

The breeding system of *Hyacinthoides non-scripta* (Asparagaceae): assessing the role of geitonogamy with captive pollinators

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Abstract

Background and aims – In self-incompatible species, geitonogamous pollen deposition can result in stigma clogging, with negative effects on cross-fertilisation and subsequently reduced fruit and seed set. In this work, using laboratory and field experiments with pollinators in captivity, we describe the reproductive system of *Hyacinthoides non-scripta* and analyse the effect of the different mechanisms of pollen transfer, trying to quantify the effect of geitonogamy on the reproductive success of the species.

Material and methods – Using laboratory and field experiments with pollinators in captivity we evaluated the effect of the different types of pollen transfer.

Key results – The results show that *H. non-scripta* is a self-incompatible species and the pollen tubes in autogamous and geitonogamous pollination are rejected at the base of the style. In the field experiments, the presence of geitonogamous pollen on the stigma did not prevent subsequent fertilization by xenogamous pollen, but it reduced seed production.

Conclusion – This study provides evidence that natural rates of geitonogamy significantly reduce female fitness in plants with large numbers of flowers per inflorescence and show how experiments with captive pollinators can help quantify the intensity of this effect.

Keywords

geitonogamy, *Hyacinthoides non-scripta*, pollen tube growth, pollinators in captivity, self-incompatibility

INTRODUCTION

Even though mixed-mating systems are frequent in plants, outcrossing and self-pollination represent two alternative reproductive pathways with important genetic and evolutionary consequences (Goodwillie et al. 2005; Barrett 2010). Geitonogamy (pollination with pollen from another flower of the same plant) is the most widespread mode of self-pollination (Lloyd and Schoen 1992), and many hermaphroditic species experience geitonogamous pollination in natural conditions (de Jong et al. 1993). In self-incompatible species, geitonogamous pollen deposition can result in stigma clogging with negative effects on cross-fertilisation and subsequently reduced fruit and seed set (Ehlers 1999; Gross 2005). In self-compatible species, geitonogamy will result in

pollen discounting (pollen used in selfing reduces the pollen available for outcrossing) and outcross-pollen interference (self-pollen interferes with outcross pollen at the stigmatic surface) (Lloyd and Webb 1986; Lloyd and Schoen 1992; Klinkhamer and de Jong 1993; Holsinger and Thomson 1994; Finer and Morgan 2003).

In many species, the selfing component of mixed mating may represent a non-adaptive cost associated with the large floral displays required to attract animal pollinators. Rates of geitonogamy are expected to be correlated with the number of flowers per individual. In self-compatible species, significant amounts of selfed seed may arise from inter flower pollen transfer (geitonogamy) and provide little benefit to fitness because of strong inbreeding depression and pollen discounting (Barrett 2003). In self-incompatible species with flowers grouped

in inflorescences, negative effects of geitonogamy depend on the degree of floral synchrony within the inflorescence and synchrony between ramets in clonal plants (Eckert 2000). Additionally, the behaviour of pollinators (directionality, fidelity, etc.) can play an important role in geitonogamy effects. Consequently, the design and floral display of the inflorescences is seen as the result of a compromise between maximizing attraction to pollinators and reducing geitonogamy levels (Harder and Barret 1995; Harder et al. 2000; Finer and Morgan 2003).

Self-incompatibility is the most important mechanism used to prevent inbreeding. There are two major classes of self-incompatibility at the genetic level: gametophytic self-incompatibility and sporophytic self-incompatibility. In gametophytic self-incompatibility, the incompatibility phenotype of the pollen is determined by its haploid genome, whereas in sporophytic self-incompatibility the pollen exhibits the incompatibility phenotype of its diploid parent. Members of the Brassicaceae, Asteraceae, and Convolvulaceae are well-known examples of the sporophytic type, and those of the Solanaceae, Asparagaceae, Poaceae, and Fabaceae are of the gametophytic type.

Our aim is to describe the breeding system and the consequences of geitonogamy in *Hyacinthoides non-scripta*, using laboratory and field experiments. Traditionally, studies on the reproductive consequences of geitonogamy are based on manual transfers of pollen from a flower of the same plant, accompanied by emasculation of the focal flowers and subsequent bagging to prevent the arrival of xenogamous pollen (Kearns and Inouye 1993). However, this can lead to an erroneous assessment of its effects because it does not adequately reproduce the behaviour of pollinators. We used a design with captive pollinators that is more appropriate since it reproduces the natural conditions in which visits are made by insects, facilitates the monitoring of visits, and prevents the interference of pollen from other species.

Specifically, we ask the following questions: (1) Is *H. non-scripta* a self-incompatible species? If so, (2) what is the effect of the different mechanisms of pollen transfer on the pollen tube growth and reproductive success of the species? Additionally, (3) in a field experiment with captive pollinators, we assess the extent to which the reproductive success of the species is affected by pollinator behaviour (xenogamous versus geitonogamous pollen transfer).

MATERIAL AND METHODS

Species description and study area

Hyacinthoides non-scripta (L.) Chouard ex Rothm. (Asparagaceae), commonly called English bluebell, is a bulbous perennial that is native to open woodland areas of western Europe. Racemes are unilateral (one-sided) with 4–16 flowers. Flowers are made up of six perianth

segments (tepals), usually violet-blue, but rarely white. The flowers are bisexual and contains six creamy-white anthers of unequal length and a persistent style. The ovary is made up of three carpels with about eight ovules each.

Hyacinthoides non-scripta has both sexual and asexual reproduction through the formation of small bulbs (Wilson 1958). Regarding this, Corbet (1998) noted that there is a degree of self-incompatibility, and cross-pollination produces more fruit and seeds than self-pollination (see also Wilson 1958). The species blooms between May and June and is pollinated in the area by Hymenoptera (*Bombus hortorum* (Linnaeus, 1761), *B. pratorum* (Linnaeus, 1761), and *B. terrestris* (Linnaeus, 1758)) and Lepidoptera (*Aglais urticae* (Linnaeus, 1758), *Aporia crataegi* (Linnaeus, 1758), and *Gonepteryx rhamni* (Linnaeus, 1758)). In a population census (120 minutes of observation and 270 visits), 14% of insects observed visited a single flower on each plant (resulting in xenogamy), while 86% made more than one visit per plant successively (with a potential for geitonogamy) (Javier Guitián unpublished data).

The study was conducted in the Alto do Couto (1400 m a.s.l.), in the Sierra de O Caurel (Lugo province), located in the NW of the Iberian Peninsula.

Experimental design

Plant breeding system

In late May and early June 2018, we collected 62 plants in population and moved them to the laboratory where they were kept under controlled temperature (day: 20°C; night: 14°C) and natural light conditions in the absence of pollinators. Plants were watered on alternate days throughout the experiment.

The treatments were:

1. Cross-pollination/facilitated xenogamy (hereafter called the xenogamy treatment) (n = 125 flowers on 21 plants). To avoid self-pollination, we emasculated each flower at the beginning of anthesis when the stamens were still immature. The stigma was pollinated with a brush, using pollen from other plants. This hand pollination was carried out in stages depending on when the flowers bloomed, and then repeated once a day for 9 days until all the flowers were open.
2. Self-pollination/facilitated geitonogamy (hereafter geitonogamy) (n = 138 flowers on 22 plants). During these 9 days, flowers were self-pollinated with brushes, using pollen from another flower on the same plant. To avoid possible contamination with pollen from other individuals a different brush was used for each plant.
3. Control/autonomous self-pollination (hereafter autogamy) (n = 108 flowers in 19 plants). Flowers were left untreated under the same conditions in the absence of pollinators.

To calculate reproductive success, 46 plants were used: 15 in the xenogamy treatment, 17 in the geitonogamy

treatment, and 14 autogamous controls, with a total of 98, 110, and 83 flowers, respectively; 16 plants died during the experimental period. At the time of fruiting, the fruits, seeds, and ovules were counted per treatment.

We calculated the Self-Compatibility Index (SCI) to describe the breeding system (as in Lloyd and Schoen 1992). SCI is assessed as the average seed set for facilitated selfing divided by the average seed set for facilitated xenogamy, and it provides information about the self-compatibility of the species. SCI values are standardized to yield values between 0 and 1 (Lloyd and Schoen 1992).

To assess the occurrence of self-pollination and fertilisation, we calculated the Auto-Fertility Index (AFI) by dividing the seed set for spontaneous autogamy by the seed set for facilitated xenogamy. AFI ranges from zero to above one and gives information about the degree of spontaneous autogamy of the species. Low levels of auto-fertility do not necessarily imply lack of self-pollination under natural conditions (Lloyd and Schoen 1992).

Pollen tube growth

To examine the pollen tubes, we removed several pistils in each treatment 7–9 days after anthesis. The total number of pistils examined was 71:27 in the geitonogamy treatment, 22 in the xenogamy treatment, and 22 in the autogamous controls. We used the epifluorescence technique with aniline blue. The excised pistils were kept in ethanol (70%) for more than 24 hours after soaking in NaOH for 24 hours and stained with aniline blue (0.1% by 0.1 mol/L K_2HPO_4) also for 24 hours (Kearns and Inouye 1993). After mounting on slides the pistils were viewed using a fluorescence microscope and photographed for further analysis. For each stigma the presence of pollen grains and pollen tubes were recorded. For pollen tubes, we counted the number of tubes in three locations: stigma, middle of the style, and at the base of the style. The counts were conducted with the aid of the image analysis program Image-Pro Plus (Media Cybernetics).

Pollen transfer effects

To study the effect of pollen transfer we performed an experiment using bumblebees in captivity. We built a cubic metallic structure (2 m × 2 m × 2 m) covered with tulle, in a plot with plants of *H. non-scripta*, before the beginning of flowering; all plants inside the structure were labelled with metal labels. The flowers were counted and their position on the inflorescence noted. In the vicinity of plots, we located nests of *Bombus pratorum*, the most frequent pollinator. Bumblebees were captured with a butterfly net at the exit of the nest. Every morning one bumblebee was released into the tulle structure and recaptured at sunset; the tulle remained closed at night, repeating the process over seven days (total 49 hours of observations). During the day, each visit to a plant and flower and the origin/destination of the bumblebee was recorded. We classified the flowers as having received: (1) a single geitonogamous visit; (2) a single xenogamous visit;

(3) several geitonogamous + subsequent xenogamous visits; (4) several xenogamous visits; (5) no visits.

The tulle structure remained in place until fruiting time and fruits were collected and transported to the laboratory, where fruit set and seed-ovule ratios were determined.

Data analysis

Differences in fruit production among pollen transfer treatments were analysed with the nonparametric Kruskal-Wallis test; the Bonferroni correction was applied in pairwise comparisons. Treatment effects in seed production were investigated using a nested ANOVA with Tukey post-test comparisons, with cases (plants) nested in treatments, treatment as a fixed factor and plant within treatment as a random factor. Spearman's rank correlation coefficient was used to identify and test the strength of the relationship between the number of flowers and the number of geitonogamous visits. The effect of the type of pollen transfer on the seed ovule ratio was analysed using a GLM. All analyses were performed using IBM SPSS (IBM 2012).

RESULTS

Plant breeding system

Plants in the geitonogamy and xenogamy treatments produced fruit, but those in the autogamy (control) treatment did not. Analyses showed significant differences between treatments in fruit set: 0.3 ± 0.28 and 0.07 ± 0.13 (mean \pm SD) in the xenogamy and geitonogamy treatments respectively (Kruskal-Wallis; $H = 17.87$; $p = 0.0001$) (Fig. 1).

Significant differences were found in seed set among the xenogamy, geitonogamy and autogamy treatments (Nested ANOVA; $F = 15.29$; $p < 0.001$). Both geitonogamy and autogamy treatments had similar seed set ($p = 1$), close to zero (Fig. 1).

The value of the self-incompatibility index (SCI = 0.05) shows that the species is clearly self-incompatible. The value of the auto-fertility index was zero.

Pollen tube growth

In the autogamy (control) treatment, the proportion of pollen tubes that germinated on the style was 0.86 ± 2.83 ; there were no pollen tubes in the middle and at the base of the style.

All flowers in the samples of geitonogamy and xenogamy treatments showed pollen tube growth. In the geitonogamy treatment, pollen tube growth was inhibited the most in the basal half of the style (Fig. 2). The number of germinated pollen grains (quantified as the number of pollen tubes at the start) varied greatly

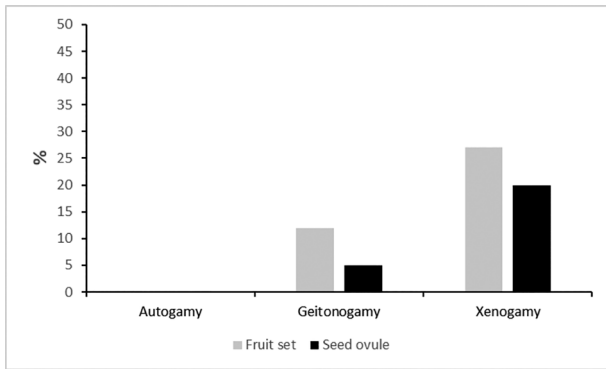


Figure 1. Levels of fruit set and seed set in the different pollen treatments in *Hyacinthoides non-scripta*.

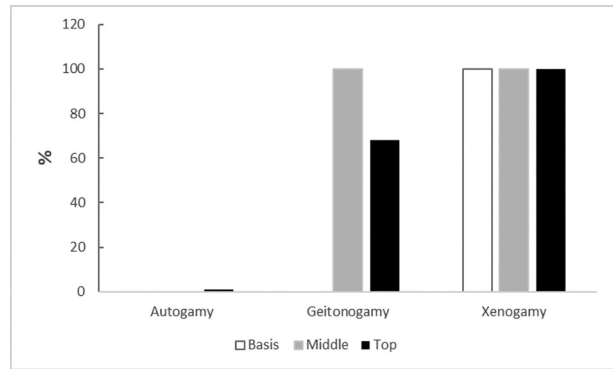


Figure 2. Proportion of pollen tubes surviving in different regions of the style in the treatments in *Hyacinthoides non-scripta*.

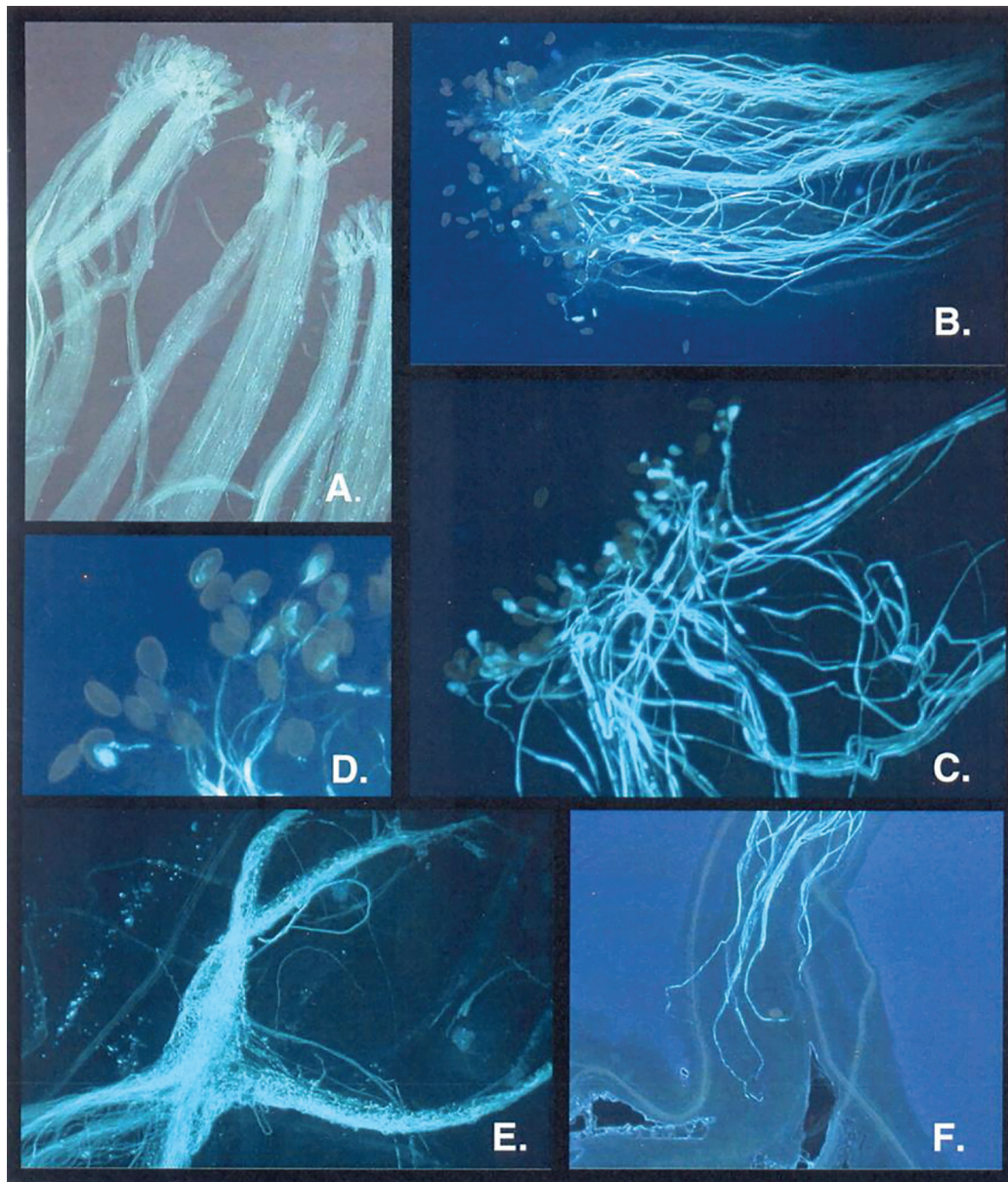


Figure 3. Fluorescence microscope images of different pollen-pistil interactions in *Hyacinthoides non-scripta* pollination. **A.** Pistils without pollen tubes from the autogamy treatment. **B–C.** Pistils with well-developed tubes from cross-pollination treatment. **D.** Germinated pollen grains. **E.** Base of style in cross-pollination treatment. **F.** Base of style in autogamy treatment showing the progressive elimination of pollen tubes.

Table 1. Effect of number of xenogamous visits in seed ovule ratio of *Hyacinthoides non-scripta*.

Effect	d.f.	SS	F	p
Intercept	1	21.940	197.465	0.000
N° of visits	1	0.6050	5.45	0.021
Error	98	10.888		

Table 2. Effect of visit type in seed-set in *Hyacinthoides non-scripta*.

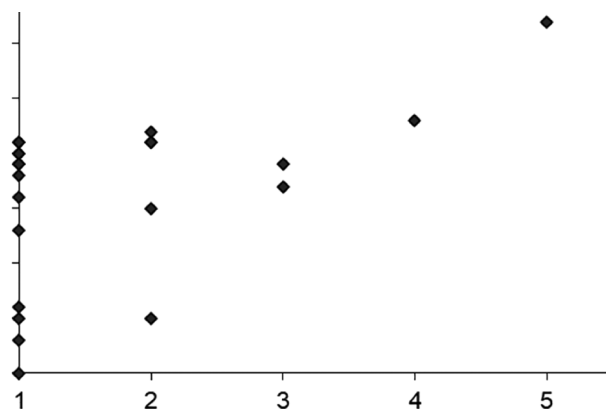
Effect	d.f.	SS	F	p
Intercept	1	4.839	47.651	0.000
Visit type	3	0.816	2.6800	0.050
Error	52	5.281		

among samples (48.06 ± 35.24) (mean \pm SD). The number of tubes decreased towards the base (middle: 37.78 ± 27.75 ; base: 1.66 ± 2.37). The proportion of rejected tubes in the style was 21% and 96% in the middle and at the base respectively, versus the number at the top (Fig. 2). In the xenogamy treatment, pollen tubes had no difficulties reaching the base of the style, forming three groups, each directed towards a carpel. Figure 3 shows the different behaviour of pollen tubes in the pollination treatments.

Pollen transfer effects

In experiments with captive pollinators, the number of visits increased with the number of flowers (Spearman rank correlation 0.48; $p = 0.08$, marginally significant). Flowers without visits did not produce any fruit.

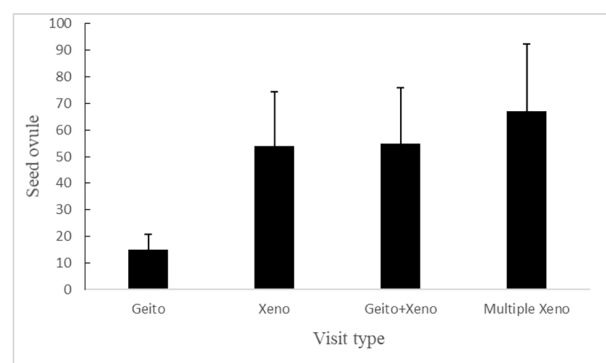
The seed-ovule ratio depended on the number of xenogamous visits (Table 1, Fig. 4). The results show that geitonogamy had a negative effect on seed set, but a single visit with self-pollen did not prevent subsequent fertilisation by xenogamous pollen. Flowers that received only one or more xenogamous visits had a seed-ovule ratio of 0.67 ± 0.3 , compared to 0.48 ± 0.34 in flowers receiving a visit with geitonogamous pollen followed by a visit with xenogamous pollen (Table 2; Fig. 5).

**Figure 4.** Relationship between the number of xenogamous visits and seed set in *Hyacinthoides non-scripta*.

DISCUSSION

Hyacinthoides non-scripta is a species pollinated by Hymenoptera that regularly make visits between flowers on the same plant, transferring self-pollen (geitonogamy). The analysis of the reproductive system in the laboratory and the values of the SCI and AFI indices show that the species is self-incompatible and only manual transfer of outcross pollen produced seeds. This result contrasts with those obtained by Corbet (1998) in the UK, which show a certain degree of self-compatibility with a low number of initiated seeds per fruit in self-pollinated (as opposed to open- or cross-pollinated) fruits.

In *H. non-scripta*, pollen rejection from the same genotype does not occur at the level of the stigma, since a high percentage of pollen grains develop pollen tubes; generally, these are arrested in the basal part of the stigma (gametophytic self-incompatibility, GSI). Sage et al (2001) showed that within the monocotyledons, self-incompatibility is frequent and can operate at different levels depending on the species. The most common type of self-incompatibility is gametophytic SI (GSI). Gibbs (2014) noted that GSI and late-acting self-incompatibility (LSI) are present in the Asparagaceae. The latter type, in which the incompatibility barriers are present in the ovary, has been documented in *Clintonia borealis* (Aiton) Raf. (Dorken and Husband 1999), *Blandfordia grandiflora*

**Figure 5.** Seed set in different pollination modes in *Hyacinthoides non-scripta*.

R.Br. (Ramsey 1993), and *Narcissus triandrus* L. (Sage et al. 2001) and dominates in monocots.

In the field experiment with *Bombus* in captivity, the transfer of geitonogamous pollen in *H. non-scripta* did not produce seeds. A single visit with self-pollen did not prevent the subsequent fertilisation by xenogamous pollen, but it reduced seed production by a third. Similar results have been obtained in the field with the self-incompatible *Polemonium viscosum* Nutt., in which self-pollen on a stigma reduced germination of subsequently applied compatible pollen by 32% and reduced seed set by 40% (Galen et al. 1989). In self-incompatible *Ipomopsis aggregata* (Pursh) V.E. Grant, self-pollen germinates, forms a pollen tube, and penetrates ovules, which subsequently abort. Compared with flowers pollinated only with outcross pollen, the application of self- and subsequent cross-pollen reduced seed set by 42% (Waser and Price 1991). Thus, pollination with self-incompatible pollen as the result of geitonogamy may interfere with cross-pollen, resulting in reduced seed set. In many species, especially those that are self-incompatible like *H. non-scripta*, prior or simultaneous deposition of pollen results in pollen limitation by stigma clogging, probably the most widespread cause of limitation in seed production (Aizen and Harder 2007).

In animal-pollinated species like *H. non-scripta*, the abundance and behaviour of pollinators and their interaction with floral design and display are the primary determinants of the relative frequency of geitonogamous selfing (see Corbet 1999). Because geitonogamy is almost never advantageous, it has most often been viewed as a nonadaptive by-product of floral display (Lloyd and Schoen 1992; Eckert 2000). A high degree of geitonogamy may lead to low fruit set because with every subsequent flower visit, the number of cross-pollen grains adhering to the body surface of the pollinator diminishes.

The “plant’s dilemma hypothesis” proposes that evolution of inflorescence size is driven by selection for pollinator attraction but constrained by higher rates of geitonogamy experienced by larger inflorescences (Klinkhamer and de Jong 1993; Finer and Morgan 2003). Our study provides more evidence that natural rates of geitonogamy can have a significant cost for female fitness.

CONCLUSION

This study provides evidence that natural rates of geitonogamy significantly reduce female fitness in *Hyacinthoides non-scripta* plants with large inflorescences and show how experiments with captive pollinators can help quantify the intensity of the effect. Further studies should determine to what extent the population structure and the ecological context influence the balance between geitonogamous selfing and outcrossing and its consequences in natural conditions.

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