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Differences in floral traits and flower visitation rates in mating systems in *Prunella vulgaris* (Lamiaceae)

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Abstract

Floral traits and the number of visitors are expected to change with different mating systems. We tested this hypothesis by comparing flowers of *Prunella vulgaris* (Lamiaceae) with inserted and exserted styles across a strongly exserted style biased, an exserted style biased, and a strongly inserted style biased subalpine population. We examined flowering phenology, floral morphology, flower visitation rate, capacity for autonomous self-pollination and visitor contribution to seed production for each style type and in each population. We also examined inbreeding depression (ID) by comparing the relative performance of progeny from self- and cross-pollination. Exserted style plants had larger and more open flowers, increased pollen production, higher amounts of nectar rewards and higher visitation and outcrossing rates than inserted style plants. Similarly on the population level, the visitation rates were higher in the exserted style-biased populations than in the inserted style-biased population. Inserted style plants provided a stronger reproductive assurance (RA) through autonomous selfing than exserted style plants. RA and outcrossing rates did not differ among populations, showing low visitation rates may be sufficient for adequate seed production in *P. vulgaris*. Although inserted style plants had a lower ID level than exserted style plants, the ID of both was less than 0.5, suggesting that an ID should not counteract the evolution of selfing in this species. Inserted style plants provide RA through autonomous selfing, and exserted style plants ensure outcrossing through pollinator services, supporting a stable mixed mating system in this subalpine plant.

Keywords autonomous selfing, floral traits, inbreeding depression, visitors, *Prunella vulgaris*, reproductive assurance

夏枯草交配系统对花特征和访花频率差异的影响

摘要：植物花特征和传粉者的访问次数与交配系统类型密切相关。唇形科植物夏枯草(*Prunella vulgaris*)存在两种植株类型，分别为柱头伸出花冠和柱头在花冠内部的植株，而且两种植株的比例在不同种群中存在差异。本研究选择柱头伸出花冠外植株占绝大多数、柱头伸出花冠外植株占多数和柱头在花冠内部植株占多数的3个种群，通过比较每个种群中两种植株类型的开花物候、花形态特征、昆虫访问频率、自

交能力、传粉者对结实的贡献以及近交衰退的水平，以检验花特征和传粉者访问次数与交配系统类型的关系。研究结果表明，与柱头在花冠内部的植株相比，柱头伸出花冠外的植株具有更大和更多的花，产生更多的花粉和花蜜，具有更高的访花频率，并主要通过异交产生种子。在种群水平，柱头伸出花冠外的植株占多数种群的访花频率显著高于柱头在花冠内部植株占多数的访花频率。柱头在花冠内部的植株比柱头伸出花冠外的植株具有更强的自动自交能力，在传粉者缺乏时为其提供了繁殖保障，但繁殖保障和异交率在不同种群中差异不显著，表明较低的昆虫访问能够满足夏枯草的授粉需求以产生种子，这可能与夏枯草较少的胚珠数量(每朵花仅有4个胚珠)有关。柱头在花冠内部植株的近交衰退水平低于柱头伸出花冠外植株的近交衰退水平，但两种植株类型的近交衰退水平均低于0.5，说明近交衰退不足以阻止该物种中自交的进化。综上所述，柱头在花冠内部的植株能够通过自交为夏枯草提供繁殖保障，而柱头伸出花冠外的植株能够利用昆虫传粉确保异交，表明混合交配系统在该物种中是一个稳定的状态。

关键词：自交，花特征，近交衰退，传粉者，夏枯草(*Prunella vulgaris*)，繁殖保障

INTRODUCTION

Alpine and subalpine conditions present significant challenges to plant species, leading to evolutionary changes in mating systems. These responses appear to be strongly associated with variations in abiotic conditions, pollinator availability and floral traits (Cutter 2019; Elle and Carney 2003; Kuriya *et al.* 2015; Mu *et al.* 2011; Sicard and Lenhard 2011). Mating strategies that are stable from an evolutionary perspective represent a balance between the chances of pollination and self-fertilization and the loss of genetic diversity (Bartoš *et al.* 2020; Chen and Zhao 2019; Lande and Schemske 1985; Ma *et al.* 2019). However, the importance of genetic and ecological mechanisms for such evolutionary stability is not known. The evolution of mating systems is usually determined by the interplay of two genetic mechanisms: automatic gene transmission advantage and inbreeding depression (ID). Evolutionary biologists suggest that a selfing allele that arises from outcrossing populations has a 50% gene transmission advantage over an outcrossing allele to the next generation, because it passes through both pollen and ovules in self-seeders, as well as through outcrossed pollen (Busch and Delph 2012; Fisher 1941). ID is the reduced fitness of self-seeders relative to outcrossed progeny, which decreases the transmission advantage of selfing, and is considered to be the major factor favoring outcrossing (Charlesworth and Charlesworth 1987; Opedal *et al.* 2015). Theoretically, when ID is > 0.5, the transmission advantage of selfing is deleterious (Abdelaziz *et al.* 2014; Ågren and Schemske 1993; Lloyd 1992; Vaughton *et al.* 2008). Therefore, complete outcrossing in mating systems should be selected when the ID is >0.5, and complete selfing when the ID is <0.5, thereby yielding an evolutionarily stable system (Charlesworth and Charlesworth 1987; Lande and Schemske 1985).

However, mixed mating systems are remarkably common in nature (Goodwillie *et al.* 2010; Panique and Caruso 2020; Totland and Schulte-Herbrüggen 2003). Therefore, we must consider ecological mechanisms (i.e. pollen/ovule discounting) and their ecological context (i.e. pollinator availability) to fully understand why selfing evolves and how ID is counteracted. Recent empirical studies suggest that ecological mechanisms associated with pollen discounting (when pollen used for selfing reduces pollen available for outcrossing) or ovule discounting (loss of cross-fertilization opportunities when ovules are disabled by self-pollination) can oppose the evolution of autonomous selfing, especially in cases where autonomous selfing does not provide reproductive assurance (RA) (Jacquemyn and Brys 2015; Lloyd 1992; Porcher and Lande 2005). RA occurs because plants have mechanisms to ensure full seed production through autonomous selfing when outcross pollen is limiting (Brys *et al.* 2011; Eckert *et al.* 2009; Ling *et al.* 2017; Zhang and Li 2008; Zhang *et al.* 2014). Thus, seed production through autonomous selfing as a mechanism of RA should be favored when pollinators are absent. If pollinators are completely absent, then there is no pollen/ovule discounting (Ling *et al.* 2017; Lloyd 1992; Zhang and Li 2008; Zhang *et al.* 2014), implying that ID should act as the only mechanism that opposes the evolution of selfing. However, when autonomous selfing occurs over time in highly self-pollinated species, plants can purge deleterious recessive alleles effectively and counteract ID (Goodwillie *et al.* 2005; Uyenoyama 1991). Therefore, the magnitude of ID in plants with mixed mating systems may vary with the RA benefit of autonomous selfing (Ansaldi *et al.* 2019), and this variation may, in turn, influence the stability of mixed mating systems (Griffin *et al.* 2019). Many studies have challenged the benefits of autonomous selfing and the strength of ID variations among populations in self-pollinated species, but the potential for differences in these mechanisms among individuals of a species with different mating systems has rarely been studied and also inconsistent results are observed (Arista *et al.* 2017; Bartoš *et al.* 2020; Opedal *et al.* 2015; Wang 2019; Winn *et al.* 2011).

Changes in floral traits as a consequence of plant adaptations to different mating systems play an important role in enhancing the beneficial effects of autonomous selfing on flower visitors (Cutter 2019; Kennedy and Elle 2008; Mu *et al.* 2011; Panique and Caruso 2020; Sicard and Lenhard 2011). Compared to cross-pollinated species, self-pollinators have reduced flower size and longevity, and fewer open flowers (Elle and Carney 2003; Hernández-Villa *et al.* 2020; Mu *et al.* 2011). This affects pollinator visitation rates, and subsequently influences the RA benefit of autonomous selfing. It is also suggested that pollinator availability is particularly low in habitats with small population size or in autonomous selfing dominant populations, enhancing the RA benefits (Elle and Carney 2003; Kennedy and Elle 2008). Therefore, low pollinator availability promotes selection for increased autonomous selfing. Increased floral traits are costly for plants and are no longer needed when the plants are capable of autonomous selfing. The reduction in floral traits may have evolved as a response. Comparing differences in floral traits and pollinator visitation rates among individuals of a single species that have different mating systems provides better information than comparing this aspect in related species. This allows us to test how intraspecific variation in floral traits and pollinator visitation rates may be related to the benefits of RA and the magnitude of ID, without the complication of phylogenetic non-independence. However, this type of system has rarely been studied. We predict that individuals that favor autonomous selfing should show reduced floral traits and pollinator visitation that increase the RA benefit, whereas those favoring outcrossing should show enhanced floral traits and pollinator visitation that increase outcrossing rate.

The majority of *Prunella* species (Lamiaceae) have been known in both alpine and subalpine environments (Egawa *et al.* 2015; Liao *et al.* 2010; Melnikov 2019), where fluctuations in conditions mean that intraspecific variation in floral traits may confer advantages to reproductive fitness. Ling *et al.* (2017) noted that a Chinese subalpine population of *Prunella vulgaris* with flowers with inserted styles (IS), where the styles were shorter than the length of long stamens, was capable of autonomous selfing that provided a certain level of RA, where flower visitation rate was relatively low. In contrast, Qu and Widrlechner (2011) observed that flowers with exserted styles (ES) of *P. vulgaris*, where the flower styles extended past open corollas when viewed from above or were longer than the length of long stamens, set almost no seeds when pollinators were excluded to examie the capacity for autonomous selfing. These authors also suggested that flowers with IS produced more seeds through autonomous selfing than those with ES as a result of their close proximity to the stigma, a common trait that enhances autonomous selfing (e.g. Zhang and Li 2008). Moreover, Kuriya *et al.* (2015) documented that in Japanese subalpine populations, the flower size of this species varied along an altitudinal gradient and was positively correlated with the tongue length of visitors to local flowers, influencing both pollen removal and deposition. However, these authors did not find the autonomous selfing mechanism in the Japanese populations of *P. vulgaris* for which flower size was also much larger than in the Chinese population of *P. vulgaris* as reported by Ling *et al.* (2017). Therefore, the mating system in this species is likely to vary with flower size or style type and pollinator availability. However, it remains unclear whether the type of style is related to flower size, coupled with other floral traits, and how differences in these traits might be associated with flower visitation rate under different environmental conditions. This could ultimately influence the RA benefit of autonomous selfing and outcrossing rate. Moreover, there is limited information regarding the effects of intraspecific variations in mating systems and adaptations to different floral traits and flower visitors on ID. To address this, *P. vulgaris,* each comprising both IS- and ES-flowered plants (Fig. 1a and b) of varying proportions: one population was strongly ES-biased (97.83% ES-flowered plants), one population was ES-biased (64.75% ES-flowered plants) and one population was strongly IS-biased (89.56% IS-flowered plants; Table 1).

We addressed three specific questions in the study: (i) What are the differences in floral traits between IS- and ES-flowered plants? (ii) Does the flower visitation rate differ between IS- and ES-flowered plants in different populations? (iii) Do IS- and ES-flowered plants differ in the level of RA through autonomous selfing among populations, and in the strength of ID? We predicted reduced floral traits in IS-flowered plants, lower flower visitation rates in both IS-flowered plants and strongly IS-biased populations and greater RA through autonomous selfing for IS-flowered plants, particularly in strongly IS-biased populations. We also predicted that the level of the accumulated ID throughout the life span would be lower for IS-flowered plants than for ES-flowered plants.

MATERIALS AND METHODS

Study species and site

Prunella vulgaris is a self-compatible, perennial herb that is broadly distributed in the Northern Hemisphere, including high elevations in the northern parts of China (Liao *et al.* 2010; Melnikov 2019; Nelson 1967). It inhabits lowlands, gravel streams, moist pasturelands and subalpine meadows. Individual plants produce an inflorescence that comprises two to six flowers per whorl, with a total of two to seven whorls per inflorescence. Individual plants can have one to 15 open flowers at a time. A single flower completed blooming after 5 days, while an individual plant completed blooming after 25 days (Ling *et al.* 2017). The corolla of this tubular flower is purple and has didynamous stamens with two anthers with short and long stamens. It has four ovules per pistil, and therefore, a maximum of four seeds per fruit. Each flower has one style that can be either an IS or an ES (Fig. 1a), a common trait that influences the mating system of *P. vulgaris* (Qu and Widrlechner 2011). A small floral nectary is situated

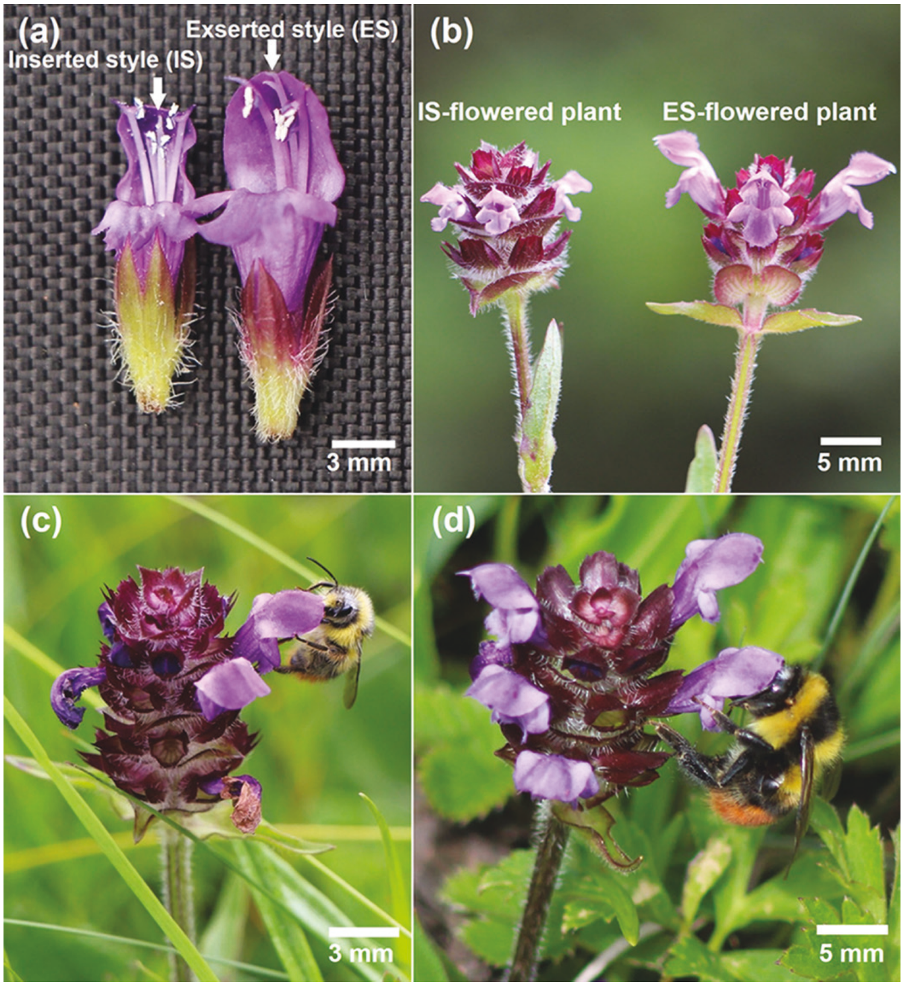

Figure 1: Flowers of *Prunella vulgaris* that are small with an inserted style (IS) versus large with an exserted style (ES); white arrows pointing to the stigmas (**a**). IS- and ES-flowered *P. vulgaris* (**b**) *Bombus lepidus* visiting an IS-flower of *P. vulgaris* (**c**) *B. friseanus* visiting an ES-flower of *P. vulgaris* (**d**).

Table 1: Populations used in this study, namely Wenhai village (WH), near Wenhai lake (WL) and Lijiang Alpine Botanical Garden (LABG); style type (IS- and ES-flowered plants); plant density (m^{-2}); area (m^2); estimated number of flowering plants and population category classification

Population	Style type	Plant density (m^{-2})	Area (m^2)	Estimated plants no.	Population category (proportion of IS- and ES-flowered plants)
WH	IS	4.33 ± 0.13	127.99	938.59	Strongly ES-biased population (IS2.17:ES97.83)
	ES	4.33 ± 0.13	3483.38	42 265.01	
WL	IS	4.34 ± 0.14	913.37	5419.33	ES-biased population (IS35.25:ES64.75)
	ES	4.43 ± 0.13	957.31	9956.02	
LABG	IS	4.20 ± 0.14	1215.40	7697.53	Strongly IS-biased population (IS89.56:ES10.44)
	ES	4.50 ± 0.11	121.21	896.95	

We estimated the total number of flowering plants by multiplying plant density by area for each style type in each population. We classified the population category by dividing the estimated total number of flowering plants of both style types by the estimated total number of either IS-flowered plants or ES-flowered plants. For each population, we defined strongly ES-biased population as the proportion of ES-flowered plants was more than 80%, ES-biased population as the proportion of ES-flowered plants was more than 50% and IS-biased population as the proportion of ES-flowered plants was less than 20%, respectively.

at the base of the corolla tube. Bumblebees are the main visitors to *P. vulgaris* flowers (Egawa *et al.* 2015; Kuriya *et al.* 2015; Ling *et al.* 2017).

All experiments were done in three natural subalpine populations of *P. vulgaris* around Lijiang (26°51′19.80″ N; 100°13′39.92″ E; 2400 m a.s.l.) in southwest China in 2015, where both IS-and ES-flowered plants grew together, but differed markedly in the proportions of the IS- and ES-flowered plants. Populations were either strongly ES-biased (IS2.17:ES97.83), located near Wenhai village (WH: 3218 m a.s.l.); ES-biased (IS35.25:ES64.75), located near Wenhai lake (WL: 3249 m a.s.l.) or strongly IS-biased (IS89.56:ES10.44), located in the Lijiang Alpine Botanical Garden (LABG: 3259 m a.s.l.) (Table 1; Supplementary Fig. S1). The geographic distance between the populations ranged from 1.5 to 2.5 km. All of the populations studied inhabited subalpine meadows.

Flower type and population classification

Our initial flower type classification was based on the style type. In the three study sites, IS- and ES-flowered plants occurred within the same subalpine meadows, although the distribution areas of the two style types within the meadows did not overlap and were 0.4 to 1 m from each other. Thus, we defined a population as plants of the two style types that shared the same meadow. A schematic representation of the population characteristics is shown in Supplementary Fig. S1.

To confirm whether the styles of IS-flowered plants were always inserted and styles of ES-flowered plants were always exserted, we recorded the number of flowers with ES from 50 flowering plants per style type in each population. We observed the total number of open flowers per inflorescence, which produced two to 14 open flowers on a daily basis, to ensure that all flowers within inflorescences had the same style type in each style type group. In total, 818 flowers (279 flowers in strongly ES-biased population, 265 flowers in ES-biased population and 274 flowers in strongly IS-biased population) were examined for the IS-flowered plant group and 1316 flowers (440 flowers in strongly ES-biased population, 442 flowers in ES-biased population and 434 flowers in strongly IS-biased population) were examined for the ES-flowered plant group. We calculated the percentage of ES by dividing the total number of flowers with ES by the total number of open flowers per inflorescence for each style type and population. Styles in the ES-flowered plants were always exserted. In IS-flowered plants, 0.72% of flowers in the strongly ES-biased population, 0.75% of flowers in the ES-biased population and 1.09% of flowers in the strongly IS-biased population had flowers with ES. Flowers with ES occurring in the IS-flowered plants were still included in the IS group for this study, because they were found on inflorescences that produced more than nine open flowers, but only had a single flower with an ES.

We justified our initial classification on population category (the proportion of both IS- and ES-flowered plants) by estimating the total number of flowering plants of each style type in each population (the number of IS- or ES-flowered plants was divided by the total number of plants). The total number of flowering plants was estimated by multiplying plant density (m^{-2}) by the distribution area (m^2) for each style type in each population. Because the distribution areas of the two style types within the populations did not completely overlap (Supplementary Fig. S1), we quantified the distribution area for each style type within the populations using a Garmin GPSMAP 639sc unit. To examine plant density, we established five squares prior to flowering for each style type in each population. The area of each square was 3 m × 3 m, and the distance between the squares for each style type in each population was approximately 10 m. Plant density was estimated by counting the number of plants for both style types within each square during the peak blooming season. For each population, we defined strongly ES-biased population as the proportion of ES-flowered plants was more than 80%, ES-biased population as the proportion of ES-flowered plants was more than 50% and strongly IS-biased population as the proportion of ES-flowered plants was less than 20%, respectively (Table 1; Supplementary Table S1 for complete overview for the populations used, plant density, area and population category).

Flowering phenology and morphological traits

To examine the flowering phenology of *P. vulgaris*, we recorded the number of open flowers in each square once a week from June to September, which is the flowering season for *P. vulgaris* in the studied area. To determine the flowering profile for each style type in each population, the total number of open flowers from all five squares in each population was recorded on each observation day and divided by the total number of open flowers from all five squares during the entire observation period.

During the peak flowering season, we measured the corolla length and width of 30 flowers for each style type and population to determine whether flower size differed in IS- and ES-flowered plants. We also determined the flower longevity of 30 flowers for each style type and population by recording every day from the day the bud opened to the day the corolla wilted. We used one flower from the middle whorl of an inflorescence per plant to prevent replication and ensure independence. We recorded 30 flowering plants per style type and population, as well as the total number of open flowers per plant. To quantify the number of pollen grains per flower, we collected one flower from 15 plants per style type and population and fixed each flower separately in a Formalin-Aceto-Alcohol (FAA) solution (formalin: acetic acid: ethanol at a ratio of 5:5:90 by volume) to determine the total number of pollen grains from each flower. All anthers in each flower bud were dissected and all pollen grains were collected in a 1.5-mL micro-centrifuge tube with a suspension of 0.5 mL of a mixed FAA solution and detergent. In each observation, 10 subsamples (10 μL each) were placed on a glass microscope slide, and the total number of pollen grains on the slide were counted under a light microscope (XSZ-0900, Wuzhou Oka Optical Instrument Co., Ltd., Wuzhou, Guangxi, China). We determined nectar volume and sugar concentration by bagging 10 flowering plants before anthesis for each style type in each population, with a total of 30 flowering plants for each style type. Individual inflorescences were bagged with fine nylon bags to exclude insect visitors. On the second day after anthesis, nectar was collected from a single flower from the middle whorl of one inflorescence per plant, using 100 × 0.5 mm glass capillary tubes (Instrument Factory of West China Medical University, Chengdu, Sichuan, China). Collections were timed for peak nectar secretion periods, between 0900 h and 1200 h. Nectar volume was determined by measuring the height to which the nectar filled tube with a digital caliper (0.01 mm precision, Guilin Guangdu Measuring Instrument Co., Ltd., Guilin, Guangxi, China) and the length measurements were then converted to microliters. The sugar concentration was measured using a hand-held, temperature-compensated refractometer (Eclipse; Bellingham & Stanley Ltd., Turnbridge Wells, Kent, UK). Published data on flower size, number of open flowers per plant, flower longevity and pollen numbers for IS-flowered plants for the LABG population (Ling *et al.* 2017) were used to statistically compare the traits of ES-flowered plants in the LABG population. A generalized linear model (GLM) was applied to test for significant differences in morphological traits, with population and style type as fixed factors. Interaction effects were also included. A normal error distribution with an identity link function was used to analyze all measurements. We also quantified the relative frequency of the size of both IS- and ES-flowered plants using histograms. All statistical analyses were conducted using IBM SPSS version 27.0 for Windows (2020, IBM Corp., Armonk, NY, USA).

Flower visitation rate

We observed visitors to flowers with both style types on clear sunny days from 0900 h to 1900 h during the peak flowering season to quantify the difference in flower visitation rates between IS- and ES-flowered plants of *P. vulgaris*. For each style type, we performed 4 days of observation at different times of the day in each population. The observations were done for all populations and both style types on either the same day or one close to the day, as differences in observation timepoints could influence the visitation rate, depending on flower visitor phenology. On each observation day, we selected 20 to 52 fresh flowers from seven to 18 individuals (two to four flowers per individual) per style type and recorded the number of individual flower visitors. All insect visitors were collected using a nylon net and were killed using ethyl acetate following observation periods. Pinned and labeled specimens were identified to the species level and preserved at the Kunming Institute of Botany, Chinese Academy of Sciences. The visitation rate per flower per hour was calculated for each species of visitor to the flowers. We used the data for visitation rates from the most frequent visitors, *Bombus lepidus* (Fig. 1c) and *B. friseanus* (Fig. 1d) to estimate whether species visiting individual flowers showed different visitation patterns to the two style types. Only the data for the *Bombus* spp. were used as the sample size for the other species was too small for meaningful statistical analyses. A GLM was applied to test for significant differences in the visitation rate, with population, style type and visitor type as fixed factors. Their interaction effects were also included. A normal error distribution with an identity link function was used to analyze visitation rates.

A previous study documented that tongue length of the local *Bombus* species was strongly correlated with the floral corolla length of *P. vulgaris* in Japanese subalpine populations (Kuriya *et al.* 2015). We measured the tongue length of *B. lepidus* and *B. friseanus,* using a digital caliper (0.01 mm precision, Guilin Guangdu Measuring Instrument Co., Ltd., Guilin, Guangxi, China). The data were used to determine if there was a relationship between tongue length and visitation rate for the *Bombus* species and the style types in the three populations studied. A GLM was applied to test for significant differences in tongue length between the two *Bombus* species, with population and visitor type as fixed factors. Their interaction effect was also included.

Pollination experiments

We performed emasculation and pollen supplementation experiments combined with pollinator exclusion to examine the capacity for autonomous self-pollination and the extent of pollinator contribution to seed production. Pollination experiments were conducted for both IS- and ES-flowered individuals of *P. vulgaris* in the three study populations during the peak flowering period. Two to four flower buds from the middle whorl of the inflorescence from 20 plants for each style type in each population were selected for the following five pollination treatments: (i) buds were covered with a fine nylon mesh bag to examine the capacity for autonomous selfing; (ii) buds were emasculated and covered with a fine nylon mesh bag before the anthers were dehisced, and then hand-pollinated with fresh pollen collected from the same plant (geitonogamy) after the corolla had opened, to test for self-compatibility; (iii) buds were emasculated and covered with a fine nylon mesh bag before the anthers were dehisced, and then manually pollinated with fresh pollen collected from multiple individuals located at least 5 m from the pollinated plant (cross-pollination within style type); (iv) buds were emasculated and left open to examine the contribution of pollinators to seed production and (v) flowers were left undisturbed for open pollination, as a control.

We used different plants for different treatments. In each treatment, we gently emasculated all anthers of non-manipulated flowers without harming floral development to prevent self-pollination resulting from pollen transfer from other flowers on the same inflorescence and then covered the whole inflorescence with a fine nylon mesh bag. Approximately 1 month later, the seed number per flower was counted. We excluded manipulated flowers that were damaged after emasculation. Therefore, the results presented are an accurate indication of the final seed production from all treatments. Data were analyzed using a GLM with a Poisson distribution with log link function to test for significant differences in seed production between treatments and between style types across different populations, with population, style type and treatment as fixed factors. Interaction effects were also included. The autofertility index was calculated as the mean seed production of flowers from autonomous self-pollination divided by the mean seed production of flowers from hand-crossed pollination (Schoen and Lloyd 1992). We calculated the increase in seed production through autonomous selfing for both style types in each population as the difference between the mean seed production of the control treatment and that of those emasculated and left open for pollinator treatment (Lloyd 1992; Schoen and Lloyd 1992). The index of RA was estimated as: 1 − (mean seed production of emasculated and left open for pollinator treatment/mean seed production of control treatment) (Brys *et al.* 2011; Eckert *et al.* 2009).

Inbreeding depression

We obtained 53 seeds from the IS-flowered plants subjected to the hand-crossed treatment in the ES-biased population and 51 seeds from the ES-flowered plants subjected to the hand-selfed treatment in the strongly IS-biased population. This sample size was too small to invoke statistical significance for ID variations among populations. Therefore, we quantified the ID differences between the two style types by combining 50 seeds from each of the three study populations for each treatment and style type. All 150 seeds were air-dried and subdivided into 15 artificial replicates (10 seeds per replicate). We weighed the seeds from each replicate using an electronic balance (0.1 mg) and calculated the seed mass. All seeds were then kept at −20 °C for 2 months, to break dormancy. For each treatment, 150 seeds were evenly separated into five replicates of 30 seeds and placed on wet filter paper in Petri dishes to measure seed germination. The seeds were then placed in an incubator at 20 °C for 14 days, ensuring that they remained moist. The number of seeds that germinated in each Petri dish was counted, and the seed germination rate for each replicate was then calculated by dividing the number of germinated seeds by 30. ID (δ) was estimated for three parameters: seed number, seed mass and seed germination. Data were analyzed using GLMs to test for significant differences in seed mass and germination between the two style types and progenies resulting from hand-selfed and hand-crossed pollination treatments, with style type and treatment as fixed factors. Interaction effects were also included. We used a normal error distribution with an identity link function to analyze seed mass and a binomial distribution with a logit link function for seed germination. The magnitude of ID at each stage was calculated as $\delta = 1 - (Ws/Wo)$, when $Ws \le Wo$, and $\delta = (Wo/Ws) - 1$, when $Ws \ge Wo$, where δ is the ID, *Ws* is the fitness of the selfed progeny and *Wo* is the fitness of the outcrossed progeny (Abdelaziz *et al.* 2014; Ågren and Schemske 1993). Cumulative ID was calculated by multiplying the fitness values for each cross-type across the life stage, and then applying the formula above.

RESULTS

Flowering phenology and morphological traits

In all three populations, the length of flowering time did not differ between IS- and ES-flowered plants, but the start, peak and end of flowering were approximately 2 weeks earlier in ES-flowered plants than in IS-flowered plants (Supplementary Fig. S2). Morphological traits differed between IS- and ES-flowered plants (Table 2), where ES-flowered plants had a larger corolla length and width, a larger number of open flowers per plant and pollen grains per flower, and a greater nectar volume and sugar concentration than IS-flowered plants. Flower longevity did not differ between the ES- and IS-flowered plants (Table 2; Supplementary Table S1; Fig. 2). Corolla length ranged from 5.90 to 9.93 mm and corolla width ranged from 2.54 to 4.84 mm in IS-flowered plants, whereas corolla length ranged from 10.13 to 12.87 mm and corolla width from 4.91 to 5.86 mm in ES-flowered plants (see Supplementary Fig. S3 for the relative frequency of corolla length and width for each style type). Morphological traits did not differ among populations, and the population × style type interaction was not significant, either (Table 2).

Flower visitation rate

In total, we recorded 107 individual insects visiting the flowers of *P. vulgaris* during our flower visitor observations. Of the total visits, 52% were from *Bombus lepidus* (Fig. 1c), followed by 41% from *B. friseanus* (Fig. 1d), 3% from *Apis cerana*, 2% from *Macroglossum phyrrhosticta* and 1% each from *B. avanus* and *B. lucorum*, respectively. Of these, *B. lepidus*, *B. friseanus* and *A. cerana* visited both IS- and ES-flowered plants, *B. avanus* visited only IS-flowered plants, and *B. lucorum* and *M. pyrrhosticta* visited only ES-flowered plants (Supplementary Fig. S4). The overall visitation rate of *B. avanus* was 0.004 ±

Table 2: Summary of GLMs analyzing different factors: style type (IS-and ES-flowered plants of *Prunella vulgaris*); population (strongly ES-biased, ES-biased, strongly IS-biased populations); treatments (AU = autonomous self-pollination; HC = hand-crossed pollination; HS = hand-selfed pollination; EO = emasculated styles of flowers and left open for pollinators; NC = flowers were left undisturbed for open pollination, as a natural control; SN = seed number; SM = seed mass; SG = seed germination and CID = cumulative ID) and their interactions with corolla length, corolla width, flower longevity, number of open flowers per plant, pollen number, nectar volume, sugar concentration, visitation rate, tongue length of flower visitor, seed number, seed mass and seed germination

	df	Wald chi-square	*P* value
Corolla length			
Population	2	0.002	0.999
Style type	1	506.269	**<0.001**
Population × Style type	2	2.423	0.298
Corolla width			
Population	2	0.410	0.815
Style type	1	628.601	**<0.001**
Population × Style type	2	1.480	0.477
Flower longevity			
Population	2	1.796	0.407
Style type	1	1.376	0.241
Population × Style type	2	0.023	0.989
Number of open flowers per plant			
Population	2	0.053	0.974
Style type	1	48.515	**<0.001**
Population × Style type	2	0.099	0.952
Pollen number			
Population	2	0.184	0.912
Style type	1	717.378	**<0.001**
Population × Style type	2	1.274	0.872
Nectar volume			
Population	2	0.116	0.944
Style type	1	23.203	**<0.001**
Population × Style type	2	0.326	0.850
Sugar concentration			
Population	2	0.076	0.962
Style type	1	54.033	**<0.001**
Population × Style type	2	0.028	0.986
Visitation rate			
Population	2	13.196	**<0.01**
Style type	1	122.820	**<0.001**

Table 2: Continued

	df	Wald chi-square	*P* value
Visitor type	1	4.989	**<0.05**
Population × Style type	2	3.648	0.161
Population × Visitor type	2	4.133	0.127
Style type × Visitor type	1	37.528	**<0.001**
Population × Style type × Visitor type	2	5.558	0.062
Tongue length of flower visitor			
Population	2	1.407	0.495
Style type	1	0.860	0.354
Visitor type	1	191.430	**<0.001**
Population × Style type	2	0.579	0.749
Population × Visitor type	2	1.275	0.529
Style type × Visitor type	1	1.025	0.311
Population × Style type × Visitor type	2	0.364	0.546
Seed number			
Population	2	0.039	0.980
Style type	1	92.037	**<0.001**
Treatment	4	395.310	**<0.001**
Population × Style type	2	0.291	0.865
Population × Treatment	8	2.208	0.974
Style type × Treatment	4	271.492	**<0.001**
Population × Style type × Treatment	8	3.090	0.929
Seed mass			
Style type	1	70.375	**<0.001**
Treatment	1	0.520	0.471
Style type × Treatment	1	13.418	**<0.001**
Seed germination			
Style type	1	0.104	0.747
Treatment	1	9.941	**<0.01**
Style type × Treatment	1	25.634	**<0.001**

Published data on flower size, number of open flowers per plant, flower longevity and pollen numbers for IS-flowered plants for the LABG population (Ling *et al.* 2017) were used to statistically compare the traits of ES-flowered plants in the LABG population. Values in bold types ($P < 0.001$; $P < 0.01$; $P < 0.05$) represent significant differences.

0.004 (mean ± SE) and that of *M. phyrrhosticta* and *B. lucorum* was 0.02 ± 0.02. The overall visitation rate of *A. cerana* was 0.01 ± 0.01 for IS-flowered plants and 0.03 ± 0.02 for ES-flowered plants.

When we compared the visitation rates of the most frequent visitors, *B. lepidus and B. friseanus,* to each style type, we found that the visitation rates varied among populations and differed between both

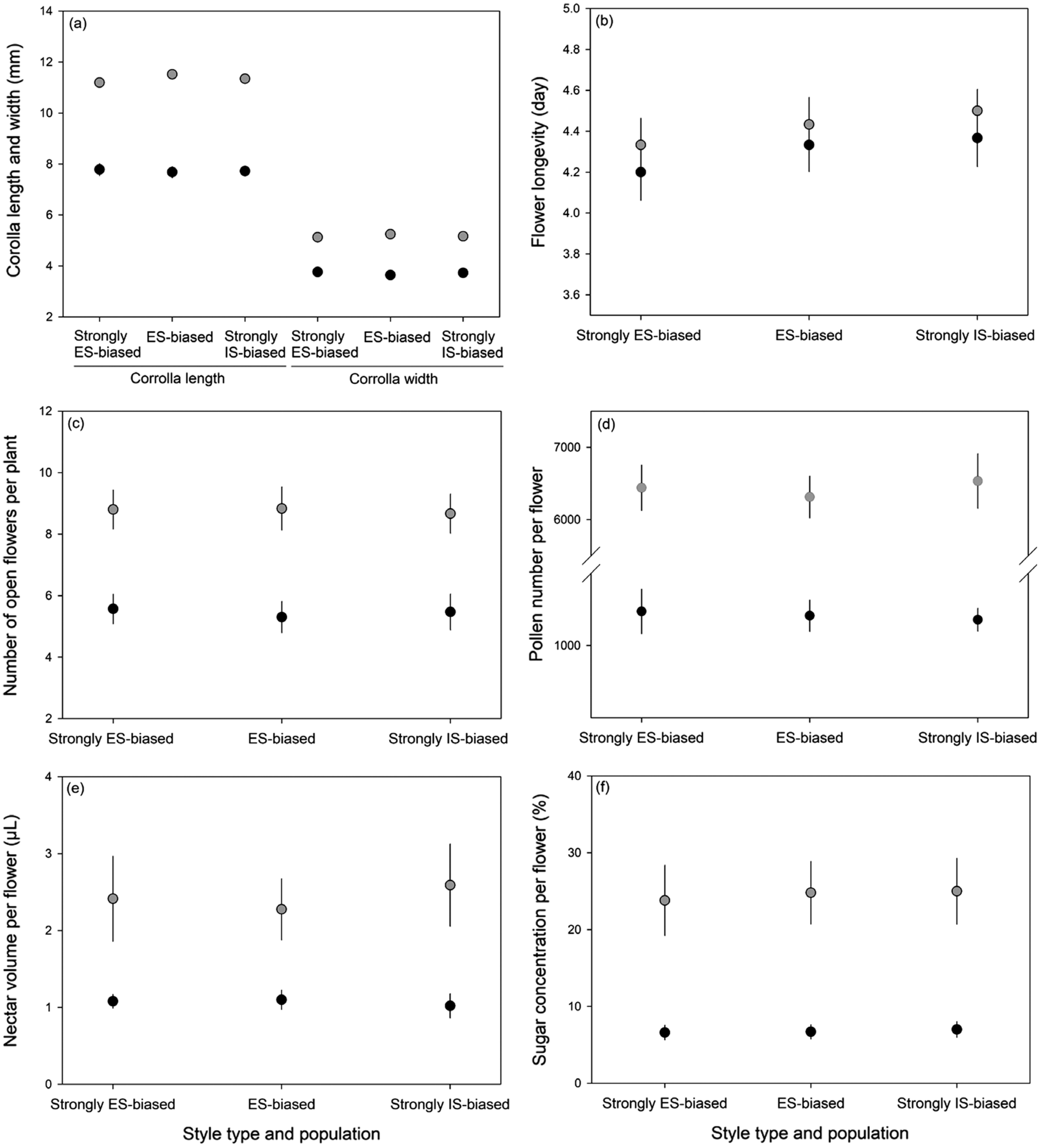

Figure 2: Corolla length and width (**a**), flower longevity (**b**), number of open flowers per plant (**c**), pollen number (**d**), nectar volume (**e**) and sugar concentration (**f**) of IS- and ES-flowered *Prunella vulgaris* examined from the strongly ES-biased, ES-biased and strongly IS-biased populations. Black and gray lines/symbols denote IS- and ES-flowered plants, respectively. Published data on flower size, number of open flowers per plant, flower longevity and pollen numbers for IS-flowered plants for the LABG population (Ling *et al.* 2017) were used to statistically compare the traits of ES-flowered plants in the LABG population. Data analyses for pollen number, nectar volume and sugar concentration are presented in Table 2 and Supplementary Table S1.

style type and visitor type (Table 2). The strongly IS-biased population had the lowest visitation rate compared to the two ES-biased populations (Supplementary Table S2; Fig. 3a). ES-flowered plants had a higher visitation rate than IS-flowered plants in all populations (Supplementary Table S2;

Fig. 3a). The overall visitation rate of *B. friseanus* (0.34 ± 0.02) was significantly higher than that of *B. friseanus* (0.26 ± 0.02). However, the style type × visitor type interaction was significant, because IS-flowered plants had a greater visitation rate from *B. lepidus*, whereas ES-flowered plants had a higher visitation rate from *B. friseanus* (Supplementary Table S2; Fig. 3a). The interactions, including population × style type, population × visitor types and population × style type × visitor type, were not significant (Table 2).

Tongue length differed between *B. lepidus* and *B. friseanus*, but not between the visitation rates to style types and populations, and the interactions were not significant (Table 2). The tongue length of *B. lepidus* (3.96 ± 0.07; *n* = 56) was significantly shorter than that of *B. friseanus* (5.97 ± 0.10, *n* = 44, Table 2; Supplementary Table S2; Fig. 3b).

Pollination experiments

Seed production differed between treatments and style types, and their interactions were also significant (Table 2). IS-flowered plants showed the highest seed production in both hand-pollination treatments, whereas seed production was intermediate in the autonomous self-pollination and natural control treatments, and the lowest in the emasculated and left open for pollinator treatments (Supplementary Table S3; Fig. 4a). In contrast, ES-flowered plants showed the highest seed production in both hand-pollination treatments, which was intermediate in the natural control, and lowest in autonomous self-pollination and emasculated and left open for pollinator treatments (Supplementary Table S3; Fig. 4a). IS-flowered plants had a significantly higher seed production in autonomous self-pollination and control treatments, whereas ES-flowered plants had a significantly higher seed production in the emasculated and left open for pollinator treatments (Supplementary Table S3; Fig. 4a). Seed production by hand-pollination treatments did not differ between the two style types. Seed production did not differ among populations, and population × style type, population × treatment and population × style type × treatment interactions were not significant, either (Table 2).

Autonomous selfing levels for IS-flowered plants were 0.89, 0.88 and 0.93 in strongly ES-biased, ES-biased and strongly IS-biased populations, respectively, whereas autonomous selfing levels for ES-flowered plants were 0.03, 0.03 and 0.04 in strongly ES-biased, ES-biased and strongly IS-biased populations, respectively. Overall, the autonomous selfing levels for both style types were 0.90 and 0.03, respectively. RA through autonomous selfing measures for IS-flowered plants were 0.80, 0.77 and 0.80 in strongly ES-biased, ES-biased and strongly IS-biased populations, respectively, whereas the RA through autonomous selfing measures for ES-flowered plants were −0.06, 0.04 and 0.12 in strongly ES-biased, ES-biased and strongly IS-biased populations, respectively. Overall, the total mean RA through autonomous selfing for both style types were 0.79 and 0.03, respectively.

Inbreeding depression

Seed numbers per flower did not differ between treatments and style types (Supplementary Table S3;

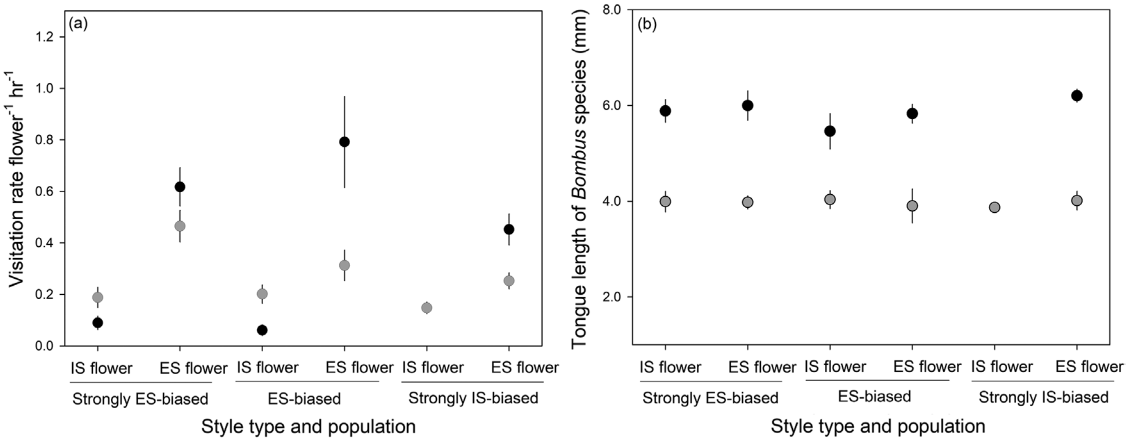

Figure 3: The visitation rate (flower⁻¹ hr⁻¹) (**a**) and the tongue length (**b**) of two focal *Bombus* species (i.e. *B. lepidus* and *B. friseanus*) to IS- and ES-flowered *Prunella. vulgaris* observed in strongly large-biased, large-biased and strongly small-biased populations. Gray and black symbols denote *B. lepidus* and *B. friseanus*, respectively. Data statistics of visitation rate are presented in Table 2 and Supplementary Table S2.

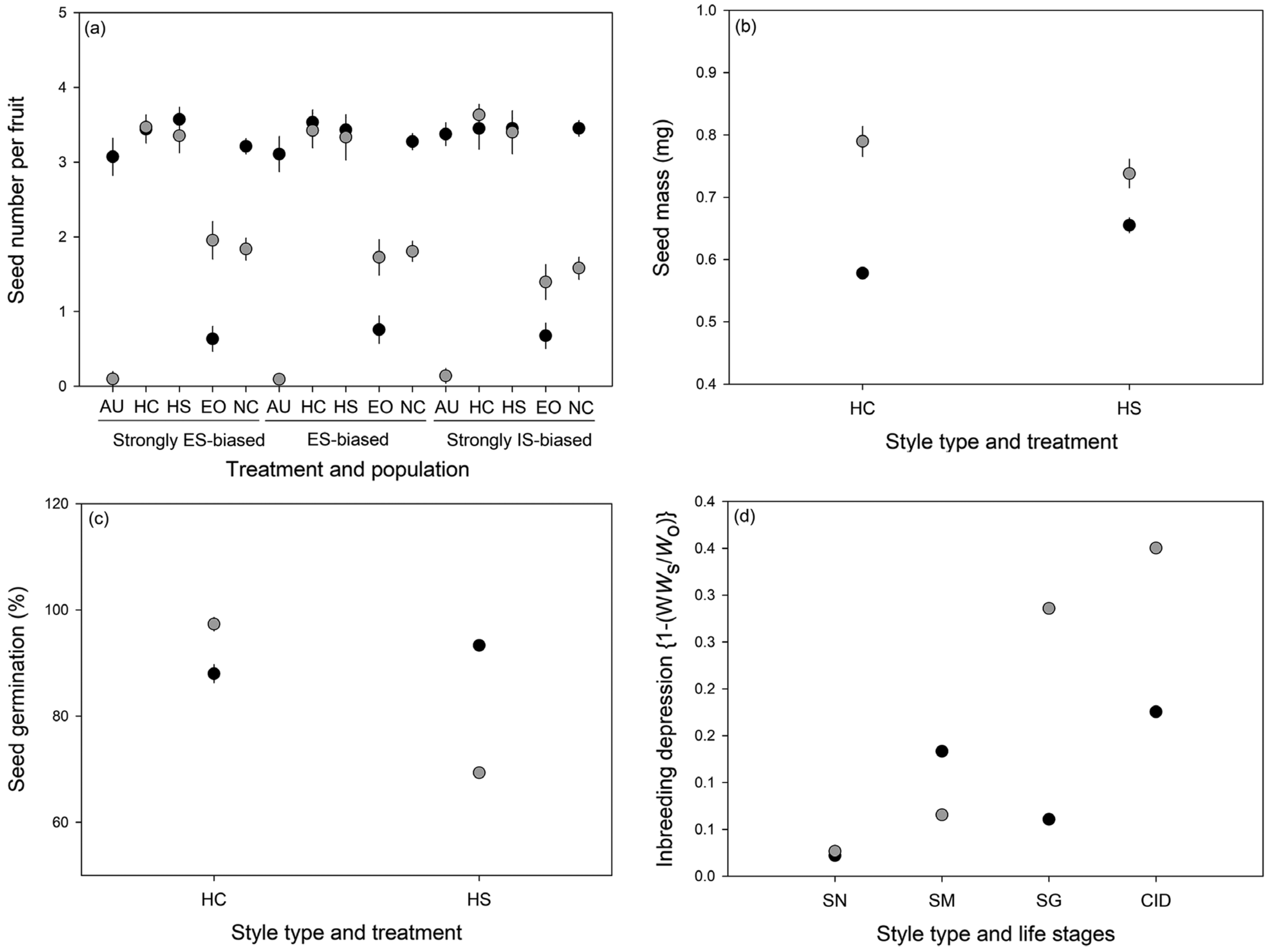

Figure 4: Seed number per flower of IS- and ES-flowered *Prunella vulgaris* subjected to different pollination treatments from the strongly ES-biased, ES-biased and strongly IS-biased populations (**a**). The comparisons of seed mass (**b**), seed germination rate (**c**) and inbreeding depression (**d**) between hand-crossed and hand-selfed pollinations for each style type. AU = autonomous self-pollination; HC = hand-crossed pollination; HS = hand-selfed pollination, EO = emasculated styles of flowers and left open for pollinators; NC = flowers were left undisturbed for open pollination, as a natural control; SN = seed number; SM = seed mass; SG = seed germination and CID = cumulative ID. Black and gray bars denote the seed number or the progeny of IS- and ES-flowered plants, respectively. Data statistics are presented in Table 2 and Supplementary Table S3.

Fig. 4a), and their interaction was not significant, either (Wald chi-square = 0.351, df = 1, $P > 0.05$ for style type; Wald chi-square = 0.205, df = 1, $P > 0.05$; Wald chi-square = 0.001, df = 1, $P > 0.05$ for style type × treatment interaction). Seed mass differed between style types (Table 2), where ES-flowered plants had a higher seed mass than IS-flowered plants for both hand-pollination treatments (Supplementary Table S3; Fig. 4b). There was no significant difference in seed mass between the treatments (Table 2). However, the treatment × style type interaction was significant (Table 2), because IS-flowered plants had a higher seed mass in the hand-selfed treatment than in the hand-crossed treatment, whereas ES-flowered plants had a lower seed mass in the hand-selfed treatment than in the hand-crossed treatment (Supplementary Table S3; Fig. 4b). Seed germination rates did not differ between style types, but differed between treatments, and their interaction was also significant (Table 2). The hand-selfed treatment had a higher germination rate than hand-crossed treatment in IS-flowered plants, whereas the hand-crossed treatment had a higher seed germination rate than the hand-selfed treatment for ES-flowered plants (Supplementary Table S3; Fig. 4c). The ID of seed number, seed mass and seed germination for IS-flowered plants were 0.133, 0.022 and 0.061, respectively, whereas those for ES-flowered plants were 0.065, 0.027 and 0.288, respectively (Fig. 4d). The cumulative ID for IS- and ES-flowered plants were 0.18 and 0.35, respectively, both of which were lower than 0.5 (Fig. 4d).

DISCUSSION

Our results indicate that IS-flowered plants of subalpine *P. vulgaris* had smaller flowers, fewer open flowers and pollen grains per flower and a lower nectar volume and sugar concentration than ES-flowered plants, but flower longevity per flower did not significantly differ. The flowering time and peak bloom of IS-flowered plants was approximately 2 weeks later than that of ES-flowered plants, while flowering length in the strongly IS-biased population was approximately 2 weeks shorter than that in the strongly ES-biased and the ES-biased populations. Flowering length did not differ between the strongly ES-biased population and the ES-biased population. As predicted, a lower flower visitation rate was found in IS-flowered plants and strongly IS-biased populations when compared with ES-flowered plants and ES-biased populations. The capacity for autonomous selfing was much greater for IS-flowered plants than for ES-flowered plants. However, RA through autonomous selfing did not differ between populations within style type, suggesting that a low visitation rate was sufficient to produce adequate seed numbers in ES-flowered plants in a strongly IS-biased population. Notably, the cumulative ID of the two style types of *P. vulgaris* was below 0.5, the threshold below which selfing should evolve. However, the ID of IS-flowered plants was even lower than that of ES-flowered plants, suggesting a higher purging of deleterious alleles for IS-flowered plants. Below, we discuss the results, acknowledge the limitations of this study and highlight potential future directions.

Floral trait and flower visitation rate differences between IS- and ES-flowered plants

Intraspecific variations in floral traits play a vital role in shaping the foraging behavior of flower visitors. Regardless of the specific mechanism, plants with larger flowers and greater nectar and pollen production rates, frequently attract more insect visitors because of increased visibility (Hernández-Villa *et al.* 2020; Mu *et al.* 2011). In this study, ES-flowered *P. vulgaris* individuals had not only larger flowers but also a greater number of open flowers, nectar volume, sugar concentration and pollen production than IS-flowered *P. vulgaris* individuals in all populations. Flower visitation rates were much lower in IS-flowered plants than in ES-flowered plants in all populations in the study, supporting our prediction. Floral trait and visitation rate differences in the two style types of *P. vulgaris* shown here are consistent with those reported in previous studies (Chen and Zhao 2019; Grindeland *et al.* 2005; Mu *et al.* 2011).

Recent studies have questioned whether reduced flower visitation rates occur at lower population size than at higher population size, particularly where the population is dominated by small-flowered plants (Devaux *et al.* 2019; Elle and Carney 2003; Kennedy and Elle 2008). In this study, compared to the ES-biased populations, the visitation rate was significantly lower in the strongly IS-biased population, which also had the smallest population size and was dominated by IS-flowered plants with reduced floral traits. The results of this study predict that *P. vulgaris* should have produced ES-flowered plants in natural populations because of enlarged floral traits that attract more flower visitors, unless there are mechanisms to produce IS-flowered plants, such as autonomous selfing, which provides RA.

Evolution of autonomous selfing and the RA benefit

Providing RA through autonomous selfing when potential pollinators are absent is a primary mechanism underlying the evolution of autonomous selfing (Zhang and Li 2008; Zhang *et al.* 2014). A change in flower size as a result of a transition to selfing has been found to result in different pollinator visitation rates, which ultimately influences the RA benefit (Kenney and Sweigart 2016; Smith-Huerta and Huerta 2015). For example, *Collinsia parviflora* were found to have higher rates of autonomous selfing, lower pollinator visitation rates and greater RA, when compared to large-flowered *C. parviflora* (Elle and Carney 2003). Consistent with this result, we found that IS-flowered *P. vulgaris* produced seeds mostly through autonomous self-pollination and provided a high level of RA because of the relatively low flower visitation rate. In contrast, ES-flowered *P. vulgaris* produced seeds mostly through pollinator services. These results demonstrate a clear selective force to maintain the mixed mating system of the two style types of this species. In addition, our finding on the capacity for autonomous selfing is consistent with a prior study on this species, in which IS-flowered plants showed up to 96% seed set through autonomous selfing, whereas the seed set of ES-flowered plants was only 6% through autonomous selfing (Qu and Widrlechner 2011). However, Qu and Widrlechner (2011) did not mention whether the two style types had different flower sizes, coupled with other traits, and received different flower visitation rates under natural conditions. Thus, our results are unique in showing how the evolutionary transition from outcrossing to selfing and the RA benefit in *P. vulgaris* occur by affecting floral traits and flower visitors.

We predicted that the greatest RA through autonomous selfing would be found in the strongly IS-biased population if the visitation rate was low. As expected, the visitation rate was significantly lower in the strongly IS-biased population. Surprisingly, however, this did not result in differences in RA through autonomous selfing among populations. This is in contrast to Elle and Carney's (2003) report on populations of self-compatible *C. parviflora*, where RA increased as either the proportion of small-flowered plants increased or the flower visitation rate decreased. We propose two possible mechanisms that might influence the benefits of RA in *P. vulgaris*. Firstly, the number of seeds per flower in the Lamiaceae family is usually four, and each style type of *P. vulgaris* produces more than 1000 pollen grains per flower. It is possible that low visitation rates in the strongly IS-biased population were sufficient for fully setting seeds. Secondly, the size matching between the tongue length of pollinators and the flower size hypothesis might also influence the benefits of RA in *P. vulgaris*. For example, a previous study on the Japanese subalpine populations of *P. vulgaris* suggested that the flower-pollinator size match influenced both male and female fitness of this species, such that short-tongued bumblebees were more effective pollinators for small-flowered plants and long-tongued bumblebees were more effective pollinators for large-flowered plants (Kuriya *et al.* 2015). In the present study, we also found that short-tongued *B. lepidus* tended to visit IS-flowered plants more often, while long-tongued *B. friseanus* mostly visited ES-flowered plants, and that this was particularly pronounced in the strongly IS-biased population. We postulated that short-tongued bumblebees might be more effective pollinators for IS-flowered plants, and long-tongued bumblebees for ES-flowered plants, which thereby results in an increased seed production in the strongly IS-biased population. However, further investigation is needed to validate the pollination effectiveness of short- and long-tongued bumblebees.

Intraspecific variation in flowering time and length may also be important traits for determining the benefits of RA in *P. vulgaris*. The time limitation hypothesis predicts that small-flowered plants that favor autonomous selfing produce mature flowers quickly and have a shorter flowering length, which can subsequently increase the benefits of RA (Snell and Aarssen 2005). In this study, although flowering length did not differ between the two style types in ES-biased populations, the flowering length of IS-flowered *P. vulgaris* in the strongly IS-biased population was approximately 2 weeks shorter than that of ES-flowered plants of *P. vulgaris*. In addition, the flowering time and peak bloom of IS-flowered plants were approximately 2 weeks later than that of ES-flowered plants in all populations. This is in contrast to a previous study on *C. parviflora* (Elle and Carney 2003), where small-flower biased populations bloomed a month earlier than large-flower biased populations. These contrasting patterns in flowering time might be associated with seasonal variations in local pollinator activity (e.g. Kudo and Ida 2013), such that pollinators are scarce early in the flowering season in *C. parviflora* but late in the flowering season in *P. vulgaris*. Such phenological mismatch with flower visitors is associated with high RA through autonomous selfing in the late flowering IS-flowered *P. vulgaris*. Differences in the visitation rate among flower visitors should also affect the RA benefits. The visitation rate of *B. lepidus* was higher for IS-flowered *P. vulgaris* and that of *B. friseanus* was higher for ES-flowered *P. vulgaris*. However, the visitation rate of *B. lepidus* was still lower than that of *B. frisanus*, particularly in the strongly IS-biased population. Therefore, delaying flowering time may result in RA benefits for IS-flowered *P. vulgaris* while there was no time for producing in ES-flowered *P. vulgaris*. In addition, intraspecific variation in flowering time might be associated with plant adaptation to different abiotic factors (i.e. drought, altitude and wind speed). For example, a previous study in the Japanese subalpine populations of *P. vulgaris* suggested that small-flowered plants are more common at higher altitudes, while large-flowered plants are more common at lower altitudes (Kuriya *et al.* 2015), although exceptions do exist (Egawa *et al.* 2015). In our study, IS-flowered *P. vulgaris* also tend to be more common at higher altitudes with less dense vegetation, which are generally more exposed to the elements and winds than the other two populations (unpublished manuscript). In contrast, Elle and Carney (2003) also noted that small-flowered *C. parviflora* occurs in drier parts of the species' range in the British Colombia than large-flowered *C. parviflora*. Nevertheless, whether the adaptation of the two style types to different flowering times and lengths is solely due to pollinators and abiotic conditions cannot be determined without studying more populations with different environmental conditions throughout the entire flowering season.

Evolution of ID

Since the publication of Darwin's classic book (Darwin 1876), ID has been considered a key mechanism that opposes the evolution and benefits of autonomous selfing (e.g. Wang 2019). It has been suggested that decreased ID levels are more common in highly self-pollinated species than in cross-pollinated species because of the more frequent purge of deleterious alleles (Bartoš *et al.* 2020; Devaux *et al.* 2014). In this study, our results indicate that ID did not differ between seed numbers for both types of styles, that ID was higher in IS-flowered plants for seed mass and that ID was higher in ES-flowered plants for seed germination. In addition, cumulative ID in IS-flowered plants was lower than that of ES-flowered plants, showing a higher purging of deleterious alleles in IS-flowered plants. However, the cumulative ID for both style types was still lower than the threshold of 0.5. Therefore, ID should not counteract the benefits of selfing in *P. vulgaris*. It is also important to note that ID gradually increased over the life stages in ES-flowered plants, suggesting that pollinators strengthen the magnitude of ID to a large degree. The strength of ID may vary with environmental conditions and is often stronger in field conditions than in greenhouse conditions. Unfortunately, we measured the ID of the two style types only up to the seed germination stage in the controlled, favorable conditions of a growth chamber, without separating the populations for seed germination rate. Further investigations on IS- and ES-flowered plants should be conducted under natural conditions with different types of soils and environmental conditions throughout plant development to better gauge survival rates in a more realistic setting that accounts for potential environmental variation. Or experiments in a growth chamber/greenhouse could be conducted under different settings of environmental conditions that can be controlled and are exactly known.

CONCLUSIONS

The present study demonstrates that selection for differences in floral traits as a result of a change in mating systems appeared to result in different flower visitation rates, which influences the RA of autonomous in *P. vulgaris*. In summary, ensuring RA through autonomous selfing by reducing floral traits and flower visitation rates, and ID in IS-flowered plants and ensuring the outcrossing rate by increasing floral traits and flower visitation rates, and ID in ES-flowered plants may enhance the stability of the mixed mating system in these subalpine populations of *P. vulgaris*. Future studies should focus on the effects of various abiotic and genetic mechanisms on the pollinator community and mating systems within different ecosystems, explore how different pollinators may respond to plants with different flower sizes throughout the bloom period and determine the efficiency of different pollinators in transferring pollen within and between style types. This will deepen our understanding of the benefits of RA and mating systems for plants with variable floral traits, such as subalpine *P. vulgaris*.

Supplementary Material

Supplementary material is available at *Journal of Plant Ecology* online.

Table S1: Summary table of generalized linear models (GLMs) showing parameter estimates (standard error (SE)) of different factors: corolla length and width; flower longevity; open flower number per plant; pollen number; nectar volume; and sugar concentration of IS- and ES-flowered plants of *Prunella vulgaris* in the strongly ES-biased, ES-biased, and strongly IS-biased populations.

Table S2: Summary table of the GLMs showing parameter estimates (SE) of visitation rate and tongue length of *Bombus friseanus* and *B. lepidus* to IS- and ES-flowered plants in the strongly ES-biased, ES-biased, and strongly IS-biased populations.

Table S3: Summary table of GLMs showing parameter estimates of seed number per flower of autonomous self-pollination (AU), hand-crossed (HC), hand-selfed (HS), emasculated and left for open-pollination (EO), and natural control (NC) treatments for IS- and ES-flowered plants of *Prunella vulgaris* in the strongly ES-biased, ES-biased, and strongly IS-biased populations.

Figure S1: A schematic representation of the population characteristics, the distribution area of IS- and ES-flowered plants of *Prunella vulgaris*, and the 3 m × 3 m squares within each population.

Figure S2: Flowering time of IS- and ES-flowered *P. vulgaris* observed in the strongly ES-biased, ES-biased, and strongly IS-biased populations.

Figure S3: Relative frequency of corolla width and length of IS- and ES-flowered *P. vulgaris*.

Figure S4: Total flower visitors observed in IS- and ES-flowered *P. vulgaris* observed in strongly ES-biased, ES-biased, and strongly IS-biased populations.

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Conflict of interest statement. The authors declare that they have no conflict of interest.

Authors' Contributions

T.C.L., Y.W.D. and Y.P.Y. conceived and designed the study. T.C.L. prepared the manuscript for publication. T.C.L. and L.L.W. performed the field experiment. Y.W.D. and Y.P.Y. revised the manuscript. All authors have read and approved the final manuscript.

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