapping stigma receptivity with pollen presentation), no seed production was recorded after a treatment which allowed for spontaneous within-flower self-pollination (treatment 1), indicating that *S. carniolica* relies on outcrossing for reproduction and the presence of self-incompatibility phenomena that prevent self-fertilization.

At the population level, the protogyny in *S. carniolica* is 'asynchronous' - flowers in the female and male sexual phases are present at the same time in multiple ramets per individual genets, which allows geitonogamous pollination (e.g.<sup>56-59</sup>). The promotion of geitonogamy by 'asynchronous protogyny' was demonstrated e.g. in species from the genus *Scrophularia*, Scrophulariaceae<sup>60,61</sup>. Indeed, in *S. carniolica*, the seeds developed after geitonogamy were non-viable as they formed no embryos and endosperm, indicating the presence of late-acting self-incompatibility.

### Breeding system: obligatory out-crossing with self-incompatibility

We analyzed various traits in order to gain broader knowledge of the plant mating system, i.e. in addition to the fruit set and size, we evaluated the degree of the seed set and estimated the ability of seeds to express vital function by measuring the metabolic activity of the embryo (63,64). However, the seed viability results assessed with the TTC test (2,3,5-triphenyl tetrazolium chloride test) have to be considered as a 'potential' for seedling establishment and do not indicate the seed and seedling performance in non-optimal environmental conditions for the species or its role in population stability<sup>62</sup>.

The results of the pollination experiment evidenced that *S. carniolica* is unable to self-pollinate effectively but relies on pollinators to ensure fertilization and successful seed and fruit development, as no fruits and seeds were set when insects were excluded. The floral attributes (e.g. showy corolla, presence of primary attractants, predominance of sucrose in nectar, changes in nectar sugar composition) described in our previous complementary article<sup>35</sup> are in concordance with the idea of entomophily in this species<sup>39,40</sup>. Another feature of our study species that is crucial for successful sexual reproduction is cross-pollination. Specifically, this assumption is supported by the highest fruit and seed set noticed in the cross-pollinated flowers (both natural and induced). Likewise, only seeds produced by the cross-pollinated flowers had fully formed embryos, indicating the outcrossed nature of species and the benefits of cross-pollination for viable offspring. As shown by literature data, an obligatory cross-pollination mating system is a reproductive strategy where cross-pollination is essential for fertilization and seed production, which indicates that the species cannot reproduce effectively through self-pollination<sup>63-66</sup>. In fact, self-pollination in *S. carniolica* (whether autonomous or induced within-flower self-pollination) consistently fails to produce seeds, indicating strong outcrossing and self-incompatibility<sup>20,67-70</sup>. Self-incompatibility is further proved by the results of induced geitonogamous self-pollination, where fruits may develop but their seeds are non-viable and have no embryos and endosperm, consequently leading to infertile offspring. The production of non-viable embryos following geitonogamous pollination may indicate a system of late-acting self-incompatibility<sup>19,28,71</sup>. Late-acting self-incompatibility is widely described in angiosperms (e.g.<sup>24</sup>) and has also been reported within the Solanaceae family, e.g. in *Lycium cestroides*<sup>72</sup>. It is a phenomenon where self-pollen tubes are allowed to reach the ovary and successfully penetrate the ovules, but the developing seeds are non-viable, leading to self-sterility<sup>10,73</sup>. Further research should include detailed histological analyses of post-pollination events on stigmas, pistils, and ovaries with self- and cross-pollen in order to identify where the self-incompatibility barrier actually occurs in *S. carniolica* and try to indicate a possible mechanism for controlling self-incompatibility at the molecular and genetic levels.

Facultative xenogamy was indicated by the P/O ratio, as its value ( $1102.1 \pm 303.1$ – $1692.5 \pm 677.8$ ) falls within the range of 1,000–10,000<sup>7</sup>. However, the P/O ratio is based only on the ‘pollination success’ and does not analyze the offspring quality.

Surprisingly, supplemental cross-pollination did not improve fruit set or seed production; instead, it considerably reduced them compared to naturally and experimentally cross-pollinated flowers in both studied populations. There are several possible causes of these unexpected results. It is possible that the amount of pollen applied during supplementation was too large (the stigmata received both insect-applied pollen, including self-pollen and hand-applied pollen) and the stigmatic surface was clogged, hence pollen germination was blocked and therefore many ovules were not fertilized. In fact, the presence of movement herkogamy (discussed above) in the flowers of *S. carniolica* may also potentially contribute to blocking the stigma with self-pollen. Research conducted by Opedal<sup>74</sup> demonstrated that the reduced anther-pistil distance during movement herkogamy can be disadvantageous - it restricts stigma’s access to cross-pollen and increases self-pollen deposition, leading to stigma clogging and increased competition between self- and cross-pollen, possibly hindering cross-pollination. In consequence, it can negatively impact the seed set, particularly in self-incompatible species<sup>75</sup>. Other studies have also shown that excessive pollen coverage on the stigma hinders the fertilization process in *Collinsia heterophylla* (Plantaginaceae)<sup>76</sup> and *Silene alba* (Caryophyllaceae)<sup>75,77</sup>, supporting the idea that over-pollination can negatively affect reproductive success. In *S. carniolica*, these mechanisms likely explain why supplemental cross-pollination reduced rather than improved fruit and seed set, highlighting the importance of pollen quality, timing, and stigma receptivity in this strongly outcrossed, self-incompatible species. These findings also raise the question of whether reproductive success in *S. carniolica* is truly limited by pollen quantity, or rather by the quality, compatibility, and timing of pollen transfer - a question addressed in the following section.

### Possibility of pollen limitation

Despite the dependence on cross-pollination, our data indicate that natural pollination provides near-optimal reproductive outcomes. However, based on our data it is not possible to clearly state whether the species is pollen limited due to insufficient pollen amount on the stigmas. In strongly self-incompatible *S. carniolica* the disparity in seed number between open cross-pollination and supplemental cross-pollination may likely reflect pollen interference, where the added pollen, potentially incompatible, disrupted fertilization by naturally deposited pollen. These results indicate that *S. carniolica* reproduction may be dependent on the quality, source, and timing of pollen transfer, rather than the total amount of pollen received. While the species is entirely cross-pollen-dependent, natural pollination appears to provide an optimal balance for successful reproduction. Given that pollen viability fluctuates throughout the floral lifespan, reproductive output is likely constrained by both pollen compatibility and temporal variation in pollen viability. Literature data indicate that differences in the timing of pollination, whether occurring at different times of day, flower maturity stages, or across the flowering season, may influence reproductive outcomes by altering pollen transfer efficiency, pollen tube growth, or fertilization success<sup>28,78–80</sup>. In the Solanaceae, the timing of pollination is a key factor determining reproductive success. Both the time of day and the age of the flower may influence pollen viability, stigma receptivity, and the rate of pollen tube growth. In *Solanum lycopersicum*, pollination performed in the morning hours (07:00–09:00) resulted in higher fruit set, by approximately 18–25%, compared to pollination conducted at midday<sup>81,82</sup>. The disparity has been attributed to lower morning temperatures, which maintain higher pollen viability and accelerate pollen tube growth, thereby increasing the probability of successful fertilization. The age of the flower may also influence reproductive outcomes. In *S. lycopersicum* and *Capsicum annuum*, pollination on the day of anthesis resulted in high fruit-set rates (70–90%), whereas a 24-hour delay reduced fruit set to 30–60%<sup>83–85</sup>. An even more stranger effect has been observed in *Nicotiana tabacum*, where a one-day delay resulted in more than a 60% reduction in seed number<sup>78</sup>. These results indicate the existence of a narrow ‘time window’ during which stigma receptivity and pollen viability are optimal. Reproductive success may also be influenced by flowering phenology. For example, in *Solanum sisymbriifolium*, fruit set reaches ~ 70% at the start of the flowering season but falls below 35% toward the end, accompanied by fewer seeds per fruit<sup>80</sup>. This decline has been attributed to reduced pollinator activity and decreasing pollen quality as the season progresses. It seems that the observed differences in seed number among *S. carniolica* populations reflect not just pollen availability, but mainly variation in pollen quality, availability of compatible pollen, and timing of transfer. However, pollination success and subsequent fruit/seed development are not solely determined by pollinator behavior or abundance and pollen traits. Various pre- and post-pollination factors, including availability of resources, can also significantly impact the pollination/fertilization outcomes<sup>86</sup>. Furthermore, the outcrossing sexual reproduction may be limited by e.g. the characteristics of the population (when the population is too small and/or too uniform genetically) or intense competition for pollinator service among individual plants<sup>87,88</sup>.

### Pollinators

We used two indicators (the number of pollen grains transferred on insect bodies and the number of pollen grains deposited on stigmas during a single visit) as a proxy for pollinator effectiveness. Bumble bees and solitary bees (mainly *Andrena fulva*) were indicated as pollinators of *S. carniolica* by Knuth<sup>39</sup>. We found that solitary bees deposited almost no pollen, demonstrating that they are not the true pollinators of our study species. Presumably, morphological and chemical adaptations in *S. carniolica* (e.g. numerous non-glandular trichomes on the inner surface of the corolla and the presence of various chemical compounds in corolla cells reported by Tymoszuk et al.<sup>35</sup> restricted the pollen deposition by short-tongued solitary bees. Indeed, morphological and chemical adaptations, alongside pollinator behavior, play a crucial role in controlling access to floral rewards and sexual organs, ultimately influencing pollinator effectiveness<sup>89</sup>.

Our research evidenced that bumble bees deposited substantially more pollen to *S. carniolica* flowers than is needed for fertilization of all ovules (ca. 9-fold, on average), while honey bees loaded slightly higher amounts

(ca. 1.5-fold, on average) than required. Therefore, it seems that both pollinator types can provide sufficient pollination, which highlights their importance in the reproductive success of *S. carniolica*, even if honey bees might be less efficient. However, adequate pollination of individual flowers does not guarantee overall pollination success at the population level. As reported by many authors, even if the average pollen load is large enough to fertilize all ovules, some flowers may still experience insufficient pollination and fail to set all possible seeds (e.g.<sup>16,90</sup>). Given the predicted decline of bumblebee populations in Europe<sup>87,88</sup>, the self-incompatibility of *S. carniolica* may potentially accelerating population declines and leading to a contraction of its geographic range.

We are aware of several limitations of this study. First, experiments were conducted in only two populations (artificial vs. natural), and observations were temporally separated, occurring in different years for each population. This was partly due to challenges in locating sufficiently large natural populations. Second, pollination treatments in the natural population were restricted to a single year because of permit limitations, which may not fully capture interannual variability in sexual reproduction, such as fluctuations in seed production. Third, seed quality was assessed solely using the tetrazolium test. While this test provides a useful measure of embryo viability, it does not fully reflect germination potential under natural conditions. Future studies should address these limitations by examining the biological and genetic basis of self-incompatibility in *S. carniolica* and validating pollination results across multiple populations to better assess the role of pollen limitation in reproductive success. Additionally, multi-year observations and complementary seed quality assessments, such as germination trials under variable environmental conditions, would provide a more comprehensive understanding of the factors regulating reproduction in this species.

In summary, reproductive success in the clonal, self-incompatible, and strongly outcrossed *S. carniolica* depends on pollen quality, compatibility, and timing rather than quantity. Natural pollination provides near-optimal outcomes, while excessive or incompatible pollen can hinder fertilization. Bumble bees and honey bees are the main effective pollinators, highlighting their key role in reproduction. In this context, the conservation of *S. carniolica* should focus on maintaining abundant and diverse pollinator communities ensuring genetic diversity and spatial mixing of clones. These factors are crucial to sustain viable populations and promote long-term reproductive success in this species.

## Data availability

The raw datasets used during the current study are available from the corresponding authors on reasonable request.

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## Author contributions

K.T., B.D. – conceptualized the study; K.T. – conducted the field observations; K.T., B.D. – performed the laboratory procedures; E.S. – performed pollen analyses; K.T., J.M., B.D. – performed the statistical analyses; K.T. – took the photographs; K.T. – designed the tables and figures; K.T., B.D. – wrote the main manuscript text; B.D. – supervised the study. All authors reviewed the manuscript.

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## Declarations

### Competing interests

The authors declare no competing interests.

### Additional information

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