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## Demographic study of a peripheral population of the rare amphicarpic species *Scrophularia arguta*

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### ABSTRACT

*Scrophularia arguta* (Scrophulariaceae) is a rare amphicarpic annual species with a fragmented distribution in North Africa, part of the Arabian Peninsula, and Macaronesia, with several peripheral populations on the Iberian Peninsula (south-western Europe) as well. The Iberian populations have a disjunct distribution: several endangered or critically endangered populations are present in the southeast versus a few non-listed ones representing a relict lineage with a unique plastid haplotype inhabiting the west-central region (Extremadura). In this study, we examined the largest and most important population of this relict lineage to assess potential threats to its survival. We evaluated pollination and reproductive systems, floral rewards, fruit and seed set from chasmogamous and cleistogamous flowers, and population dynamics (i.e., phenology, survival, reproductive success, and predation level). We found that the main threats to the population are predation by wild herbivores, such as red deer and, to a lesser extent, *Cucullia scrophulariae* caterpillars. These threats decrease reproductive success to such an extent that population survival may be in jeopardy if no action is taken to reduce the effects of herbivores. In addition, the existence of nearby beehives alters the presence and density of wild pollinators that contribute to gene flow via pollen transfer and increase genetic variability. Cleistogamous seeds may help maintain the population in specific microhabitats (mainly quartzite rock cracks), although at the cost of reducing genetic variability. Finally, we recommend that regional/national administrations responsible for biodiversity matters adopt measures to ensure the conservation and survival of west-central populations.

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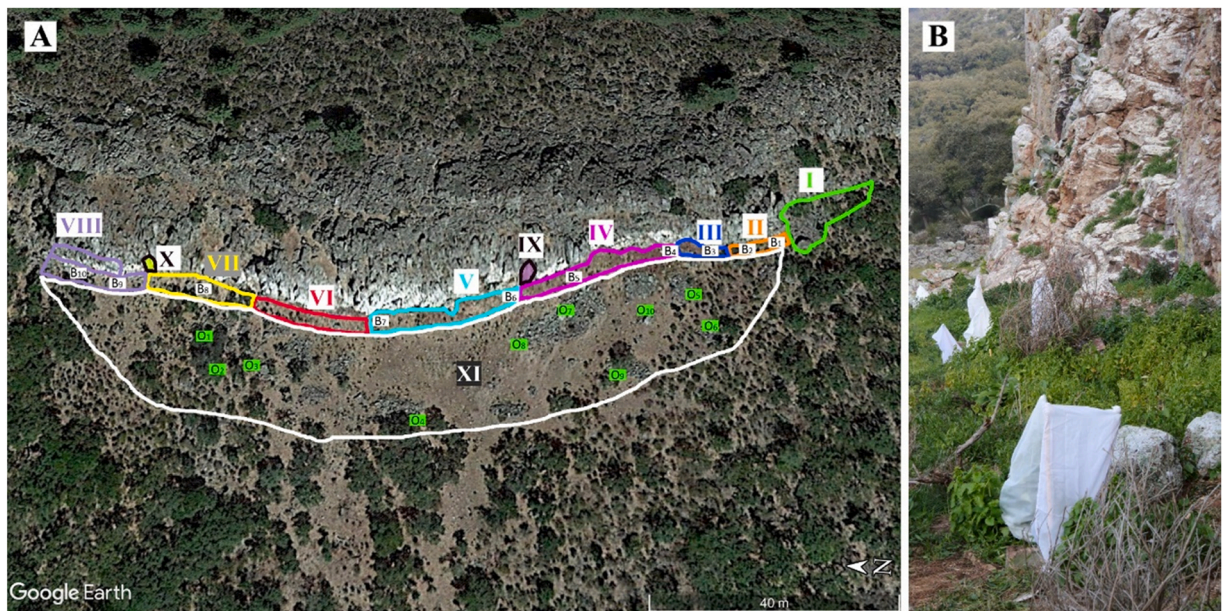
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## 1. Introduction

The first required step in the conservation of a species is determining the unique circumstances of each population in its distribution area, as local conditions may necessitate different conservation measures for a given population. The need for such locality-specific investigations has already become evident during the course of recent studies (Abeli et al., 2014; Fenu et al., 2020; Sulis et al., 2021). Local-level studies should be reinforced not only for the threatened species (Fenu et al., 2020), whether endemic or not, but also address peripheral populations, even if the latter are not obviously endangered. Compared with core populations, peripheral populations are expected to be exposed to more extreme environments and tend to be smaller, less genetically diverse, more demographically variable, less reproductively successful, and more prone to extinction (Channell, 2004; Reed, 2004), although no general pattern exists (Sagarin and Gaines, 2002; Sexton et al., 2009). Alternatively, these populations can be thought of as having a singular genetic diversity while at the same time exhibiting an increasing number of diverse local adaptations and harbouring unique ecotypes (Safriel et al., 1994; García-Ramos and Kirkpatrick, 1997; Hampe and Petit, 2005; Parmesan, 2006; Sexton et al., 2009; Thompson et al., 2010; Rehm et al., 2015). Given this consideration, a focus on peripheral populations, no matter how small, that inhabit very different environments from other populations of a species should be a high research priority. Demographic and/or genetic studies of peripheral populations should be used to identify the best management practices for enhancing their long- and short-term survival (Menges and Dolan, 1998 and references therein).

Plant populations are subject to numerous short- and long-term threats that endanger their continuity. The assessment of threats, including their identification and quantification, is essential for conservation (Domínguez-Lozano et al., 2020). Some of the most widespread threats, especially in Spain, are those derived from human activities, such as livestock grazing (Simon et al., 2001; Domínguez-Lozano et al., 2013; López-Sánchez et al., 2016), and, to a lesser extent, grazing by wildlife (López-Sánchez et al., 2016, but see Simon et al., 2001; Domínguez-Lozano et al., 2013).

In this study, we focused on the demographics (e.g., reproductive and pollination biology, survival, and predation) of a peripheral population of the rare amphicarpic annual *Scrophularia arguta*. Amphicarpic is a very rare phenomenon worldwide occurring in only ca. 67 herbaceous species of 13 families of angiosperms according to the latest estimate (Zhang et al., 2020). Through this phenomenon fruits originate both above and belowground. This species is distributed in Macaronesia, North and Northeast Africa, the Arabian Peninsula, and Socotra Island, with a few, mostly small populations present in south-western Europe (Spain) (Ortega-Olivencia et al., 2006; Ortega-Olivencia, 2009). *Scrophularia arguta* has a disjunct distribution in peninsular Spain, with several populations found in the southeast (Andalusia -Granada and Almería- and Murcia) and a few in the west-central region (Extremadura) (Ortega-Olivencia et al., 2006; Valtueña et al., 2020). Of these Iberian populations, those from Andalusia are catalogued as “critically endangered” (Cabezudo et al., 2005) and those from Murcia as “endangered” (Anon BORM, 2003), but the species is not included in the Spanish Floral Vascular Red List (Moreno, 2008). A species recovery plan for the Murcian populations, including biological and demographic



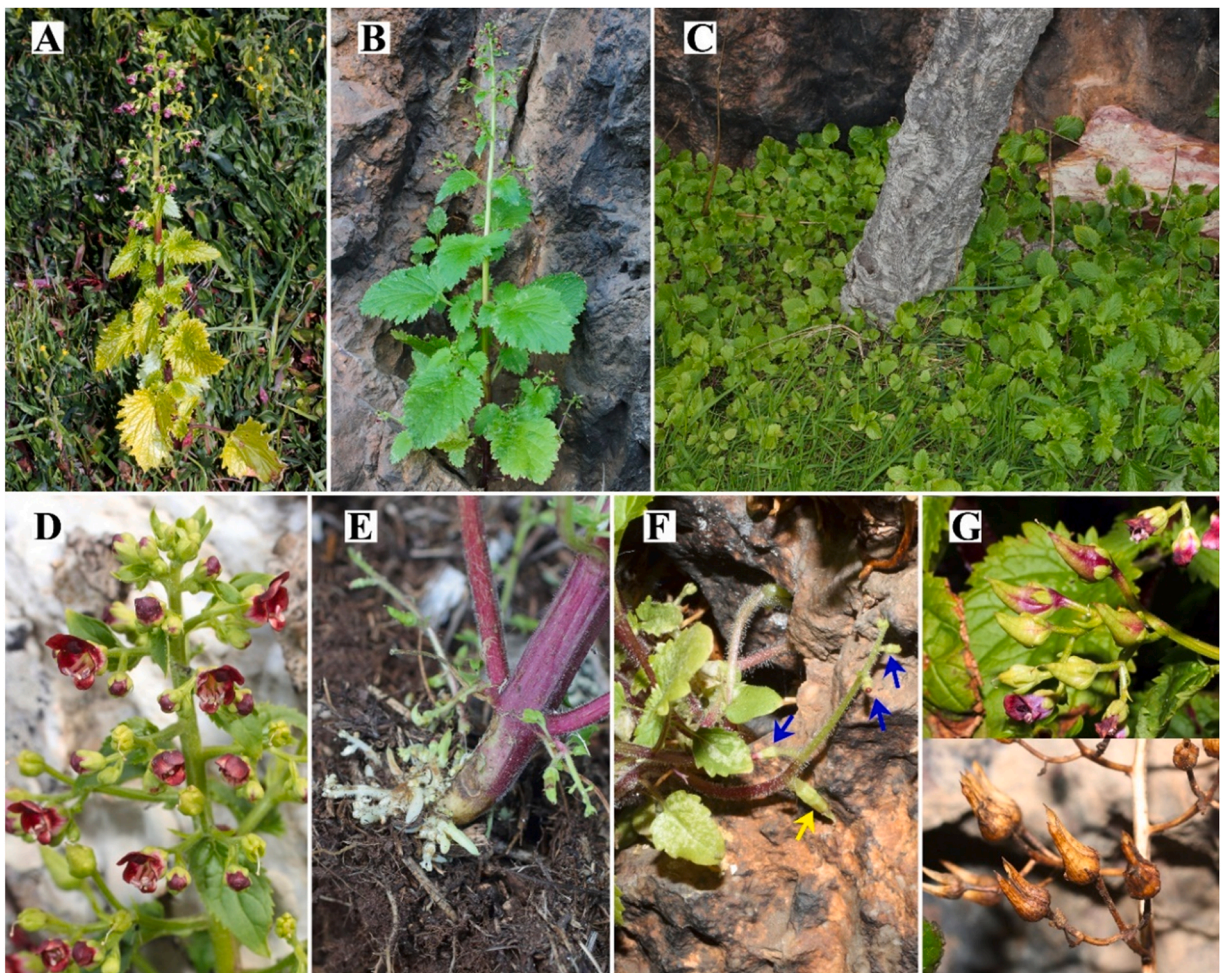
**Fig. 1.** The *Scrophularia arguta* population in this study. (A) Aerial view of the population showing its division into sectors and sections (sector A: sections I–X; sector B: section XI). Sector A is located at the base of the wall and extends approximately 3 m outward, and sector B is the open area located more than 3 m away from the base of the wall. Sector A is divided into 10 sections corresponding to different ecological conditions. Plots at the base of the wall and in the open area are designated as B<sub>1–10</sub> and O<sub>1–10</sub>, respectively. (B) Photograph of the population at the base of the wall in sector A showing several individuals, some of them bagged from reproduction experiments, mixed with companion species (e.g., *Mercuialis ambigua*).



monitoring for early detection of possible threats, is underway (Anon BORM, 2015).

According to Valtueña et al. (2016, 2020), the west-central Iberian (Extremadura) populations of *S. arguta* constitute a well-differentiated lineage for the following reasons: (i) they are isolated from the rest of populations in the phylogenetic tree, (ii) possess a unique plastid haplotype, (iii) constitute an independent genetic group based on AFLP clustering analyses, and (iv) exhibit some degree of ecological differentiation. These results indicated that the dispersal event giving rise to the Extremaduran populations was the oldest involving the Iberian Peninsula; these populations constitute an evolutionary significant unit and can be considered peripheral relicts (Valtueña et al., 2016, 2020). The Iberian populations occupy contrasting habitats: the central-west populations inhabit mesic areas on soils of quarzitic rocky areas over 500 m asl, whereas the south-eastern ones are found in xeric environments on calcareous or dolomitic substrates below 400 m asl (Ortega-Olivencia et al., 2006). In addition, the main western Iberian population comprises a larger number of individuals (1000–ca. 5000) than any other population (Ortega-Olivencia et al., 2006; Valtueña et al., 2020).

As pointed out by Valtueña et al. (2020), the peripheral populations are undeniably of conservation interest; this is especially true of the Extremaduran ones, which lack the protected status of those in the southeast yet constitute one of the five major intraspecific clades of *S. arguta*. In addition, the west-central populations likely harbour alleles adapted to more mesic environments, which makes them unique; ensuring that the genetic variants of these populations do not disappear is important. Consequently, our primary aim was to study the main Extremaduran population from a reproductive and demographic perspective to propose solutions for its conservation. Our specific goals were to investigate the following: (i) pollination and reproductive systems; (ii) population dynamics, including phenology, reproductive success, survival, and persistence of seeds in rock cracks; and (iii) predation levels and other factors related to threats to the population.



**Fig. 2.** *Scrophularia arguta*. (A) Flowering individual growing in an open area. (B) Flowering individual growing in a wall crack. (C) Small individuals in a shaded area producing only cleistogamous flowers. (D) Chasmogamous flowers. (E) Subterranean and basal (aerial) cleistogamous flowers and fruits. (F) Whitish basal stems with cleistogamous flowers (blue arrows) and fruits (yellow arrow). (G) Fruits from chasmogamous flowers. Upper and lower panels are immature and mature fruits, respectively.

## 2. Materials and methods

### 2.1. Study area

The main population of *S. arguta* in western Europe (Iberian Peninsula, Extremadura) is in the Regional Area of Interest of Sierra de San Pedro, which is included in the Network of Protected Natural Areas of Extremadura and the Natura 2000 Network. The site is not protected from cattle or wild animal grazing or from human disturbance. The population, which occupies an area of approximately 0.32 ha, is situated on the Mesomediterranean floor, mainly at the base of a large west-facing quartzite wall on deep, nitrified soils, on the forest road to Vica de Barreros above 490 m asl (39°35′13.59″ N-7°12′53.60″ W) (see Fig. 1) in the Sierra de Santiago (Santiago de Alcántara, Cáceres, Spain) (Ortega-Olivencia et al., 2006) (see Fig. A.1). The annual mean temperature is approximately 15.5 °C, with temperatures ranging from (−3.0) 2.0 °C in January to 33.0 (38.0) °C in July. The total annual precipitation is approximately 606 mm. The maximum precipitation occurs in November (101.0 mm), and the minimum is in July (4 mm). Approximately 65% and 25% of precipitation respectively falls between October and February (with a monthly average above 60 mm) and between March and May (with a monthly average between 40 and 60 mm) (Anon Weatherspark, 2021).

The zonal vegetation in the study area is constituted by scrublands, mainly “jaguarzales and jaral-heaths”, that are supplanting a cork oak forest (Ortega-Olivencia et al., 2006). Most of the *S. arguta* individuals grow at the base of a large, nearly vertical wall, with others found between the cracks of the wall or, if located away from the base, usually nestled in cavities under large rocks or stone piles. *Scrophularia arguta* individuals at the base of the wall coexist with annual herbaceous species, mainly *Mercurialis ambigua* and *Lamarckia aurea*, and/or perennials such as *Digitalis thapsi* (Fig. 1). Much of this area is fertilized by the droppings of a griffon vulture colony nesting on the upper part of the wall.

### 2.2. Species studied

*Scrophularia arguta* (Scrophulariaceae) is the only amphicarpic member of the genus *Scrophularia* as well as the family Scrophulariaceae. This annual species (Fig. 2) is distributed in Macaronesia (Selvagens and Canary Islands, and Cape Verde), North Africa, the Horn of Africa, the Arabian Peninsula, Socotra Island, and rarely in south-western Europe (Spain) (Fig. A.1). In peninsular Spain, the species is located in two disjunct areas (i.e., southeast and west-central regions, Fig. A.1), where it usually constitutes a few small populations (Ortega-Olivencia et al., 2006; Ortega-Olivencia, 2009; Valtueña et al., 2020). Its rarity and scarcity on the Iberian Peninsula are such that this species has been granted protected status in two regions of the southeast (Andalusia and Murcia), but not in the west-central (Extremadura).

The species has dimorphic flowers: (i) aerial chasmogamous flowers (Fig. 2A, B, D) and (ii) basal (aerial) and/or underground cleistogamous flowers (Fig. 2E), with aerial cleistogamous flowers sometimes appearing on whitish basal stems that generally lack leaves or have very small ones (Fig. 2F). Fruits therefore originate both above and belowground. The aerial flowers on whitish stems may be intermediate between the two types of flowers in various characteristics, such as size and pollen production, but they function like cleistogamous flowers. If stems are elongated, then the flowers located at the apex may act like chasmogamous ones. As in most other members of the genus, the chasmogamous flowers are hermaphroditic and pentamerous, with a reddish bilabiate corolla (Fig. 2D), four stamens plus a staminode, and a bicarpellary gynoeceium (Ortega-Olivencia, 2009). In contrast, the cleistogamous flowers are smaller and whitish (Fig. 2E) and lack a staminode. *Scrophularia arguta*, which has capsule-like fruits, is heterocarpic, with cleistogamous fruits (Fig. 2E) that are narrower than its chasmogamous ones (Fig. 2G).

### 2.3. Pollination and reproductive systems

To evaluate the pollination system of the *S. arguta* population, we carried out pollinator censuses during 2015 and 2016 seasons. The censuses, which were conducted at a distance of 0.5 m from plants, consisted of 10-min evenly spaced observation periods during daylight hours throughout the entire flowering period. Night censuses were also carried out for 1–2 h before sunrise and for 1–2 h starting at sunset. Whenever possible, the number of flowers and inflorescences visited per flight were recorded. For each functional animal group, the visitation rate (VR) was calculated as the number of flowers visited per inflorescence and per minute. More specifically, we calculated ideal VR, in which only those censuses with visits were taken into account, and real VR, in which all censuses were included, even those without visits (in which case, the VR for that census was equated to zero).

The nectar volume, concentration, and sugar composition of 90 flowers from 10 individuals were measured in the morning (10:00–11:00 h) during the 2016 season. Nectar was extracted with a graduated micropipette, and concentration was measured using a hand refractometer. The nectar sugar composition of a total of 76 flowers from 10 individuals was determined using a Whatman No. 1 paper strip. The strips were introduced into chasmogamous flowers to collect the small amount of accumulated nectar and kept cold before being transported to the laboratory and frozen at −40 °C for subsequent analysis. The strips were analysed by the Research Support Service of the University of Extremadura (SAIUEX) using an ion chromatograph with amperometric detection coupled to a Metrosep Carb 1 (150/4.0) column, with the separations carried out using a mobile phase of 8 mM sodium hydroxide and a gradient of 75 mM sodium hydroxide at a flow rate of 1 ml/min.

During the 2015 season, we estimated the pollen–ovule (P/O) ratio, fruit set (fruit/flower ratio), and seed set (seed/ovule ratio). The P/O ratio was calculated for both flower types (chasmogamous and cleistogamous) using four to five flowers from 10 individuals of each flower type. The total production of pollen grains in chasmogamous (CH) flowers was calculated using the dilution method (Cruden, 1977), while that of cleistogamous (CL) flowers was determined by counting the four anthers separately. The ovule



production of ovaries from the same flowers used to calculate pollen production was counted under binocular vision. We calculated the CH fruit and seed set of 10 control (natural pollination) and 10 bagged (spontaneous self-pollination, SSP) plants. We also carried out manual self- and cross-pollination experiments, but most of the plants did not survive because of external factors (e.g., grazing and/or trampling by wild animals) and have thus not been considered. In addition, we estimated the seed set of basal/underground CL flowers. Finally, 24 and 28 groups of 30 seeds of each type (CH and CL, respectively) were randomly selected and weighed on a precision scale ( $\pm 0.1$  mg).

We similarly studied the features of CH and CL seeds using the 30 seeds from each of the above-mentioned groups. We measured the length and width of CH seeds (from aerial chasmogamous fruits), basal/underground CL seeds (from basal/underground cleistogamous fruits), and aerial CL seeds (from aerial cleistogamous fruits on whitish basal stems) of seven individuals. In addition, we calculated seed volume. For this calculation, the seed was treated as a spheroid, with its three axes based on the length and width of the seed.

#### 2.4. Population dynamics

To carry out this portion of the study, the population was divided into two sectors: sector A, at the base of the great wall extending approximately 3 m outward, and sector B, the open area located more than 3 m away from the base of the wall. Because most of the individuals in the population were growing along the base of the wall (sector A), this sector was divided into 10 sections (Fig. 1). Six sections (II–VII) encompassed most of the plants growing in the ground. Sections II and III were the shadiest sections, with individuals shaded most of the day and nearly devoid of other plant species. In contrast, sections IV–VII were located in sunny areas and included a high density of other species (e.g., *Mercurialis ambigua*, *Lamarckia aurea*, and/or *Digitalis thapsi*). Sections I and VIII harboured most of the *S. arguta* individuals growing respectively on overhangs or in cracks in the wall. Finally, two sections (IX and X) covered plants in small caves or inlets in the wall that were somewhat elevated from the ground. In contrast to sector A, sector B was considered as a single section (XI) because of the low density of individuals of *S. arguta* growing within it (Fig. 1).

To investigate population dynamics, we examined plant vegetative and reproductive development and the effect of predation by different biotic agents. The following developmental stages were monitored: 1–2 pairs of leaves (2pl); 3–4 pairs of leaves (4pl); more than 4 pairs of leaves or the immature stage ( $>4$ pl); flowering (Fl, individuals presenting flowers), and fruiting (Fr, individuals with mature fruits). Observations began on November 20, 2018, and ended on May 30, 2019, which roughly coincided, respectively, with the emergence of seedlings and wilting of individuals in the population. We visited plants approximately every 14 days to make observations.

##### 2.4.1. Population phenology and reproductive success

As mentioned above, the population was divided into two sectors (A and B) comprising 11 sections (10 in sector A and 1 in sector B) (Fig. 1). For this part of the study, observations began on November 20, 2018, corresponding to seedling emergence or the presence of 2pl individuals. During each visit, the number of individuals in each section was counted, and their developmental stage (see above), except for cotyledons, was noted. To minimize damage (breakage) to large plants, which had to be pushed aside to uncover smaller ones, we observed the following restrictions when counting: (i) starting on February 7, we only counted small plants  $< 4$  pl, immature plants (adult plants without flowers), and mature plants (flowering or fruiting adult plants); (ii) starting on February 21, we only considered immature and mature adult plants; and (iii) starting from March 8, we only counted mature plants. We also estimated reproductive success (fruit and seed set) at section, sector, and population levels. To estimate seed set, we considered seed set per fruit to be the mean value of control plants from the reproductive biology experiment (see above).

##### 2.4.2. Survival and life table analysis

On January 24, 2019, 20 plots (50 cm  $\times$  50 cm) were selected and marked in the population: 10 at the base of the wall in sections II–VII (sector A), and 10 distributed in the open area (section XI, sector B; Fig. 1). These plots were all far enough apart to be considered independent of each other. The minimum between-plot distance in sectors A and B was approximately 7 and 9 m, respectively. At each visit, we noted the number of individuals, their developmental stage, and the presence or absence of signs of predation by herbivorous vertebrates and/or invertebrates (see Plant predation section). We waited until each of the seedlings had at least one pair of leaves before starting monitoring to consider they all belonged to the same generation.

##### 2.4.3. Persistence of cleistogamous seeds in rock cracks

Given the hardness of the quartzite rock and the narrow width of most cracks, we only searched for evidence of a possible cleistogamous seed bank associated with cracks in the wall on a coarse scale. For this purpose, we selected and marked 50 cracks containing dry plants from the current season with white latex paint in September 2017. Except for 2020, these locations were analysed every year until 2021 to check for new individuals potentially derived from cleistogamous seeds. We also collected 24 mature plants growing in the ground with chasmogamous flowers and 30 small, young plants (no more than 10 cm in height) without chasmogamous flowers and counted the number of individuals with cleistogamous fruits.

##### 2.4.4. Plant predation

To ascertain the degree of plant predation by different agents (herbivorous vertebrates and invertebrates), we categorized each plot according to the observed predation level. In the case of vertebrates, each plot was assigned to one of five categories based on the percentage of plants exhibiting signs of predation as follows: (0) 0%, (1)  $> 0\%$  and  $\leq 25\%$ , (2)  $> 25\%$  and  $\leq 50\%$ , (3)  $> 50\%$  and  $\leq 75\%$ , and (4)  $> 75\%$  and  $\leq 100\%$ . Plants showing evidence of vertebrate predation usually eventually died and were often uprooted.

In the case of predation by invertebrates, levels were defined according to the degree of damage to vegetative and reproductive plant parts as follows: (0) little or no damage, (1) low predation (only a few leaves affected and/or leaves with less than 25% of the blade damaged; no damaged flowers or fruits on mature individuals); (2) medium predation (some, but fewer than 50% of leaves damaged and/or leaves with 25–50% of the blade damaged; no damaged flowers or fruits on mature individuals), (3) high predation (leaf damage as in case 2, or higher; slight damage [i.e.,  $\leq 50\%$ ] to flowers and fruits of mature individuals), and (4) very high predation (leaf damage as in case 2, or higher; severe damage [i.e.,  $> 50\%$ ] to flowers and fruits of mature individuals).

## 2.5. Statistical analyses

All statistical analyses were performed using IBM Statistics SPSS 27 (IBM Corp, 2020). In pollinator-related analyses, two functional groups (wasps and other pollinators) were not considered given their low incidence. Presence of pollinators, which was measured as the number of censuses in which a pollinator was observed, was analysed using a Chi-squared test. After square-root normalization, ideal VR was analysed by a one-way analysis of variance (ANOVA), with post-hoc comparisons carried out using Tukey's HSD test to assess the effects of season, pollinator, and their interaction. In contrast, real VR was analysed using a generalized linear model fitted to a Tweedie distribution with a logarithmic link. In both analyses, season, pollinator, and their interaction were used as principal factors. Differences among ideal and real VR were analysed using the non-parametric Wilcoxon test. All parameters related to pollen and ovule production, fruit and seed set, and seed size were analysed using the non-parametric Kruskal–Wallis test (more than two independent variables) or the Wilcoxon test (two independent variables) with false discovery rate (FDR) adjustment of  $P$ -values. Survival analyses were carried out by the method of Kaplan–Meier, with the log-rank (Mantel–Cox) test used to analyse intra- and inter-sector differences. In plant survival analyses, censored plants were individuals that survived until the end of the experiment; in analyses of flowering and fruiting survival, censored plants were flowering or fruiting individuals who died before leaving offspring. Finally, the relationship between predation level and plant density was analysed using Spearman's correlation coefficient ( $r_s$ ) with FDR adjustment of  $P$ -values.

## 3. Results

### 3.1. Pollination and reproductive systems

Over 2 years, the total time formally spent observing floral visitors was 78 h and 7 min (2015: 30 h 06 min; 2016: 48 h 01 min) for diurnal observations and 13 h for nocturnal observations. Insects recorded acting as pollinators fell into five functional groups: (i) bees (*Apis mellifera* and *Andrenidae*), (ii) small bees (*Lasioglossum viride*), (iii) wasps (*Polistes* spp. and *Alastor atropos*), (iv) hoverflies (*Chrysotoxum* spp., *Eupeodes* spp. and *Scaeva* spp.), and (v) others (flies) (Fig. 3). Pollinator presence was significantly different in both seasons (2015:  $\chi^2 = 822.94$ ,  $P < 0.05$ ; 2016:  $\chi^2 = 319.24$ ,  $P < 0.05$ ). In 2015, bees (mainly *Apis mellifera*) were the significantly most abundant pollinators, followed by hoverflies and small bees, with no wasps or other pollinators observed (Fig. 3). In 2016, in contrast, small bees were significantly the most abundant, surpassing the number of bees and hoverflies, and small numbers of wasps and flies were recorded (Fig. 3). In the nocturnal censuses, we only observed a small moth visiting two flowers, for one second each.

In regard to visitation rate, ideal VR (i.e., considering only censuses with pollinator visits) was significantly affected by season and pollinator but not their interaction; in contrast, real VR (i.e., including all censuses, even those without visits) was affected by



**Fig. 3.** Relative abundance of different functional groups of pollinators visiting *Scrophularia arguta* flowers in Sierra de Santiago de Alcántara (Cáceres, Spain) during 2015 and 2016.



pollinator and its interaction with season (Table 1). The bee ideal VR was significantly higher than those of small bees and hoverflies, whereas real VR was significantly different among all pollinators (Fig. 4).

The mean volume of nectar secreted by flower was  $0.60 \pm 0.71 \mu\text{l}$  (mean  $\pm$  SD), with a sugar concentration of  $42.0 \pm 24.0\%$ . This nectar was sucrose rich, with almost no glucose (Fig. A.2), and the sucrose/hexose ratio was  $8.45 \pm 4.03$ . Pollen grain production per flower (CH =  $47,420.21 \pm 11,874.97$  vs CL =  $1,755.73 \pm 1,604.61$ ), ovule production per flower (CH =  $130.49 \pm 20.95$  vs CL =  $17.40 \pm 5.11$ ), and the P/O ratio per flower (CH =  $367.38 \pm 85.66$  vs CL =  $93.25 \pm 57.33$ ) were significantly higher in CH flowers compared with those from CL flowers (Fig. 5A).

With respect to fruit set, no significant difference was observed between control and SSP plants ( $68.68 \pm 12.87$  vs  $68.23 \pm 17.29$ , respectively; Fig. 5B), whereas seed set was significantly higher in control plants, indicating the importance of pollinators in plant reproduction. Seed set from CH flowers (control and SSP plants) were significantly lower than those from CL flowers (Fig. 5B), thereby showing pollinator limitation in chasmogamous flowers. The number of seeds per fruit was significantly lower in CL fruits than in CH fruits (Fig. 5B).

In regard to seed features, CL seeds were significantly heavier (based on 30-seed weight) than CH seeds ( $0.0061 \pm 0.0009$  mg vs  $0.0036 \pm 0.0004$  mg,  $W = 300.00$ ,  $P < 0.001$ ). In terms of seed size (length, width, and volume), underground and/or basal CL seeds were significantly larger than CH seeds (length:  $1.18 \pm 0.14$  mm vs  $0.87 \pm 0.13$  mm; width:  $0.54 \pm 0.08$  mm vs  $0.44 \pm 0.05$  mm; volume:  $1.45 \pm 0.46$  mm<sup>3</sup> vs  $0.72 \pm 0.25$  mm<sup>3</sup>) (Fig. 5C). Finally, aerial CL seeds, i.e., those from aerial flowers on whitish basal stems (length =  $1.06 \pm 0.14$  mm; width =  $0.49 \pm 0.05$  mm; volume =  $1.09 \pm 0.27$  mm<sup>3</sup>), were significantly larger than CH seeds and smaller than underground and/or basal CL seeds (Fig. 5C).

### 3.2. Population dynamics

#### 3.2.1. Population phenology and reproductive success

Seed germination and seedling development in the *S. arguta* population started in mid-November. Flowering began in early to mid-February, and fruiting commenced in mid-March, with all plants having withered by late May. We counted nearly 7,000 individuals at the peak of the 2pl phase (Fig. 6). During this phase, the number of plants increased in a very strong linear fashion starting at the beginning of December and then experienced a more-or-less pronounced decrease in mid-December upon the arrival of the next phase (4pl) (Fig. 6). At the beginning of the 4pl phase, the number of individuals slowly rose and then rapidly increased in mid- to late January. The number of individuals in the > 4pl phase (immature plants) followed a trend similar to the previous phase, that is, an initial slow rise followed by a dramatic increase, with a peak in mid-February (Fig. 6). From mid-February until mid-March (flowering peak), the number of flowering individuals increased substantially and then rapidly decreased. This steep decline became less pronounced in mid-April. (More information on section-level trends is provided in Appendix A Fig. A.3).

At the peak of flowering, we observed 529 flowering individuals, which corresponded to 8.24% of the total number of individuals in the population. The fruiting phase (CH fruits) began at this time. The number of fruiting plants grew rapidly until mid-April, dipped slightly until early May, and then rose again until mid-May, when a second fruiting peak similar to the previous one took place (Fig. 6). The number of fruiting individuals corresponded approximately to 4.39% of the total number of individuals in the population and approximately to 53% of the individuals had already initiated flowering.

The population was divided into two well-differentiated areas: the base of the wall (ca. 531 m<sup>2</sup>) and the open area (ca. 2,600 m<sup>2</sup>). Almost 87% of individuals in the population were located at the base of the wall. An examination of *S. arguta* plants growing in the different sections of these two areas (Fig. 1) revealed that shady areas generally harboured small individuals, most of which produced basal or buried CL flowers but no CH flowers, and practically no companion species (Fig. 2C). In contrast, more sunny areas (sections IV-VII) were characterized by the presence of generally large individuals, a large percentage of which produced CH and CL flowers. The CL flowers were frequently present on more-or-less long, whitish basal stems. These areas were densely covered with individuals of other plant species that sometimes masked *S. arguta* individuals from view.

Out of the 6,856 individuals constituting the population, only 690 (just under 10%) produced chasmogamous flowers, and 259 (slightly over 4%) fruited. Taking into account estimates of populational fruit set (10.34 fruits/individual) and seed set (80.38 seeds/fruit), the reproductive success of this population would be slightly under 71,000 fruits and approximately 5,700,000 seeds if all individuals in the population fruited, and approximately 7,100 fruits and slightly above 573,000 seeds if all blooming individuals fruited. The actual estimated production, however, was approximately 2,678 fruits, with an approximate total production of 215,000 seeds.

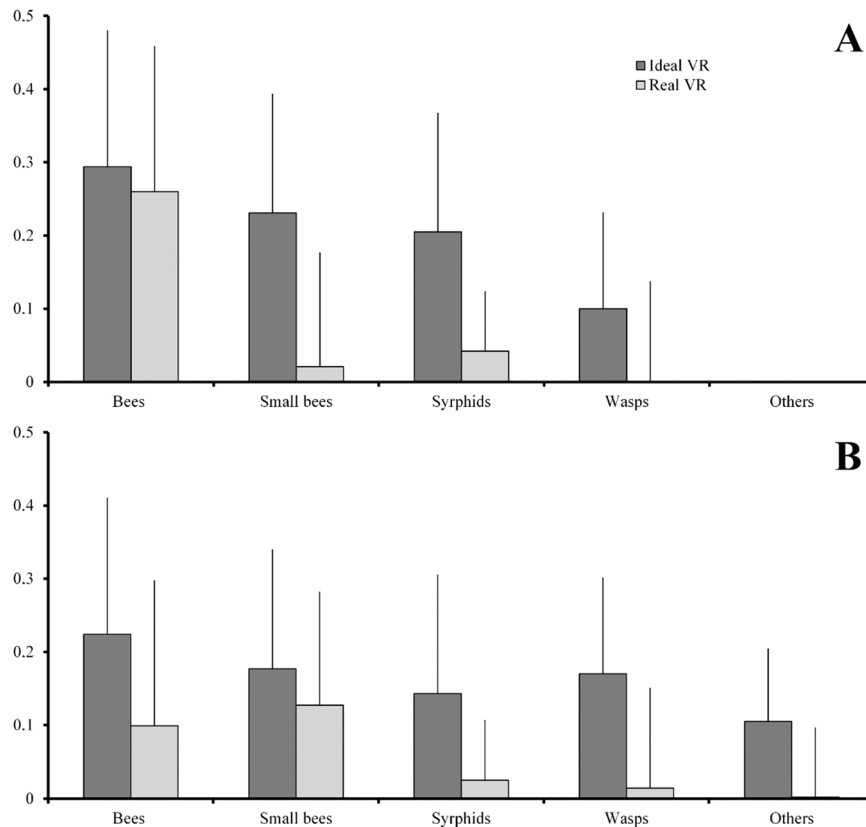
#### 3.2.2. Survival and life table analyses

A Kaplan–Meier survival analysis of the studied population revealed that plant death was significantly higher in the open area than

**Table 1**  
One way ANOVA (F) and generalized linear model ( $\chi^2$  Wald) for ideal and real visitation rates (RV).

	Ideal RV (F)	Real RV ( $\chi^2$ Wald)
Season	15.254 ***	0.710 ns
Pollinator	12.947 ***	151.268 ***
Season * pollinator	0.095 ns	102.211 ***

\*\*\*  $P < 0.001$ ; ns,  $P > 0.05$



**Fig. 4.** Floral visitation rates (mean  $\pm$  SD) of different functional pollinator groups. The visitation rate (VR) for each functional group is the number of flowers visited per inflorescence and per minute. Ideal VR, which was based only on the pollinator censuses that recorded visits by the specific pollinator group, and real VR, in which all censuses were included, are shown for 2015 (A) and 2016 (B) seasons.

at the base of the wall ( $\chi^2 = 22.47$ ,  $P < 0.001$ ; Fig. 7A). The survival percentage of fruiting plants was very low in both sectors (3.53% at the base of the wall and 3.43% in the open area). Within the two sectors (i.e., the base of the wall and the open area), survival percentages also differed significantly among plots, especially among those at the base of the wall ( $\chi^2 = 90.56$ ,  $P < 0.001$  vs  $\chi^2 = 19.57$ ,  $P = 0.021$ ; Fig. 7B, C).

The survival of flowering and fruiting individuals during the season was not significantly different between the two sectors (flowering:  $\chi^2 = 0.56$ ,  $P = 0.454$ ; fruiting:  $\chi^2 = 0.02$ ,  $P = 0.902$ ). When plots by sectors were considered, however, a significant difference was observed in the survival percentage of flowering plants (sector A:  $\chi^2 = 13.79$ ,  $P = 0.032$ ; sector B:  $\chi^2 = 17.10$ ,  $P = 0.017$ ) but not fruiting ones (sector A:  $\chi^2 = 4.66$ ,  $P = 0.588$ ; sector B:  $\chi^2 = 2.37$ ,  $P = 0.500$ ).

### 3.2.3. Persistence of cleistogamous seeds in rock cracks

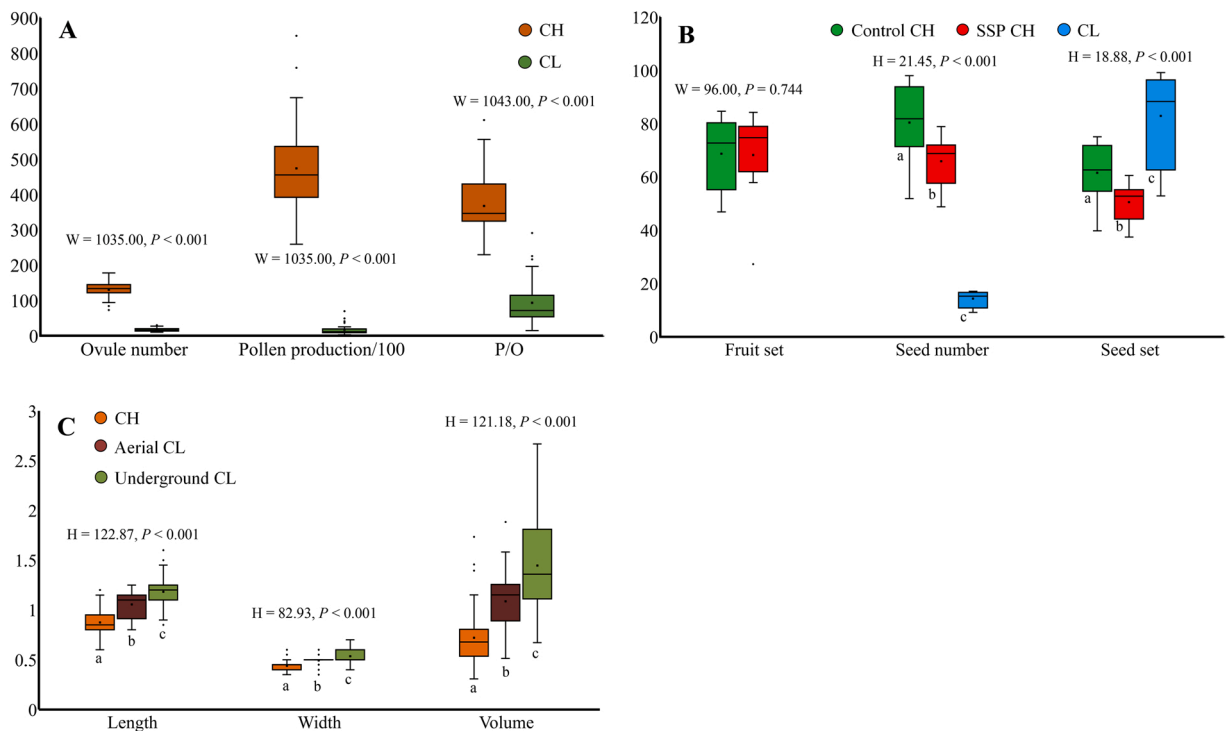
The number of cracks occupied by plants decreased from 50 in 2017–18 and then 13 in 2018 and 2019, respectively, and subsequently increased to 43 in 2021. (No data were obtainable in 2020 because of COVID-19 pandemic restrictions). In 2018, almost all plants occupying cracks produced CL fruits, mostly on short cleistogamous stems, but only 24.14% had CH fruits. In 2021, most individuals had CL fruits, and the percentage of individuals with CH fruits (51.16%) was twice that of 2018.

Among plants growing in the ground, 90% of immature individuals ( $\leq 10$  cm tall, generally with  $<6$  pairs of leaves) and 91.67% of mature individuals (with CH flowers) had CL fruits.

### 3.2.4. Plant predation

The main form of predation of *S. arguta* individuals in the studied population was herbivory by vertebrates and invertebrates. Herbivory in the first case was due to wild herbivores, specifically red deer (*Cervus elaphus* L.), with no evidence of cattle grazing. Eleven of the plots in this study exhibited signs of herbivory, and three of the five grazed plots at the base of the wall and all six in the open area were completely destroyed by red deer. In contrast, five plots at the base of the wall and four in the open area showed no evidence of herbivory (Fig. 8). The degree of predation was inversely related to plant density, but this relationship was only statistically significant at the base of the wall (base:  $r_s = -0.796$ ,  $P = 0.012$ ; open:  $r_s = -0.178$ ,  $P = 0.856$ ) (Fig. 8). The main damage to plants by red deer was due to grazing, which caused stem breakage; this activity also generally killed individual plants, as they were essentially eaten to the base, with only the main stem and sometimes a few basal leaves remaining (Fig. 9A), and were often even completely





**Fig. 5.** Reproductive features of *Scrophularia arguta*. (A) Ovule and pollen grain production and the pollen–ovule (P/O) ratio per chasmogamous (CH) and cleistogamous (CL) flowers. (B) Fruit and seed set and number of seeds/fruit of control chasmogamous flowers (control CH), spontaneous chasmogamous self-pollinated flowers (SSP CH), and cleistogamous flowers (CL). (C) Seed features of chasmogamous (CH) and cleistogamous (CL; aerial and underground) flowers.

uprooted. Although less frequent, *S. arguta* individuals were also crushed and killed when these herbivores laid down in areas with plants.

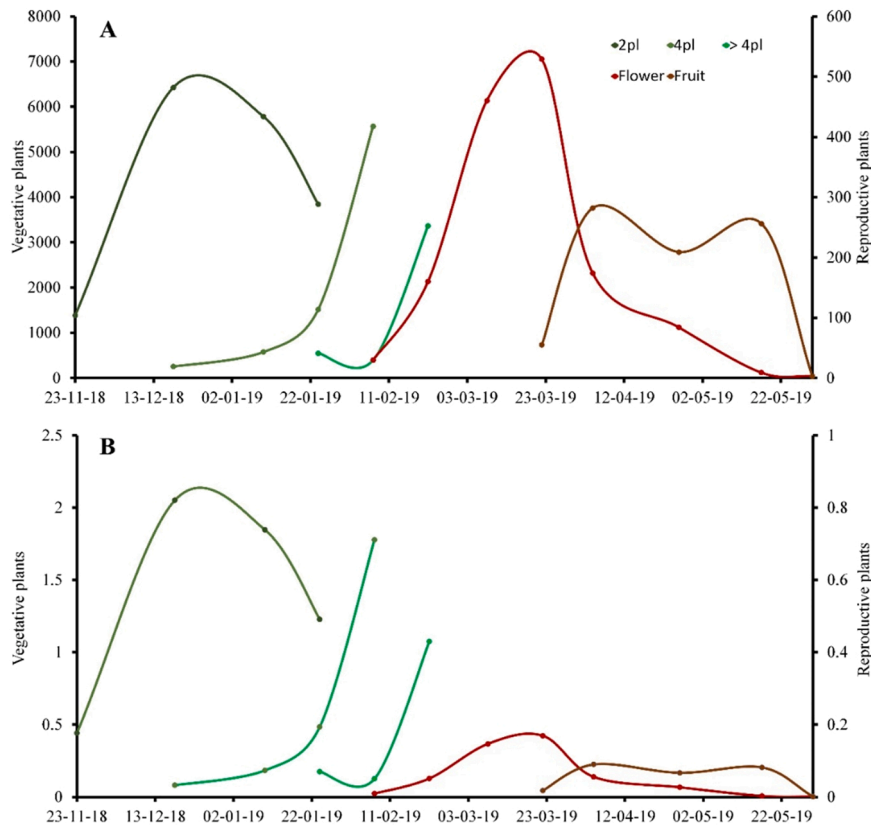
Predation due to invertebrates was mainly by *Cucullia scrophulariae* caterpillars (Fig. 9B). The level of predation was not significantly correlated to the density of plants, either at the base or away from the wall ( $r_s = 0.062$ ,  $P > 0.865$ ;  $r_s = 0.066$ ,  $P = 0.856$ , respectively), and no trend was detected (Fig. 8). Although predation by invertebrates affected a greater number of individuals than that carried out by vertebrates, it was usually less harmful. In most cases, this predation did not result in the death of the individual; instead, plants were only partially defoliated and able to survive, with their reproductive success considerably diminished only in the most severe cases or when flowers and fruits were partially or totally consumed. Plants only died when totally defoliated.

Herbivory by vertebrates as well as by invertebrates was more extensive on large individuals—both immature plants and flowering and/or fruiting ones. This form of predation usually affected reproductive success negatively.

#### 4. Discussion

*Scrophularia arguta* possesses a mixed breeding system, both selfing (CH and CL flowers) and outcrossing (CH flowers) and produces mixed progeny (i.e., heterocarpy and heterospermy). The species also has two dispersal strategies: in situ dispersal via underground or basal CL seeds, and dispersal away from the plant via CH seeds. The population in this study belongs to a genetically differentiated lineage within *S. arguta* derived from a dispersal event, probably from NW Africa and likely the oldest into the Iberian Peninsula (Valtueña et al., 2020). According to our study, the main threat to this population is predation by wild herbivores (vertebrates and invertebrates). The persistence of CL seeds may help maintain the population in specific microhabitats (mostly quartzite rock cracks). If this threat persists, however, the long-term fate of the population is uncertain; in particular, a large number of CH flowers and fruits associated with gene exchange via pollinators and wind, respectively, and also many CL fruits responsible for population persistence may be lost after plants are uprooted by red deer.

The peripheral Extremaduran population of *S. arguta* is larger and denser than populations from south-eastern Spain and the western part of the species' range (Canary Islands and Morocco), although with a similar genetic diversity. In peninsular Spain, the Extremaduran population inhabits higher-elevation, mesic habitats on quartzitic substrate, whereas Andalusian and Murcian populations are typically found on lower-elevation, drier habitats on calcareous or dolomite substrates (Cabezudo et al., 2010; Mota et al., 2005; Ortega-Olivencia, 2009; Ortega-Olivencia et al., 2006; Valtueña et al., 2020; Anon BORM, 2015; Lahora et al., 2005). The mesic Extremaduran habitat produces larger individuals, with a higher reproductive success rate (i.e., more flowers and fruits per plant), than those of Andalusian and Murcian populations, which are usually small and consist of a few scattered, short individuals (Lahora



**Fig. 6.** Phenology of *Scrophularia arguta* at the population level. (A–B) Number (A) and density (number of individuals per m<sup>2</sup>) (B) of plants in different vegetative and reproductive stages. The following vegetative stages are indicated: 2pl (1–2 pairs of leaves), 4pl (3–4 pairs of leaves), and > 4pl (immature plants with more than 4 pairs of leaves). Plant reproductive stages are as follows: Flower (flowering individuals); Fruit (fruiting individuals).

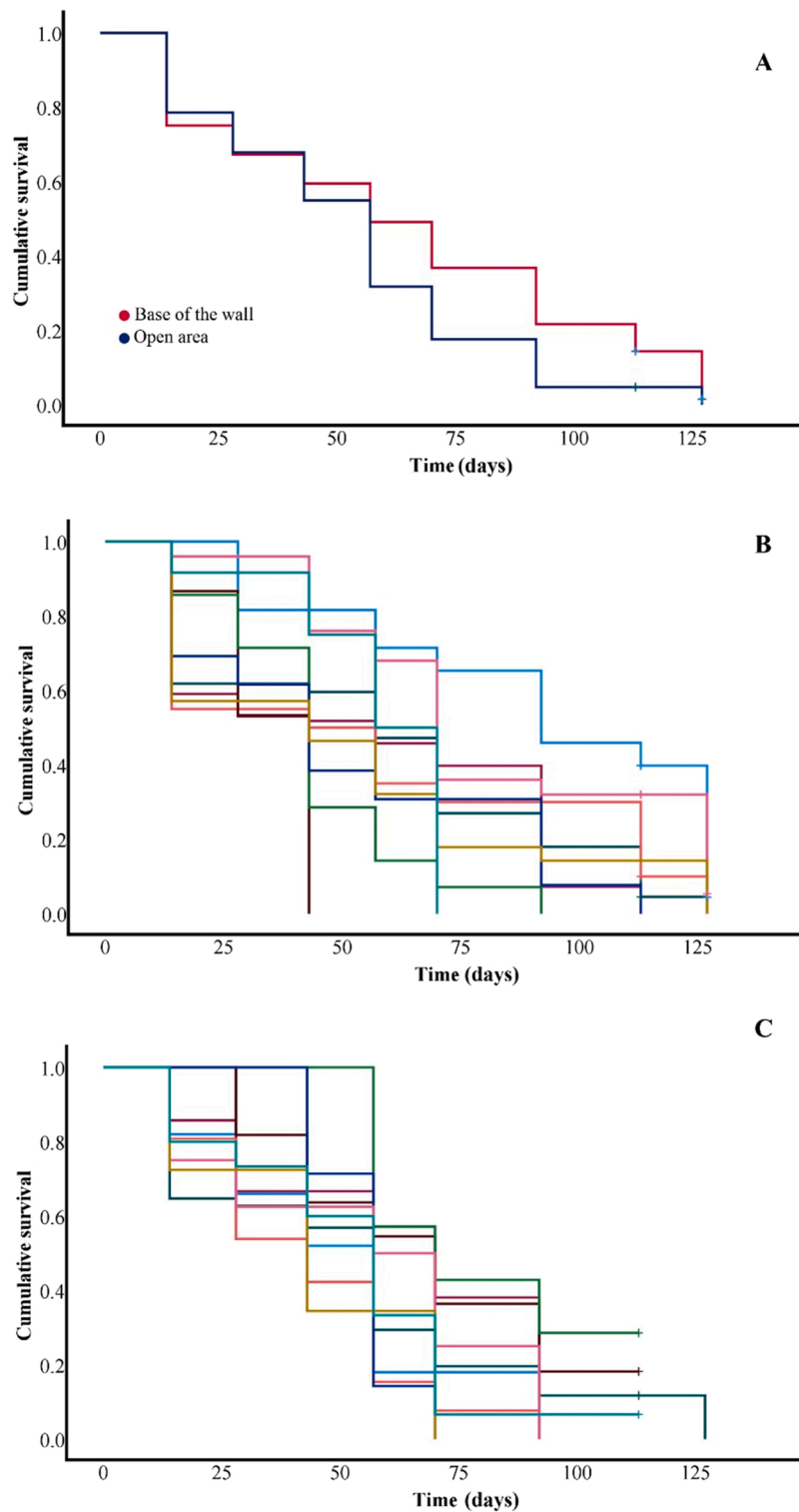
et al., 2005; Anon BORM, 2015; authors' personal observation). Two populations not addressed in the present study, however, are located near our studied population and harbour a small number of mostly small individuals (Ortega-Olivencia et al., 2006; García Alonso and Márquez García, 2011).

The population in this study exhibited dramatic demographic fluctuations from year to year (i.e., ca. 4,591 individuals in 2006; 1,285 in 2017; 1,000 in 2018; and  $\geq 7,000$  in 2019; Ortega-Olivencia et al., 2006; Valtueña et al., 2020; this paper). These fluctuations seem to be inherent to the species, as the number of individuals in Murcian populations also vary between years (DGMN, 2021; Anon, 2015; Anon DGMN, 2020). This phenomenon is probably due to the strong influence of climatic factors on this annual species, whose germination is linked to periods of rain (Anon DGMN, 2020).

Most *Scrophularia* species are self-compatible and protogynous (Shaw, 1962; Dalgaard, 1979; Ortega-Olivencia and Devesa, 1993; Navarro-Pérez et al., 2017). According to our study results and other research (e.g., Stiefelbogen, 1910; Dalgaard, 1979), *S. arguta* is not protogynous. In addition, our study has been the first to analyse functional groups of pollinators of this species, although hoverflies have previously been noted as floral visitors of *S. arguta* populations in the Savage Islands (Macaronesia, Portugal) (Pita et al., 2009). Plants in our studied population were found to suffer from pollen limitation in the absence of pollinators (i.e., lower CH seed set after SSP). Because pollinator presence is crucial for maintaining the total genetic variability of the populations through gene flow via pollen, any factor reducing the presence or density of wild pollinators (e.g., honeybee competition, habitat degradation, and loss of floral rewards [nectar and pollen]) may represent a threat to reproductive success (Goulson et al., 2015; Lindström et al., 2016). The use of managed honeybees for honey production is one such factor that can lower wild pollinator density (Breeze et al., 2011). Because honeybees have a different pollination behaviour than wild ones, their displacement of wild pollinators can have negative effects on pollination services provided to wild plants (Morales et al., 2009; Ortega-Olivencia et al., 2012; Agüero et al., 2018). In our study, the presence of honeybee hives approximately 500 m away from our population in 2015 clearly had a negative effect on wild pollinator presence and density. Although the density of hoverflies was unchanged, that of small bees, and, to a lesser extent, wasps, was significantly reduced. This effect was reflected in the real VR of each pollinator group: in 2016, when honeybee hives were absent, small bees were not only the main group pollinator, but also had the highest real VR.

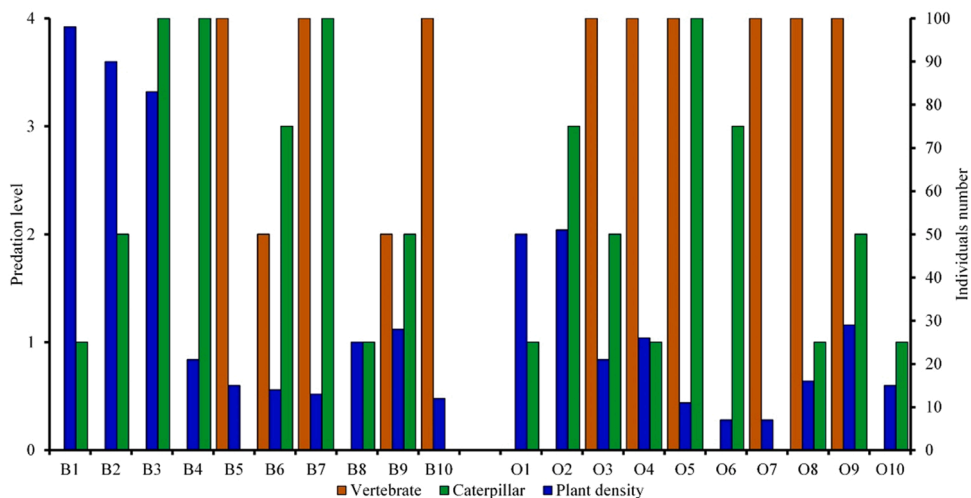
The main floral rewards sought by pollinators are pollen and nectar, the latter of which is sucrose rich (sucrose/hexose ratio  $> 1$ ) in *S. arguta*, as in other *Scrophularia* species. This result supports the hypothesis of Rodríguez-Riño et al. (2014) that sugar composition in



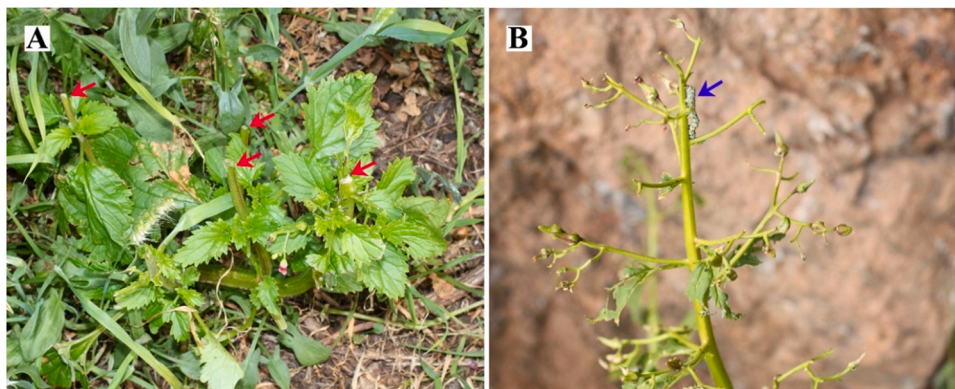


**Fig. 7.** Cumulative survival of *Scrophularia arguta*. (A) Fraction of surviving individuals in all plots in sector A (base of the wall) and sector B (open area). (B–C) Fraction of surviving individuals in each of the 10 plots analysed (indicated by different colours) in sector A (B) and sector B (C).

the genus is a conserved, phylogenetically constrained trait. This sucrose-rich nectar should increase the attraction of *Apis mellifera* and other insect pollinators. Honeybees have many characteristics (e.g., large colony sizes, high energetic requirements, large foraging ranges, and a complex communication system) that enhance their foraging efficiency; when these insects are present in high densities,



**Fig. 8.** Levels of vertebrate and lepidopteran caterpillar predation and the number of *Scrophularia* individuals in individual plots in the two sectors in this study. B<sub>1-10</sub>, base of the wall; O<sub>1-10</sub>, open area.



**Fig. 9.** *Scrophularia arguta* plants exhibiting signs of predation. (A) Evidence of predation by deer (red arrows show eaten stems). (B) Damage due to *Cucullia scrophulariae* caterpillars (blue arrow).

they thus have a competitive advantage over other wild pollinators (Torné-Noguera et al., 2016 and references therein).

In regard to fruit production, we estimate that about 2700 CH fruits and approximately 215,000 seeds were actually produced in the studied population. These numbers seem too low to allow the population to persist, especially in the face of various threats (see below), but the persistence of CL seeds in the fissures of the large quartzite wall must be taken into account. Although population size fluctuates between years, this annual species acts, in one sense, like a perennial, namely, by producing CL progeny for the following season in already colonized cracks. Along with the possible dispersal of CH seeds by wind (see Supplementary material), this perennial-like behaviour should guarantee the short-term survival of the population, at least in the cracks and/or ledges of the wall. CH seeds, being significantly lighter than CL seeds (30-seed weight of 0.0036 mg vs. 0.0061 mg), are dust-like; they are most likely wind-dispersed under natural conditions, although two other mechanisms may also be factors: ombrohydrochory, at least in sloped areas, and barochory (gravitational fall), especially in plants located on upper and middle parts of the quartzite wall.

Vertebrate and invertebrate feeding can affect flower, fruit, and seed production in two ways: directly—by consuming flowers and/or fruits—and indirectly—by feeding on vegetative parts (i.e., leaves and bracts), thereby reducing resources available for flower and/or fruit formation (Domínguez-Lozano et al., 2020; García Alonso and Márquez García, 2011; López-Sánchez et al., 2016; Simon et al., 2001; Anon DGMN, 2020). In our study, we observed both types of predation, although grazing by wildlife had the most intense impact. In other populations of *S. arguta*, wildlife and livestock grazing, trampling, digging, and damage due to roosting have been considered to be the main threats (García Alonso and Márquez García, 2011; Anon DGMN, 2020). The second most serious threat to our studied population is defoliation due to various lepidopteran caterpillars, such as members of family Noctuidae (e.g., *Shargacullia scrophulariae* (Denis and Schiffermüller, 1775) = *Cucullia scrophulariae*). This type of predation seems to be common to most species of *Scrophularia* (e.g., *S. frutescens*, Huertas, 2007; *S. canina*, *S. oxyrhyncha*, *S. lyrata*, *S. nodosa*, *S. xanthoglossa*, etc., authors' personal observations).



Another major challenge to populations in drier areas (e.g., Murcia) is water scarcity, which leads to wilting and considerably reduces reproductive success and can in turn cause seed bank depletion and possible population collapse (Anon DGMN, 2020). We have observed this phenomenon in our studied population, although not to the same extent, during years of intense drought (e.g., 2016; see Fig. A.4). Another potential issue is competition from other species, especially exotic ones, which can pose a serious threat by reducing habitat availability (Anon DGMN, 2020). In our case, *Opuntia ficus-indica*, found on elevated areas of the wall, was the only detected exotic species, and we do not believe that its impact, at least currently, is high enough to take over any of the habitat. Instead, companion native species are more likely to compete with *S. arguta* reducing the number of seedlings reaching adulthood in sunny areas, which could considerably reduce their habitat and confine these individuals to rock crevices.

With respect to grazing by wild vertebrates, no positive correlation between this form of predation and plant density has been uncovered by some authors (Domínguez-Lozano et al., 2020). This was true in our study as well; we even observed a negative trend (i.e., a lower plant density was associated with more predation). Predation is affected by factors other than plant density. One such factor in our studied population is the density of neighbouring plant species eagerly consumed by vertebrates. Predation on *S. arguta* in the study area may be an indirect effect of animals grazing on companion species (e.g., *Lamarckia aurea* and *Mercurialis ambigua*). Another factor that may account for greater predation at low densities is the fact that uncrowded *S. arguta* individuals are larger and therefore more appetizing.

*Scrophularia arguta* is a pioneer plant with a low capacity for interspecific competition. To avoid being outcompeted by neighbouring species, *S. arguta* undergoes earlier development and flowering. In mostly permanently shaded areas, high densities of small *S. arguta* individuals, generally no more than 10 cm tall and lacking CH flowers, were present with no accompanying species. In sunny areas, in contrast, high densities of large individuals of *S. arguta* producing large blooms were accompanied by high densities of neighbouring species. To vertebrates, these sunny sites are more attractive grazing areas than shady ones. The current distribution of the population, with most individuals close to the wall, is therefore probably a consequence of intrinsic and extrinsic factors, i.e., low competition capacity and wildlife grazing, respectively.

In this study, our aim was not to quantify each threat. Instead, our goal was to detect threats and assess their potential impacts on the studied population, with the ultimate goal of communicating to the appropriate authorities that the disappearance of this peripheral population would be a serious loss to the biodiversity of *S. arguta*. Because the study location is a genetic refuge for *S. arguta*, conservation of this site would not only protect the main northern peripheral population of the species, but also preserve a significant evolutionary unit. Peripheral populations such as these, which have long been isolated for various reasons from other Iberian and extra-Iberian populations, are unique and usually require different conservation strategies than those applied to the bulk of the species (Fenu et al., 2020; Sulis et al., 2021). The west-central Iberian population in this study constitutes a well differentiated lineage exhibiting local adaptations (demographic compensation; Doak and Morris, 2010) and containing a unique plastid haplotype (Valtueña et al., 2016, 2020), which is similar to peripheral populations of other species (Safriel et al., 1994; García-Ramos and Kirkpatrick, 1997; Hampe and Petit, 2005; Parmesan, 2006; Sexton et al., 2009; Thompson et al., 2010; Rehm et al., 2015). In addition, this peripheral population of *S. arguta* can be considered relictual (Valtueña et al., 2016, 2020) and even more valuable and in need of protection.

Additionally, we note the conflict between plant and animal conservation pointed out by Simon et al. (2001, and references therein). In Extremadura, animal conservation efforts, which aim to eliminate selective culling, are dramatically increasing populations of herbivorous vertebrates. This may cause irreversible damage to many plant populations (endangered or not) and their pollinators, including those of *S. arguta*. More specifically, this increase in herbivorous vertebrates may result in more predation of *S. arguta* individuals and endanger the population of this species, in turn markedly reducing available nectar and pollen and presumably decreasing or displacing solitary bees and other natural pollinators of *S. arguta*.

Finally, at a global level, the protection of the populations of this species, not only the one studied, would entail the preservation of one of the few known amphicarpic species (ca. 67, Zhang et al., 2020); those populations, as mentioned above, are scarce and, generally, made up of few individuals. Despite its wide albeit fragmented distribution, *S. arguta* is subject to heavy predation by cattle due to limited pasture in many countries and regions (authors' personal observation). In fact, the species has probably already disappeared from some areas in which it was previously recorded.

## 5. Conclusions

Several factors potentially threaten the Extremaduran peripheral relict population of *S. arguta* and endanger its survival. First, wild vertebrate grazing and caterpillar defoliation, which are the main threats, are responsible for a marked decrease in reproductive success that may lead to population collapse. Second, the existence of beehives near this population is altering the presence and density of the wild pollinators responsible for gene flow via pollen transfer. Third, competition with other companion species is supplanting individuals of *S. arguta* in the most favourable areas and confining them to the most unfavourable ones, namely, continuously shaded areas and rock crevices, where they persist mainly because of the CL seeds. Fourth, prolonged periods of drought can lead to the death of a large number of individuals. Finally, the uprooting of plants, a secondary effect of vertebrate grazing, may become serious if it increases considerably over time, as this activity decreases the reproductive success of CL flowers, the species' main strategy against the above-mentioned threats.

We recommend that regional/national administrations responsible for biodiversity matters take the following actions to conserve the studied population. Although fencing does not always have a positive conservation effect (Lorite et al., 2021), we first advise that some population sectors be temporarily fenced off and monitored to see if vertebrate predation, the greatest threat, is reduced compared with unfenced sectors. Second, beehives should be located away from this population. Third, the population should be

preserved *ex situ* in regional and national germplasm banks. Fourth, the other two small, known populations in Extremadura should be monitored. Finally, a biological follow-up study should be carried out on the studied population over time.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Data Availability

Data will be made available on request.

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### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.gecco.2022.e02265](https://doi.org/10.1016/j.gecco.2022.e02265).

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