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# Bumblebees, the proficient mountain pollinators: evidence from *Stachys germanica* (Lamiaceae) along the altitudinal gradient of Mount Olympus, Greece

KONSTANTINOS MINACHILIS<sup>1,\*</sup>, APHRODITE KANTSA<sup>1,2</sup> and THEODORA PETANIDOU<sup>1</sup>

<sup>1</sup>Laboratory of Biogeography and Ecology, Department of Geography, University of the Aegean, Mytilene 81100, Greece

<sup>2</sup>Department of Environmental Systems Science, ETH Zürich, Zürich 8092, Switzerland

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Pollen limitation and its drivers along altitudinal clines form a controversial topic, highlighting the need for more studies and in different biomes. We tested the hypothesis that the populations of a predominantly bee-pollinated plant are pollen-limited along elevations and examined whether this is related to bee visitation rate. For two years, we studied flower visitation, pollen limitation and mating system using five populations of *Stachys germanica* L. occurring between 327 and 1936 m a.s.l. on Mt. Olympus, Greece. *S. germanica* showed moderate to high self-compatibility, low spontaneous self-pollination and low pollen limitation across all altitudes and years. Bumblebees were the dominant pollinators; their visitation increased with altitude and was positively correlated with seed set and negatively correlated with pollen limitation. The opposite trend was found for the visitation by other pollinators. Seed mass was independent of visitation. Low pollen limitation is evidently due to high bumblebee visitation along the altitude, whereas seed mass could be linked to resource availability or environmental conditions. Our findings underline the functional role of bumblebees on Mediterranean mountains, and the need to focus on bumblebee conservation on this legendary mountain.

ADDITIONAL KEYWORDS: altitude – Mediterranean – pollen limitation – pollination effectiveness – pollination treatments.

## INTRODUCTION

The reproductive success of flowering plants may be affected by the transfer of low pollen quantity or/and quality to stigmas, resulting in pollen limitation (Ashman *et al.*, 2004; Dauber *et al.*, 2010). Especially in insect-pollinated plants, pollen limitation occurs when insect availability or visitation are reduced (Totland, 1997), followed by a decrease in fruit or/and seed production. In mountain ecosystems, pollen limitation has been shown to increase with altitude (Lundemo & Totland, 2007; Dai *et al.*, 2017; Yun *et al.*, 2019). Especially in alpine zones, low temperature, overcast skies and strong winds may affect pollinator diversity, abundance and/or flight activity, and subsequently, flower visitation and pollination effectiveness (Arroyo *et al.*, 1985; McCall & Primack, 1992; Totland, 1994).

Furthermore, abiotic factors prevailing at high altitudes, such as low temperatures and nutrient availability, could affect post-pollination events, viz. ovule fertilization, seed maturation and number (Totland & Birks, 1996; Totland, 1997).

Despite the predisposition for higher pollen limitation at higher elevations, an overestimation of such a limitation along an altitudinal gradient is not unheard of: a meta-analysis of 18 studies, although confirming pollen limitation in alpine plants, did not find a significant difference in pollen limitation between different alpine and lowland plants (viz. five alpine species, 19 lowland species) (García-Camacho & Totland, 2009). Plants may indeed possess mechanisms to alleviate the adverse effects of pollinator scarcity and/or efficiency and abiotic stress that may occur with increasing altitude. First, because plant species, especially the alpine ones, may respond to pollen limitation with a shift towards autogamy, resulting in high self-compatibility and autofertility (Medan

\*Corresponding author. E-mail: [k.minachilis@aegean.gr](mailto:k.minachilis@aegean.gr)

*et al.*, 2002; Seguí *et al.*, 2017). Second, alpine plants may become asexually reproduced by adopting clonal reproduction or apomixis (Richards, 1997; Körner, 2003). Regarding pollination mode, alpine plants may evolve into wind-pollinated or ambophilous (i.e. pollinated both by wind and animals; Culley *et al.*, 2002), expand longevity of their flowers (Blionis & Vokou, 2001; Fabbro & Körner, 2004; Trunschke & Stöcklin, 2017) or display larger flower size (Galen, 1996; Maad *et al.*, 2013) to ensure visitation by pollinators. Besides, alpine plants have been shown to physiologically address this condition by allocating more resources into flowers as compared with lowland plants (Fabbro & Körner, 2004).

Few of the above will happen if pollinators are available and effective. This may be the case of alpine environments where bumblebees play a dominant role as pollinators (Bingham & Orthner, 1998; Blionis & Vokou, 2001). Indeed, bumblebees are capable of transferring large quantities and high-quality pollen to stigmas due to their large hairy bodies and foraging behaviour (Bingham & Orthner, 1998; Koski *et al.*, 2018; Gallagher & Campbell, 2020). Several studies have associated differences in pollen limitation among distinct sites or years with bumblebee visitation, highlighting the bumblebee's high pollination effectiveness (Theobald *et al.*, 2016; Dai *et al.*, 2017). In support of this, pollen limitation was found to be very low or even absent in cases where bumblebees were dominant or the only pollinators available (Hargreaves *et al.*, 2015; Semwal *et al.*, 2019). In addition, high bumblebee pollination effectiveness was found to balance overall visitation decrease through the season (Gallagher & Campbell, 2020) and compensate for increased pollen limitation due to small bees (Lau & Galloway, 2004; Koski *et al.*, 2018).

Efforts to systematically investigate pollen limitation along wide altitudinal gradients in the Mediterranean Basin remain scarce, despite the area being a hotspot for wild bee diversity (Michener, 1979, 2000) with a remarkable and biogeographically important highland bumblebee fauna (Iserbyt *et al.*, 2008; Manino *et al.*, 2010; Minachilis *et al.*, 2020). Thus far, research was conducted to restricted altitudinal ranges each time, including lower altitudinal ranges [Bandera & Traveset (2005) and Guliás & Traveset (2012), altitudes up to 1250 m, Mallorca], medium altitudes [Vokou *et al.* (1990) and Blionis and Vokou (2002), altitudes between 900 m and 1850 m, Mt. Olympus, Greece; Sanz and Pulido (2015) between 1300 and 1400 m, Central Spain], and higher altitudinal ranges [Seguí *et al.* (2017), between 2400 and 3500 m, El Teide, Tenerife; Gómez (2002) at 2600 m, Sierra Nevada, Spain]. Recently, Herrera (2020), in a 19-year community study in a mountain region (altitudes 770–1920 m) in south-eastern Spain, explored the causes of pollen limitation albeit without considering altitudinal gradient.

This work is part of a wider project aiming at investigating pollination ecology in the habitats occurring along the altitudinal gradient of Mt. Olympus. Up until recently, mainly plant-focused studies have been conducted on the mountain without considering the altitudinal cline (Vokou *et al.*, 1990; Blionis & Vokou, 2005 and references cited therein). Our first published results disclosed a rich bumblebee fauna, with bumblebee diversity found to increase along the altitudinal gradient, peaking at 1900–2000 m (Minachilis *et al.*, 2020). Here we particularly aim to explore the mating system [*sensu* Neal & Anderson (2005)] and the rates of pollen limitation of a predominantly bee-pollinated plant along the altitudinal gradient of Mt. Olympus. As a model plant and phytometer we selected *Stachys germanica* L. (Lamiaceae), an obligatory insect-pollinated perennial herb with hermaphrodite flowers that do not reproduce asexually; the plant sets seed every year (Dunn, 1997) and occurs on a wide altitudinal range of Mt. Olympus. Specifically, we addressed the following questions regarding *S. germanica*:

- (i) What is the altitudinal effect on the mating system?
- (ii) Is the plant pollen-limited and is this related to altitude?
- (iii) Is flower visitation of different pollinator guilds related to altitude?
- (iv) Do seed set, seed mass and pollen limitation depend on visitation rate of different pollinator guilds along the altitudinal range of the mountain?

## MATERIAL AND METHODS

### STUDY AREA

Mount Olympus (2918 m), a legendary landmark, constitutes a circular massif of 25 km diameter on average, situated near the central-east coast of Greece. Its flora consists of > 1700 plant species, encompassing c. 25% of the Greek flora, including 58 Greek endemics of which 25 are endemic to Mt. Olympus itself (Strid, 1980; Strid & Tan, 1986, 1991). Because of its high altitude, the rich zonation along its elevation gradient, separation from other mountain ranges, high diversity of flora and fauna, and glacial history, Mt. Olympus is considered a model system for ecological, evolutionary and biogeographical studies (Strid, 1980).

The study was conducted on the north-eastern slope of Mt. Olympus, following the path route from Litochoro town to the Mousses Plateau. Along this route and at different altitudes and vegetation zones (see below), we established five experimental sites with a north-north-eastern aspect (Fig. 1; Supporting Information, Table S1). Our study sites are located within three



**Figure 1.** Map of the location of the study sites along the altitudinal gradient of Mt. Olympus, Greece (own spatial data depicted on Google Earth background).

of the four major vegetation zones of Mt. Olympus, according to [Strid \(1980\)](#): (i) evergreen–sclerophyllous (Mediterranean) scrub (300–600 m), (ii) mixed beech and montane coniferous forests (700–1500 m) and (iii) cool temperate coniferous forests (1500–2500 m). The tree line along this path lies at *c.* 2200 m (K. Minachilis, pers. obs.). The climate is Mediterranean at lower altitudes and temperate at intermediate to higher ones, with snow covering areas above 2000 m from late October to late May ([Strid, 1980](#)).

#### STUDY SPECIES

*Stachys germanica* L. (Lamiaceae) is a perennial herb densely clothed in long white silky hairs, resulting in a silvery, grey-green appearance. The corolla is pale pink, pinkish-mauve, or purple with yellow anthers, with the 4-ovuled ovary ending up to a white hairless forked stigma. Shoots develop in April or May and flowers begin to open in the first or second week of July. According to [Dunn \(1997\)](#), the flowers are protandrous, not cleistogamous and open from the bottom of the spike upward, producing nectar at the base of the ovary. Pollination in the UK is performed principally by bumblebees visiting the flowers for nectar ([Dunn, 1997](#)). The species has a wide distribution from the Canary Islands to Europe and Caucasus and can be

found at altitudes 100–2200 m a.s.l. ([Dunn, 1997](#); [Govaerts, 2003](#)).

#### POLLINATION TREATMENTS

The study took place in two different years, 2014 and 2016. In order to investigate the mating system and pollen limitation at different altitudes, we applied different pollination treatments to the flowers including measurement of free pollination in each population. In 2014, pollination treatments were applied in sites S-327, S-1485 and S-1936, while in 2016, pollination treatments were applied in sites S-327, S-1162, S-1485, S-1838 and S-1936 ([Supporting Information, Tables S1–S3](#)). The pollination treatments applied in each population were as follows:

1. Free Pollination (FP): Two flower buds per plant were marked and left uncovered to be freely visited by insects.
2. Cross Pollination (CP): Two flower buds per plant were hand-pollinated with fresh pollen from another conspecific plant situated at least 1 m in distance and then left uncovered (supplementary pollination).
3. Self-Pollination (SP): Two flower buds per plant were hand-pollinated with fresh pollen from the same plant

and then covered with fine bridal veil to exclude them from being visited by insects until flower withering.

4. Spontaneous Self-Pollination (SSP): Two flower buds per plant were covered with fine bridal veil excluding them from being visited by insects until flower withering.

To minimize among-plant effects, all treatments were carried out on the same individual plants which were chosen haphazardly within a population ([Supporting Information, Table S1](#)). Hand pollinations were done by gently rubbing anthers against the recipient stigmas using fine forceps ([Dauber \*et al.\*, 2010](#); [Petanidou \*et al.\*, 2012](#)). After maturation (*c.* 4 weeks after treatment), fruits were collected and brought to the laboratory, where the number of seeds per flower treated were counted. Seed mass of the flowers treated per plant and treatment were measured in 2016 using a balance of high sensitivity. Seed set (= number of seeds) per treatment and site was calculated as follows: first we took the average number of seeds per flower within a plant, then the average number of seeds across all plants. Statistical analyses were made on a per plant basis. Similarly, seed mass per treatment and site was calculated as follows: first we weighed the total number of seeds for each plant and treatment (accuracy 0.001 g), then we calculated the average seed weight per plant and treatment across all plants.

#### FLOWER VISITATION BY INSECTS

Flower visitation was documented in 2016. At each site, we haphazardly selected two plots of 1 × 1 m, and we counted the total number of *S. germanica* flowers in each of them. Then, we recorded the number of pollinator visits on the flowers for 60 min per plot during the peak of insect activity in calm and sunny days: 30 min in the morning (9.00 to 12.00) and 30 min in the afternoon (12.00 to 16.00), the observation per site conducted within the same day. Due to persistent afternoon thunderstorms at the highest elevations, each of the sites S-1838 and S-1936 was surveyed for two consecutive mornings. Flower visitors were identified on the wing to genus (bees), family (flies) or order (butterflies). Visitation rates were expressed as numbers of visits per flower and hour.

#### POLLINATION INDICES

Self-compatibility, spontaneous self-pollination and pollination effectiveness of *S. germanica* were calculated using, respectively, the following indices: Self-Compatibility Index (SCI) ([Petanidou \*et al.\*, 2012](#)), Spontaneous Self-Compatibility Index (SSI) ([Lloyd & Schoen, 1992](#)) and Pollen Limitation Index (PLI) ([Petanidou \*et al.\*, 2018](#)):

$$SCI = \frac{\text{average \# seeds set after SP}}{\text{average \# seeds set after CP}}$$

$$SSI = \frac{\text{average \# seeds set after SSP}}{\text{average \# seeds set after CP}}$$

$$PLI = 1 - \frac{\text{average \# seeds set after FP}}{\text{average \# seeds set after CP}}$$

where SP = Self-Pollination, SSP = Spontaneous Self-Pollination, FP = Free Pollination and CP = Cross Pollination treatment. To calculate each index per site, we first calculated each index per plant and then we took the average across all plants in a site.

SCI ranges from zero to ≥ 1, with 1 representing full self-compatibility ([Petanidou \*et al.\*, 2012](#)).

SSI ranges from zero to ≥ 1, with 1 representing full spontaneous self-pollination ([Lloyd & Schoen, 1992](#)).

PLI ranges from 0 (free pollination is maximally effective) to 1 (complete pollen limitation) ([Petanidou \*et al.\*, 2018](#)). In cases the values of the above indices were < 0 or > 1 before analysis they were set respectively to 0 and 1.

#### STATISTICAL ANALYSIS

To compare seed set of each pollination treatment among the different study sites, we used linear regression after we arcsine-transformed the percentage of mature seeds produced per flower (#seeds/4) to produce normally distributed samples. We used Levene's tests to confirm the homogeneity of variance of the response variable in all study sites. Statistical significance was assessed with ANOVA, followed by Tukey's range test, applied with the R function *TukeyHSD()*.

To investigate the relationships between visitation rate of bumblebees or non-bumblebee visitors and altitude, we used linear regression, including both the linear and the quadratic term of altitude as explanatory variables. The quadratic term was standardized ( $\bar{x} = 0$ ,  $\sigma = 1$ ) to avoid collinearity. To test for relationships between the seed set per flower treated (i.e. the arcsine-transformed percentage of mature seeds produced per flower) or the seed mass per plant and visitation rate of bumblebees or non-bumblebee visitors, we used linear models using the seed variables as dependent and the visitation rate as explanatory variable. Again, the quadratic term of the standardized version of the independent variable was also included in the model. For the latter analysis, we used the *lme()* function of the R package nlme 3.1–149, using site ID as a random factor.

To test variation of pollination indices (viz. SCI, SSI and PLI) among different sampling sites in 2014 or 2016 considered separately, we used generalized linear models with quasi-binomial distribution (link = 'logit')

to account for zero-inflation (Zuur *et al.*, 2009), using the function *glm()* in R (we should note that all these are proportion variables). The same model was used to test the relationship of PLI and visitation rate by bumblebees or non-bumblebee visitors. As visitation rate was measured in 2016, we used the PLI data obtained in that year. Calculation of PLI values were based on the results of pollination treatments, and recordings of pollinator visits provided the values of visitation rates to *S. germanica* (see above) in S-327, S-1162 and S-1838.

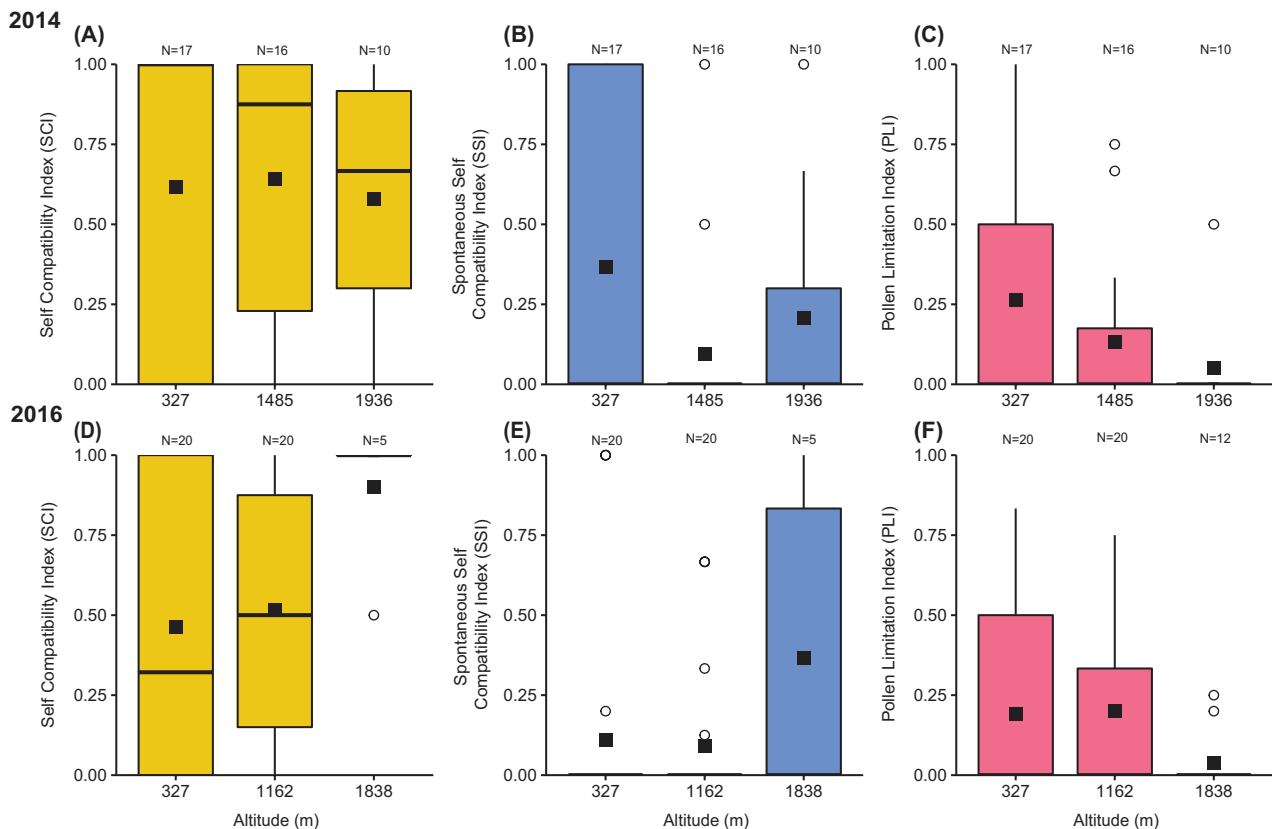
## RESULTS

### SEED SET AFTER POLLINATION TREATMENTS AND MATING SYSTEM

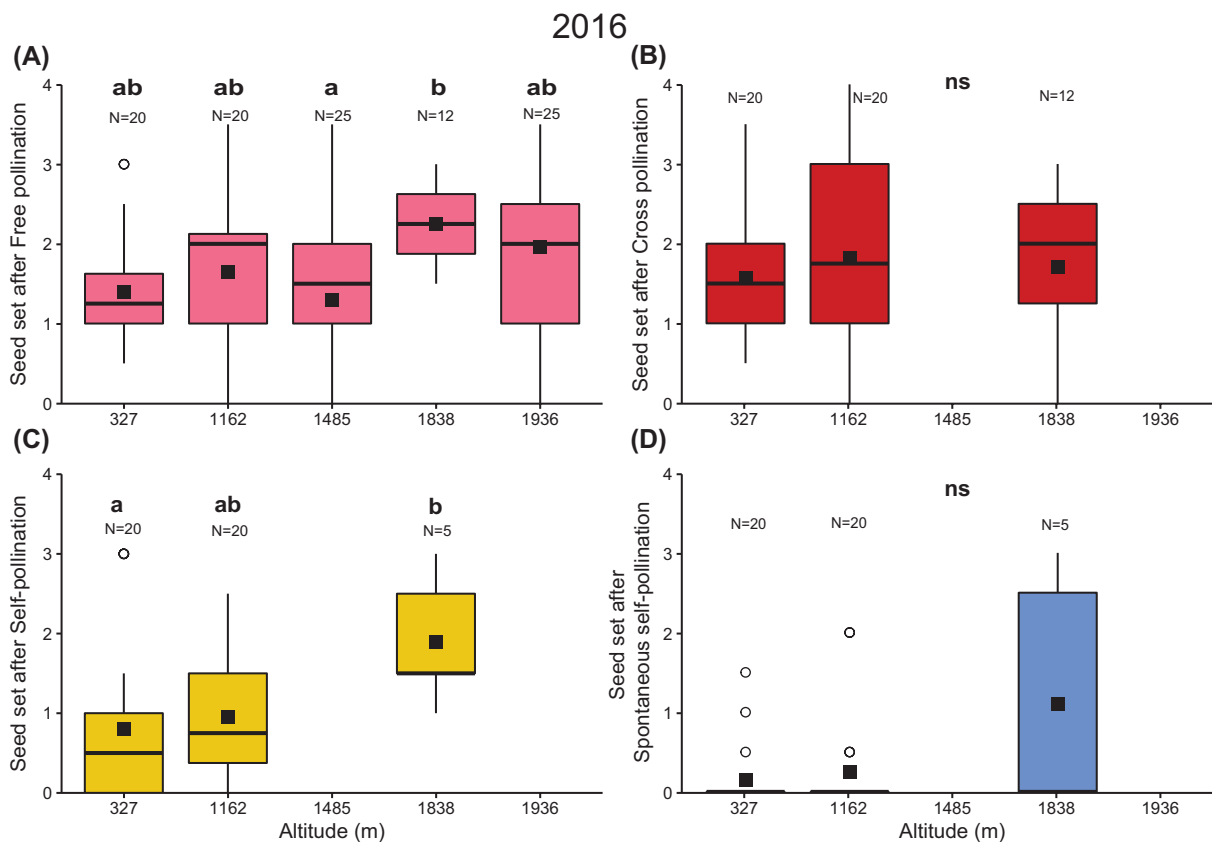
*S. germanica* was found to be moderately to highly self-compatible at all altitudes tested ( $0.46 \leq \text{SCI} \leq 0.90$ ) in both years of treatment (Fig. 2A, D; Supporting Information, Tables S2, S3); interestingly, it was found

to be fully self-compatible at 1838 m (SCI = 0.90) in 2016, a result, however, based on a limited number of plants ( $N = 5$ ). Spontaneous self-pollination was generally low in both study years, although not null ( $\text{SSI} \leq 0.37$ ; Fig. 2B, E; Supporting Information, Tables S2, S3). We found no statistical significance in altitudinal variation of either index in both sampling years.

Seed set after self-pollination marginally increased with altitude in 2016 (ANOVA,  $F_{2,42} = 2.94$ ,  $P = 0.064$ ) (Fig. 3C), but not in 2014 (Fig. 4C). Seed set after spontaneous self-pollination was not altitude-dependent in either year (Figs 3D, 4D). Seed set after free pollination increased with altitude in 2014 (ANOVA,  $F_{2,40} = 4.38$ ,  $P = 0.019$ ), the highest yield being recorded at S-1936 (Fig. 4A). In 2016, the highest seed set was observed, again, in a high population (viz. S-1838), although the difference was significant only to S-1485 and not to the remaining three populations measured (ANOVA,  $F_{4,97} = 2.86$ ,  $P = 0.027$ ) (Fig. 3A).



**Figure 2.** Pollination indices of *S. germanica* per altitudinal interval on Mt. Olympus measured in 2014 and 2016. A, D, Self-Compatibility Index (SCI). B, E, Spontaneous Self-Pollination Index (SSI). C, F, Pollen Limitation Index (PLI). Within the boxes, squares denote the mean values and black lines denote the median values. Open circles above and below the whiskers indicate outliers outside the highest and lowest value, respectively. Text above each box plot indicates sample (plant) number. Statistical differences of each index between sampling sites were tested with generalized linear models (see *Material and Methods*), but none was found significant.



**Figure 3.** Variation of seed set of *S. germanica* at the sites (indicated by the altitude) in 2016. Within the boxes, squares denote the mean values and black lines denote the median values. Open circles above and below the whiskers indicate outliers outside the highest and lowest value, respectively. Text above each box plot indicates sample (plant) number. Different letters denote significant statistical difference at the 0.05 level. Among-class differences have been acquired with ANOVA, followed by Tukey's range test.

#### POLLINATORS AND VISITS TO FLOWERS

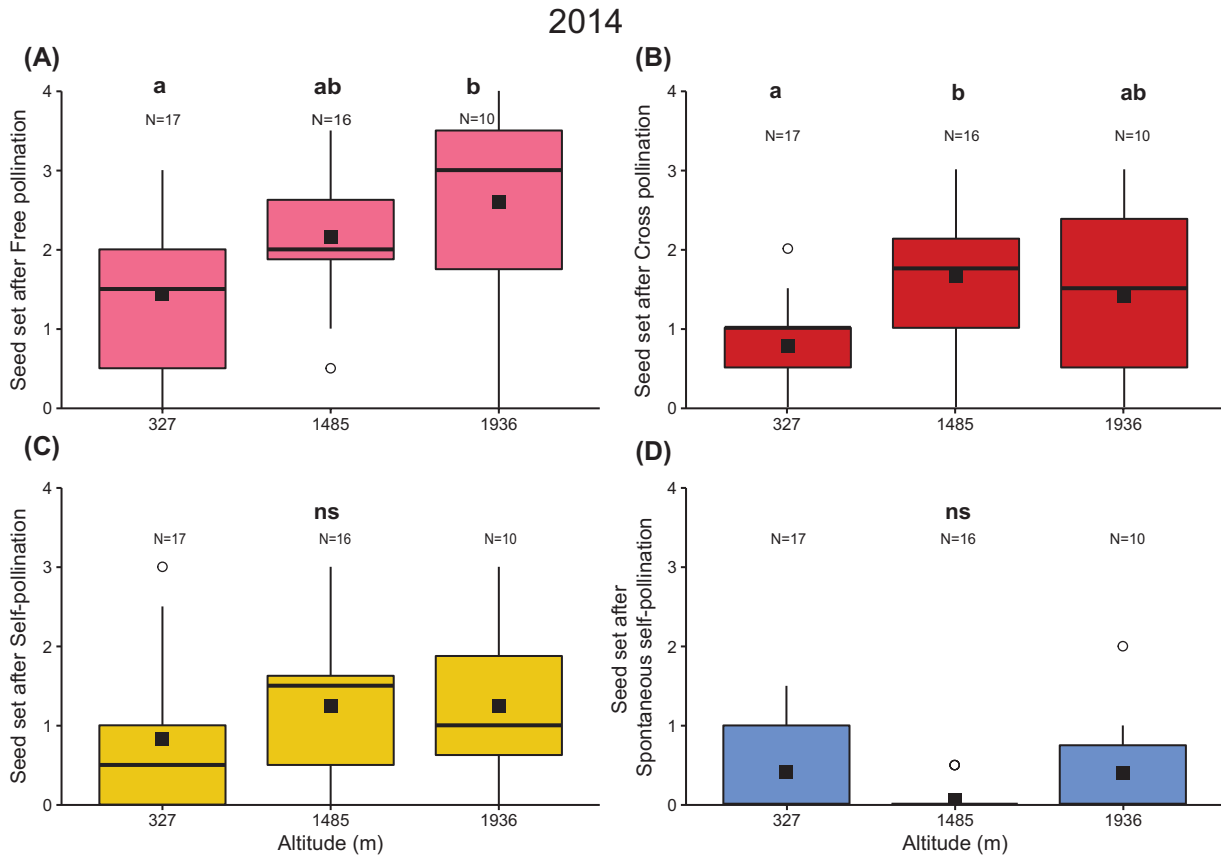
*S. germanica* was visited mainly by bees: bumblebees accounted for 84.7% of the total visits per flower while the other bee visits were paid mainly by *Anthidium* spp. (7.0%) and *Xylocopa* spp. (4.5%) and the non-bee visits paid by flies (1.2%) and butterflies (0.2%) (Supporting Information, Table S4). A marginal positive relationship was found between altitude and bumblebee visitation rate (LM  $F_{1,2} = 13.67$ ,  $P = 0.066$ ,  $R^2 = 0.79$ ) (Fig. 5A). The visitation rate by non-bumblebee visitors was altitude-independent (LM  $F_{1,2} = 0.10$ ,  $P = 0.777$ ) (Fig. 5A). Seed set per flower was positively correlated (although marginally) with bumblebee visitation (LME  $F_{1,2} = 9.89$ ,  $P = 0.088$ , marginal pseudo- $R^2 = 0.77$ , conditional pseudo- $R^2 = 0.97$ ) and negatively correlated with non-bumblebee visitation (LME  $F_{1,2} = 127.15$ ,  $P = 0.008$ , marginal pseudo- $R^2 = 0.62$ , conditional pseudo- $R^2 = 0.95$ ) (Fig. 5B). Seed mass per plant was affected neither by bumblebee visitation nor by non-bumblebee visitation (Fig. 5C).

#### POLLEN LIMITATION

*S. germanica* presented low pollen limitation across all altitudes and years (PLI  $\leq 0.26$ ) (Fig. 2C, F; Supporting Information, Tables S2, S3). We found no PLI variation along the altitudinal gradient in both sampling years. On the contrary, we found a statistically significant negative relationship between PLI and bumblebee visitation (GLM  $F_{2,51} = 4.94$ ,  $P = 0.031$ ), and a marginal positive relationship between PLI and visitation by non-bumblebee insects (GLM  $F_{2,51} = 3.55$ ,  $P = 0.065$ ) (Fig. 5D).

#### DISCUSSION

In this study, we investigated pollination effectiveness along the altitudinal gradient of a high Mediterranean mountain. To do so, we used pollen limitation of a phytometer occurring in a wide range of different elevations along the altitudinal gradient of Mt.



**Figure 4.** Variation of seed set of *S. germanica* at the sites (indicated by the altitude) in 2014. Within the boxes, squares denote the mean values and black lines denote the median values. Open circles above and below the whiskers indicate outliers outside the highest and lowest value, respectively. Text above each box plot indicates sample (plant) number. Different letters denote significant statistical difference at the 0.05 level. Among-class differences have been acquired with ANOVA, followed by Tukey's range test.

Olympus, viz. *S. germanica*, an obligatorily insect-pollinated plant with hermaphrodite flowers, in five populations occurring between 327 and 1936 m in altitude.

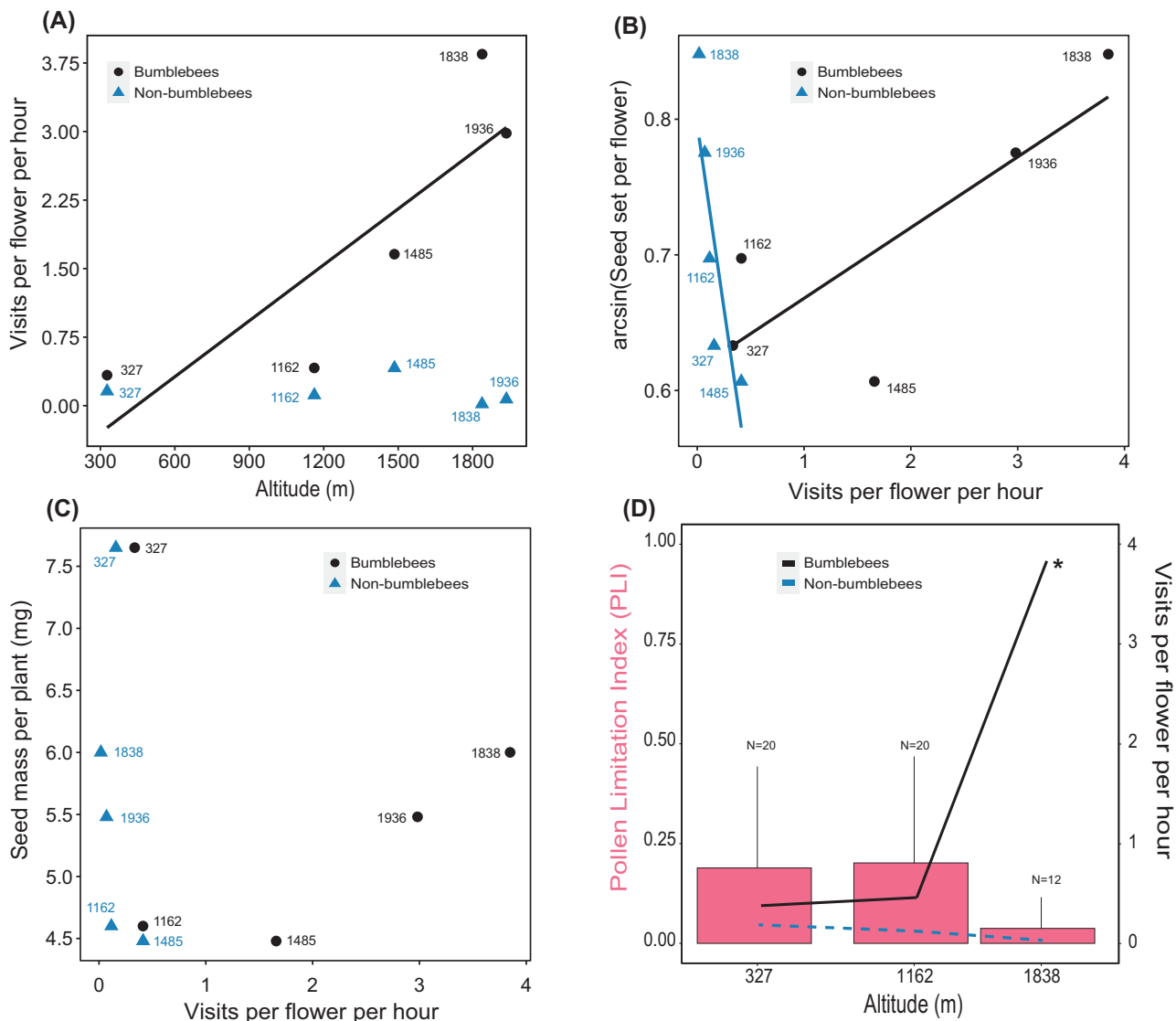
#### MATING SYSTEM AND ALTITUDE

*S. germanica* showed moderate to high self-compatibility and low ability for spontaneous self-pollination, both not varying with altitude in both study years. A few studies showed no altitude-dependence of autogamy, as in our case (Totland & Schulte-Herbrüggen, 2003; Arroyo *et al.*, 2006) whereas others found autogamy to be positively dependent on altitude (Medan *et al.*, 2002; Seguí *et al.*, 2017). The positive dependence was interpreted as an adaptation of the plants to low pollinator diversity, abundance and flight activity at higher altitudes. A mixed mating system including outcrossing, self-compatibility and an ability for spontaneous self-pollination has been shown as an assurance strategy to overcome low pollinator activity

and pollen limitation in harsh (alpine) environmental conditions (Larson & Barrett, 2000; Kalisz *et al.*, 2004; Eckert *et al.*, 2010). Therefore, we believe that the observed moderate to high self-compatibility and to the low-degree spontaneous self-pollination, both not altitude-dependent, denote the plant's mixed mating system and may act as a safety plan to maintain mountain populations of this obligatory seeding plant.

Several Lamiaceae taxa are self-compatible, but spontaneous self-pollination is infrequent due to preventing mechanisms, e.g. dichogamy (protandry) or herkogamy (Owens & Ubera-Jiménez, 1992). Other examples of spontaneously self-pollinated Lamiaceae species include *Ajuga chamaepitys*, *Salvia sclareoides* and *Salvia verbenaca* (De Clavijo, 1997; Navarro, 1997; Jorge *et al.*, 2015), *Cedronella canariensis* (Olesen *et al.*, 1998), *Dracocephalum austriacum* (Castro *et al.*, 2015), *Synandra hispidula* (Cantino, 1985), *Lepachinia floribunda* (Roldán & Ashworth, 2018), *Prunella vulgaris* (Ling *et al.*, 2017) and *Mesosphaerum suaveolens* (Aluri, 1990). Spontaneous self-pollination





**Figure 5.** Pollinator visitation to *S. germanica* flowers along the altitudinal gradient of Mt. Olympus in 2016: (A) bumblebee and non-bumblebee visitation rate along the gradient; (B, C) seed set per flower and seed mass per plant in relation to visitation by pollinators. Trend lines are shown only for the statistically significant relationships assessed with generalized linear mixed-effects models. (D) Pollen limitation Index (PLI  $\pm$  SD) (bars) and the visitation rates (line) per site sampled (S-327, S-1162 and S-1838, indicated by the altitude). The asterisk denotes the significant relationship between PLI and bumblebee visitation rates ( $P = 0.031$ ) tested with a generalized linear model (see *Material and Methods*). Y-axis label colour corresponds to the data with the same colour (PLI: pink, visitation rate: black).

has also been found in the genus *Stachys* by Rusterholz and Baur (2010) who reported > 20% seed set deriving from spontaneous self-pollination in *S. officinalis*. Our *S. germanica* also follows this pattern to some extent, with a higher rate of spontaneous self-pollination shown at a higher altitude. This result seems to be sporadic rather than a regularity at the high altitudes, as it was found only in one of the study years and only in one of the sites among all highest altitudes (S-1838). Therefore, we assume this is not due to a differential

flower structure along the elevation gradient (e.g. more elongated style at higher altitudes). Instead, we think it may be a result of a harsher weather (e.g. stronger winds) or other disturbances occurring at the highest altitudes. All in all, even though erratic, this result indicates the existing possibility for such a mountain species to backup seed setting in years that are especially adverse due to extremely bad weather conditions. This is especially enhanced in higher altitudes, where weather vicissitudes are most likely.

## POLLEN LIMITATION AND ALTITUDE

To our knowledge, our study represents the widest altitudinal investigation of pollen limitation of a single species to date (327–1936 m). Along this cline, pollen limitation of *S. germanica* was very low and almost null at high altitudes. Findings of other studies were similar, although conducted at higher altitudes and for different plant species [*Cineraria erodioides*, 2275–2675 m, South Africa (Black *et al.*, 2019); *Rhinanthus minor*, 1400–2200 m, Canada (Hargreaves *et al.*, 2015); *Viola cheiranthifolia*, 2400–3500 m, Tenerife (Seguí *et al.*, 2017); *Saussurea obvallata*, 3496–4335 m, India (Semwal *et al.*, 2019)]. In their meta-analysis, García-Camacho and Totland (2009) found no significant difference in pollen limitation between different alpine and lowland plants (viz. five alpine species, 19 lowland species); similar results were reported by Lázaro *et al.* (2015) in a community study in Norway, although different factors predicted pollen limitation, thus indicating an idiosyncratic community response. The aforementioned authors attributed the low pollen limitation to (i) pollinator-related factors [the outstanding performance of bumblebees (García-Camacho & Totland, 2009), reduced pollinator competition or higher visitation rates (Seguí *et al.*, 2017; Black *et al.*, 2019)], (ii) plant-related factors [higher autogamy (Hargreaves *et al.*, 2015; Semwal *et al.*, 2019) and higher flower longevity in the highlands (García-Camacho & Totland, 2009), resource limitation and environmental constraints (García-Camacho & Totland, 2009; Seguí *et al.*, 2017; Black *et al.*, 2019)], (iii) an idiosyncratic, multivariate and perhaps too complex to predict community response (Lázaro *et al.*, 2015) and (iv) sampling-related artefacts or technical issues (Black *et al.*, 2019; Semwal *et al.*, 2019).

Among the above factors, those that might be relevant to our study may be (i) pollinator-related factors and (iv) sampling-related artefacts or technical issues. Regarding the latter, poor hand-pollination performance may produce artefacts, especially when the flowers of the study plant are complicated to handle, such as Asteraceae heads (Black *et al.*, 2019; Semwal *et al.*, 2019). Unlike species of the Asteraceae, *S. germanica* has less challenging flowers, that in our study have been handled by the same experienced person, therefore we are confident that our sampling was artefact-free. Although plant-related factors (cf. above: ii) and an idiosyncratic community response (iii) may also be relevant, we lack empirical data to estimate the significance of these factors, so further research is definitely needed in these fields. On the other hand, we believe that the low pollen limitation of *S. germanica* on Mt. Olympus is largely explained by bumblebee visitation rate vis-à-vis the extraordinary

bumblebee diversity (Minachilis *et al.*, 2020). Both factors imply high pollination effectiveness of these insects: we found that bumblebees paid 84.7% of the total visits received by *S. germanica* flowers; their visitation was positively related with seed set, negatively related to PLI, and increased with altitude. Indeed, the highest bumblebee visitation was recorded in the sites with the highest bumblebee diversity, i.e. around 1900 m (Minachilis *et al.*, 2020).

Several other studies in which bumblebees were the main pollinators also found that pollen limitation varied with bumblebee visitation along the altitudinal gradient. Examples with no pollen limitation include *R. minor* in the Canadian Rocky Mountains [1400–2200 m; Hargreaves *et al.* (2015)] and *S. obvallata*, a ‘glass-house’ plant in the Indian Himalayas [3496–4335 m; Semwal *et al.* (2019)]. Theobald *et al.* (2016), however, reported higher pollen limitation in *Erythronium montanum* on Mount Rainier, USA (1490–1800 m), in years with low bumblebee visitation; again, *Pedicularis siphonantha*, a bumblebee-pollinated species in Yunnan, China (3200–4300 m), was found to suffer lower seed set and higher pollen limitation at higher altitudes, where bumblebee visitation rate was lower (Dai *et al.*, 2017). To sum up, our findings are in agreement with the aforementioned studies, all highlighting bumblebees’ outstanding performance as pollinators along altitudinal gradients worldwide.

Besides bumblebees, *S. germanica* was visited at a very low rate by other bees, flies and butterflies. Their visitation was negatively correlated with seed set and tended to co-vary with pollen limitation. A possible explanation is that some of these insects act as pollen thieves reducing seed set by removing pollen, thus increasing pollen limitation (Hargreaves *et al.*, 2009). Recent studies showed that visitation by small bees (Halictidae) increased pollen limitation due to pollen depletion, acting thus as functional parasites (Lau & Galloway, 2004; Koski *et al.*, 2018). As we did not observe pollen thieving in the field during our study and considering that pollen limitation was very low along the altitudinal gradient of Olympus, we believe that larceny was only sporadic if any. On the other hand, we are confident that non-bumblebee visitation, which was mainly due to *Xylocopa* and *Anthidium* bees, is complementary to low bumblebee visitation at low and intermediate altitudes, driving the observed low pollen limitation along the altitudinal gradient. Taking the above into account, we should underline the importance of assessing the composition of the pollinator community and especially the differential effectiveness of the various pollinator groups to explain the true drivers of pollen limitation and to provide a deeper understanding of the pollen limitation status of a given population (Koski *et al.*, 2018; Gallagher & Campbell, 2020; Herrera, 2020).

Seed mass per plant did not correlate with visitation. Pollen loads higher than the pollen limitation threshold (i.e. pollen load delivered to stigmas under which pollen limitation occurs) lead to high competition among pollen grains for fertilization, resulting in increased seed mass and progeny quality (Labouche *et al.*, 2017; Hildesheim *et al.*, 2019). However, in our study, higher visitation is linked to more but not heavier seeds, which was also found in other studies (Richardson & Stephenson, 1991; Niesenbaum, 1999). According to previous studies, this may be owed to either an abundance of high-quality pollen grains on stigmas which may mask the effect of pollen competition (Hildesheim *et al.*, 2019) or to a trade-off between seed number and seed size (Labouche *et al.*, 2017; Lázaro & Larrinaga, 2018; Hildesheim *et al.*, 2019). We have no empirical data to attribute our findings to any of the above reasons, but we also can add an additional possible reason, i.e. the shortage of resources such as soil fertility and shorter period for seed maturation; indeed, such resource limitation is likely especially at high altitudes, which may in turn limit the seed mass produced (Totland, 1997).

## CONCLUSION

Our study on *S. germanica* revealed the plant's mating system along the altitudinal gradient of Mt. Olympus and confirmed the clear predominance of bumblebees as its major pollinators. The observed low pollen limitation along the altitudinal gradient of Mt. Olympus is the result of the high pollination effectiveness of bumblebees documented here vis-à-vis their high diversity and abundance documented in an earlier study of ours (Minachilis *et al.*, 2020). Given that *S. germanica* is highly attractive and dependent on bumblebees, as well as its wide native distribution range across Europe (Dunn, 1997), we propose that this plant is used as an efficient and useful phytometer for monitoring bumblebee pollination activity across Europe; in addition, and at a smaller scale, this is a plant to be used in 'Bee Gardens' both for bumblebee conservation and for citizen science projects (Goulson *et al.*, 2011; Koffler *et al.*, 2021).

Bumblebees were proven to be the predominant pollinators for an obligatorily bee-pollinated plant along the altitudinal gradient of a high Mediterranean mountain and this may be the case for several other mountain plant species. In this context, a need for monitoring pollination services along the altitudinal gradient of Mediterranean mountain systems arises as imminent due to climate change effects. Indeed, climate change has been predicted to tremendously impact bumblebees, driving them upslope and possibly to extinction (Kerr *et al.*, 2015; Soroye *et al.*, 2020),

implying that conservation measures must be taken promptly. For our focal mountain, this entails the extension of the Olympus National Park area and the protection against invasive species and tourism activities, many of them planned, although with serious repercussions foreseen for the mountain's biota (Minachilis *et al.*, 2020).

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## DATA AVAILABILITY

The data underlying this article are available in the [Supporting Information](#).

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#### SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

- Table S1.** Study site description and type of pollination treatments conducted in 2014 and 2016 on Mt. Olympus.
- Table S2.** Pollination treatments performed in 2016 in *S. germanica* along the altitudinal gradient of Mt. Olympus.
- Table S3.** Pollination treatments performed in 2014 in *S. germanica* along the altitudinal gradient of Mt. Olympus.
- Table S4.** Flower visitation recorded in 2016 in *S. germanica* along the altitudinal gradient of Mt. Olympus.