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RESEARCH ARTICLE

REVISED Ecological implications of reduced pollen supply in the alpine: a case study using a dominant cushion plant species [version 2; peer review: 3 approved]

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Abstract

The reproductive assurance hypothesis states that self-incompatible female plants must produce twice the number of seeds relative to their self-compatible hermaphroditic counterparts to persist in gynodioecious populations. This is a viable life-history strategy, provided that pollination rates are sufficiently high. However, reduced pollination rates in alpine plants are likely due to climate induced plant-pollinator mismatches and general declines in pollinators. Using a gynodioecious population of the dominant plant *Silene acaulis* (Caryophyllaceae), we tested the reproductive assurance hypothesis and also the stress gradient hypothesis with a series of pollinator exclusion trials and extensive measurements of subsequent reproductive output (gender ratio, plant size, percent fruit-set, fruit weight, seeds per fruit, total seeds, seed weight, and seed germination). The reproductive assurance hypothesis was supported with female plants being more sensitive to and less likely to be viable under reductions in pollination rates. These findings are the first to show that the stress gradient hypothesis is also supported under a gradient of pollen supply instead of environmental limitations. Beneficiary abundance was negatively correlated to percent fruit-set under current pollen supply, but became positive under reduced pollen supply suggesting that there are important plant-plant-pollinator interactions related to reproduction in these alpine plant species.

Keywords

alpine, cushion plant, pollination, climate induced mismatch, pollinator decline

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REVISED **Amendments from Version 1**

We have addressed all suggestions made by the three referees. No major findings have changed but the flow and clarity of the paper has been improved.

See referee reports

Introduction

Two future scenarios in which pollination rates may be directly reduced include pollinator declines (Potts *et al.*, 2010) and plant-pollinator mismatch (Hegland *et al.*, 2009). Recently there has been concern over general global trends of reduced pollinator species abundance and diversity that are both predicted to reduce pollination rates to plants (Memmott *et al.*, 2007; Potts *et al.*, 2010). Climate induced plant-pollinator mismatch can reduce pollination rates by creating a temporal mismatch in pollinator emergence and plant flowering times (Hegland *et al.*, 2009). Pollinator emergence is regulated by temperature, whereas plant bloom time is regulated by photoperiod (Hegland *et al.*, 2009). If climate warming shifts pollinator emergence but not plant bloom time, then a temporal mismatch between plants and pollinators occurs (Hegland *et al.*, 2009). This scenario is likely more pronounced in alpine and polar environments that are experiencing a more rapid increase in annual temperature than the global average (very high confidence; Intergovernmental Panel on Climate Change [IPCC], 2013). Conceivably, both of these reductions in pollination rates occur simultaneously and thus adaptability of different sexual morphs in alpine plants can be an important consideration in predicting responsiveness and variation in reproductive output.

Hypotheses associated with pollen availability in alpine environments are controversial. It has been assumed that pollination rates are inherently low in alpine environments (Larson & Barrett, 2000; Totland & Sottocornola, 2001; Torres-Díaz *et al.*, 2011). This is attributed to low temperatures, overcast conditions, strong winds, and relatively unpredictable weather being challenging for insect pollinators (Körner, 1999). These harsh conditions generally lead to lower pollinator diversity, abundance, and activity in alpine ecosystems relative to milder ecosystems (Kevan, 1972; Primack, 1978; Moldenke & Lincoln, 1979; Arroyo *et al.*, 1982; Primack, 1983; Billings, 1987; Totland, 1993). Alternatively, pollination rates can increase with elevations suggesting adequate pollen availability under current conditions (Arroyo *et al.*, 1985; Arroyo & Squeo, 1990; Bingham & Orthner, 1998; Utelli & Roy, 2000).

The reproductive assurance hypothesis (RAH) and the stress gradient hypothesis (SGH) are thus highly relevant hypotheses to explore in the alpine. The RAH proposes that when pollen supply is low, self-compatible plants are favored over self-incompatible plants (Lloyd, 1992; Lloyd & Schoen, 1992). This is because self-compatible plants create their own pollen thereby being more adapted to low or variable pollination rates (Larson & Barrett, 2000; Muñoz & Arroyo, 2006; García-Camacho & Totland, 2009). Further, it has been proposed that self-compatible plants are less likely to become extinct if pollinators drastically decrease or disappear from a given system (Richards, 1997; Morgan *et al.*, 2005). Therefore, self-compatible plants may become favored in the future if pollen supply declines. The SHG is also an important ecological theory to consider in the possibility of reduced pollination rates and therefore reduce pollen supply. The SHG states that facilitation between two plant species is more common when resources are limited (Bertness & Callaway, 1994). Typically, the stress gradient hypothesis is tested using a gradient of environmental condition such as altitude (Cavieres & Badano, 2009) or moisture (Cavieres *et al.*, 2006), but has not been applied to the concept of pollen supply limitations which could be categorized as a quantifiable limiting resource (Maestre *et al.*, 2009). Taken together, these sets of ecological theories provide a solid platform to build pollen limitation studies upon and provide a set of potential ecological drivers that can help predict pollination rate changes in the alpine.

Here, we use a gynodioecious population of *Silene acaulis* to assess the sensitivity of different genders to pollen limitation and the potential effect of beneficiary plant species on these cushions. We test the following predictions associated with the reproductive assurance hypothesis: (1) that the reproduction of self-incompatible plants is more sensitive to reduced pollen deposition than that of self-compatible plants and (2) self-incompatible plants will be less viable under experimentally reduced pollen deposition. In doing so, we also explore whether pollen supply can be viewed as a resource stress within the stress gradient hypothesis. Specifically, we predict that facilitation between cushions and beneficiaries to increase when pollen supply is reduced.

Materials and methods

Study species

S. acaulis (L.) Jacq. (Caryophyllacae), commonly known as moss campion, is a common long-lived evergreen cushion that is found throughout the northern hemisphere (Hitchcock & Maguire, 1947). Each plant has a single strong taproot, and there is no clonal reproduction (Morris & Doak, 1998). Small pink flowers can be abundant. *S. acaulis* is visited by bumblebees (Shykoff, 1988; Shykoff, 1992; Marr, 1997; Delph *et al.*, 1999; Delph & Carroll, 2001), moths, beetles, ants (Marr, 1997; Delph & Carroll, 2001), flies (Totland, 1993; Delph & Carroll, 2001), butterflies and *Osmia* bees (Reid & Lortie, 2012). *S. acaulis* is a nurse plant species that, like many other cushion forming plants, benefits other plant species (called beneficiaries) by reducing abiotic stress (Arroyo *et al.*, 2003; Bertness & Callaway, 1994; Callaway & Walker, 1997; Cavieres *et al.*, 2006). Recent beneficiary removal studies suggest by facilitating the beneficiaries, cushions bear a cost in reduced reproductive success (Cranston *et al.*, 2012; Schöb *et al.*, 2013).

S. acaulis is sexually polymorphic (Hitchcock & Maguire, 1947) with this study population of *S. acaulis* is gynodioecious with plants that only have hermaphrodite flowers and other that only have female flowers. Female flowers have three styles with stigmatic lobes and hermaphrodite flowers have ten stamens. Male-sterility in female morphs of *S. acaulis* is predominantly under nuclear-cytoplasmic control (Delph & Carroll, 2001); the gene for male-sterility is passed on through the female gamete (Lewis, 1941). In addition to *S. acaulis,* the flowering plant species *Antennaria alpina, Antennaria* sp., *Arnica* sp., *Carex* sp., *Erigeron* sp., *Luzula* sp., *Phacelia sericea, Phlox diffusa, Phyllodoce* sp., *Poa alpina*, *Potentilla diversifolia, P. heptaphila, P. villosa, Ranunculus eschsoltzii, Salix* sp., *Saxifraga bronchialis,* and *Solidago multiradiata* were present at relatively high densities on and around the cushions.

Study site

The experiments were conducted on Whistler Mountain in British Columbia 50°03′31.68″N, 122°57′22.53″W, 2168m elevation), Canada during the snow-free season from June 26th to August 29th, 2010. This area is classified as alpine tundra with 10-months of snow cover a year (Pojar *et al.*, 1987). A total of 271 *S. acaulis* plants were measured. Three *S. acaulis* plants were excluded from the study because they were infected with the pollinator-transmitted anther smut-fungus *Microbotryum violaceum* that renders the flowers of both genders sterile (Baker, 1947; Alexander & Antonovics, 1988; Hermanutz & Innes, 1994; Marr, 1997).

Treatments

To experimentally reduce plant pollination, plants were covered with mesh and hand pollinated three times over the duration of the snow-free season. Before bud-burst, 60 *S. acaulis* plants were covered with fine cloth mesh to prevent pollinators contacting the flowers (Donnelly *et al.*, 1998). As plant gender was unknown when initially covering, 60 plants were covered to ensure that there would be 20 replicate plants of each gender. Plant gender was established after bud-burst, and at that time, plants were randomly assigned a treatment and marked with a unique identification code. Reduced pollination treatments were conducted on 20 hermaphrodite and 20 female plants. The first 20 female and hermaphrodite plants found where used as replicates with the additional 20 plants being uncovered. The 40 plants (20 of each gender) selected for the reduced pollination treatments remained covered with mesh for the entire flowering season to exclude all insect pollination. It is assumed that plants that are covered and hand pollinated three times receive less pollen than the plant open to natural pollination during the 33 snow-free days.

All flowers of the reduced-pollination treatment plants were hand pollinated with pollen collected from *S. acaulis* plants within 10 m from the treatment plants. Hermaphrodite flowers with mature anthers were collected in the morning of the hand pollination days. Pollen was then applied using small paintbrushes or by directly touching the anthers to the stigmas of all the treatment-plant flowers. We found direct contact of the anthers to the stigmas the most effective method of hand pollination. The exact amount of pollen applied to each flower at each hand-pollination event was not quantified. Hand pollination was repeated three times between July 20th and August 1st, 2010.

Reproductive output measures

Reproductive output measures were collected from the 40 hand-pollinated treatment plants as well as 231 naturally pollinated *S. acaulis* plants of which 124 were female and 107 were hermaphrodites. These measures include total number of flowers, total number of fruits, percent fruit-set, seeds per fruit, fruit weight, and seed weight. Percent germination was calculated on a subset of 60 plants, including the 40 treatment plants and 20 naturally pollinated plants.

Total number of flowers was counted during fruit collection including both successfully and unsuccessfully pollinated flowers. Fruit were collected when mature but not yet dehiscing so that the seed remained in the fruit capsule. This occurred between August 11th and 25th. All fruits were place in small-labeled paper envelopes and allowed to dry at room temperature to avoid decomposition. Percent fruit-set was calculated using the measures of total number of fruit and total number of flowers. Mean fruit weight (g) was calculated by averaging the weight of five randomly selected fruits per plant. These five fruits were dissected and the seed counted. Mean seed number per fruit was calculated from the seed counts. Total seed number per plant was estimated by multiplying total fruit with mean seed number per fruit. Mean seed weight (mg) was calculated by averaging the weight of 10 randomly selected seeds per plant. All weighing was done to four significant digits. When a plant produced less than five fruits or 10 seeds, the average was based on the maximum number of fruit or seed produced. Weighed seed was stored separately and cold stratified at 4°C for two months. A test germination trial was conducted with limited success likely because the cold stratification was not sufficient. Therefore, seeds were then stored at 0°C for two additional months in preparation for germination trials.

Germination trials were conducted on the weighed and cold stratified seeds from the 40 *S. acaulis* plants in reduced pollination treatments and the remaining 20 labeled plants that were left open to natural pollination. Growth chambers were set to standard optimum growing conditions of 20°C and light for 12 hours then 10°C and dark for the remaining 12 hours of the day (Baskin & Baskin, 1998). Relative humidity was set to 90%. The 10 seeds from an individual plant were placed on a labeled filter paper in a Petri dish. Seeds were checked weekly for three months, after which germination is rare (Milbau *et al.*, 2009). Germination was considered to have occurred when the radical breaks open the seed (Milbau *et al.*, 2009). Germinated seeds were removed to speed-up counting during the subsequent weeks and reduce counting errors (Milbau *et al.*, 2009). Percent germination was expressed as the fraction of total number of germinated seeds with respect to the total number of seeds per Petri dish.

Cushion area and floral density were measured because of their possible effect on reproductive output. Cushion area was defined by the external boundary of vegetation and calculated as an ellipse with the formula,

$$\text{cushion area} = (a/2)*(b/2)*\pi$$

where a is the longest diameter of the plant and b the diameter perpendicular to a. We calculated floral density by dividing total flower number by cushion area.

Beneficiary abundance

To test if facilitation became more common under reduced pollen loads (stress gradient hypothesis) we measured beneficiary abundance on all cushion plants. Beneficiary abundance is the total number of individual plants living on the cushions.

Data analysis

To assess the sensitivity of female and hermaphrodite reproductive output under reduced pollen deposition, we calculated percent change of reproductive success measures within each gender between current and reduced pollen deposition. Percent change was calculated using the following equation:

$$\text{percent change} = (T\text{-}C)/C*100$$

where T is the reproductive output measures under the reduced pollination treatments and C is the reproductive output measures under the current pollination rates (Ayres, 1993). Negative numbers

indicate that reduced pollination treatments decrease reproductive success and positive numbers indicate that reduced pollination treatments increase reproductive success. This method facilitates comparisons of the direction and magnitude of change.

To statistically test if reduced pollination, gender, and their interaction effects significantly explained variation in the reproductive output measures, we used a generalized linear model (GLM) with Poisson distribution and a log link function. Covariate measures of included *S. acaulis* surface area, *S. acaulis* floral density, and beneficiary abundance.

Hermaphrodites provide one-half of the genetic material to the population through pollen production. Therefore, female plants must produce at least twice the number of seeds as hermaphrodite plants to persist in the population (Charnov, 1982) or have offspring that are more fit (Lewis, 1941). To statistically analyze the viability of females under current and reduced pollen deposition levels we compared female reproductive output to twice that of hermaphrodite reproductive output. In this way, if female reproduction (F) is greater than two times hermaphrodite reproduction (2H), then females are viable in the population. For females to be viable, not all measures of reproductive success need to be twice that of hermaphrodites, but all measures are shown to be comprehensive.

GLMs were also used to test if gender significantly affected reproductive output measures under the current ambient and experimentally reduced pollination regimes.

Instead of testing the effect of beneficiaries on measures of reproductive success over a range of environmental gradients (Bertness & Callaway, 1994) we test this effect under two pollen regimes: current and reduced. As there was no experimental removal of beneficiaries, interactions are inferred by association. To test the stress gradient hypothesis in this plant-pollinator system we conducted correlation analysis and tested for significance in the interaction term between the effect pollination and beneficiary abundance on percent fruit set in a GLM. A significant p-value ($p<0.05$) indicates that the response of percent fruit set to beneficiary abundance significantly differs between plants in the current and reduce pollination regimes. All analyses are appropriate for dealing with the unbalanced number of replicates between the current and reduced pollination regimes and were done in JMP 10 (SAS, 2012).

Results

Pollination regime significantly influenced percent fruit-set, seeds per fruit, fruit weight and percent germination (Table 1). Percent fruit-set, seeds per fruit, fruit weight, and percent germination were decreased in both genders with reduced pollen deposition (Figure 1).

Table 1. Summary of GLM results testing the effect of gender, pollination regime, and the gender by pollination regime interaction on measures of reproductive success with covariate measures of *S. acaulis* surface area (SA), *S. acaulis* floral density, and beneficiary abundance indicated by *. Significance is considered at <0.05 and indicated in bold.

		% fruit-set		seeds/fruit		fruit weight		seed weight		% germination	
Factor	**DF**	**ChiSq**	**p>ChiSq**	**ChiSq**	**p>ChiSq**	**ChiSq**	**p>ChiSq**	**ChiSq**	**p>ChiSq**	**ChiSq**	**p>ChiSq**
Gender	1	193.10	**<.0001**	6.92	**0.0085**	0.66	0.4163	0.81	0.3672	0.02	0.8865
Pollination regime	1	279.27	**<.0001**	32.43	**<.0001**	11.60	**0.0007**	1.32	0.2505	14.49	**0.0001**
Gender*pollination regime	1	240.93	**<.0001**	3.80	0.0513	0.31	0.5753	0.05	0.8213	40.02	**<.0001**
S. acaulis SA*	1	20.79	**<.0001**	6.89	**0.0087**	0.70	0.4022	0.21	0.6489	142.30	**<.0001**
S. acaulis floral density*	1	18.64	**<.0001**	0.16	0.6914	0.75	0.3862	0.20	0.6521	28.80	**<.0001**
Beneficiary abundance*	1	8.72	**0.0031**	1.12	0.2897	4.39	0.0362	3.96	**0.0466**	3.91	**0.0479**

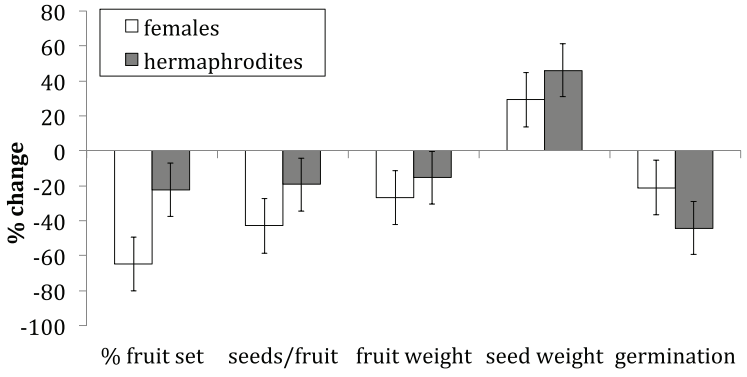

Figure 1. Percent change of female (white) and hermaphrodite (grey) reproductive output measures between current and reduced pollination regimes. ± 1 standard error bars shown.

There was a significant interaction effect between gender and pollination regime for percent fruit set, and percent germination (Table 1) indicating that these measures differed in their response. The direction of these differences is illustrated in the percent change calculations. Percent fruit-set reduced to a greater in females compared to hermaphrodites (Figure 1). Whereas, percent germination reduced to a greater in hermaphrodites compared to females (Figure 1).

Under current pollination rates, female plants had more than twice (2.98 times) the percent fruit-set compared to hermaphrodites (Figure 2). Female plants had less than twice the seeds/fruit, fruit weight, seed weight and percent germination compared to hermaphrodites (Figure 2). Under current pollination rates, all reproductive output measures were significantly different between females and two times hermaphrodite reproductive measures (Table 2).

Under reduced pollination rates, none of the female reproductive measures were greater than two times that of hermaphrodites (Figure 2). Under reduced pollination rates, all reproductive output measures, except seed weight and percent germination, were significantly different between females and two times hermaphrodite reproductive measures (Table 3).

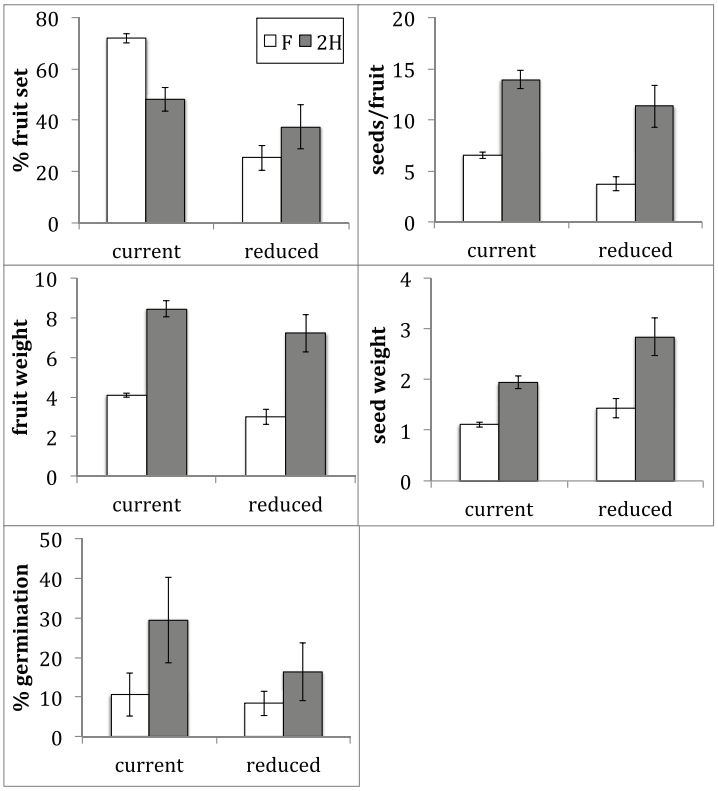

Figure 2. Reproductive output measures for female plants (white) and two times that of hermaphrodite plants (grey) under current and reduced pollination regimes. ± 1 standard error bars shown.

Table 2. Summary of GLM results testing the effect of gender on measures of reproductive success under the **current** pollination regime with covariate measures of *S. acaulis* surface area (SA), *S. acaulis* floral density, and beneficiary abundance indicated by *. Reproductive measures for hermaphrodites are doubled. Significance is considered at p<0.05 and indicated in bold.

		% fruit-set		seeds/fruit		fruit weight		seed weight		% germination	
Factor	**DF**	**ChiSq**	**p>ChiSq**	**ChiSq**	**p>ChiSq**	**ChiSq**	**p>ChiSq**	**ChiSq**	**p>ChiSq**	**ChiSq**	**p>ChiSq**
Gender (F and 2H)	1	394.83	**<.0001**	288.96	**<.0001**	159.83	**<.0001**	20.42	**<.0001**	68.09	**<.0001**
S. acaulis SA*	1	10.63	**0.0011**	6.64	**0.0099**	0.52	0.469	0.11	0.7395	13.05	**0.0003**
S. acaulis floral density*	1	28.74	**<.0001**	0.47	0.4912	0.31	0.5793	0.02	0.892	2.42	0.1198
Beneficiary abundance*	1	69.30	**<.0001**	1.82	0.1775	2.28	0.1313	6.89	**0.0087**	6.99	**0.0082**

Beneficiary abundance had a significant effect on percent fruit-set (Table 1). Under the current pollination rates, percent fruit-set and beneficiary abundance are negatively related (slope = -0.48, R^2 = 0.04, Figure 3). In contrast, under reduced pollination rates, percent fruit-set and beneficiary abundance are positively related (slope = 0.24, R^2 = 0.06, Figure 3). The slopes of these lines significantly differ (Chi^2 161.25, p-value <0.0001).

Dataset 1. The reproductive effects of reduced pollen deposition via exclosures and hand pollination on the cushion plant *Silene acaulis*

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Silene acaulis is an important alpine plant species commonly demonstrated to have positive impacts on other plants and insects. In this experiment, we covered sets of plants (gynodioecious species with hermaphrodite and female flowers) with mesh with hand pollination only to examine the relative importance of pollen load as a potential stressor in alpine ecosystems.

Discussion

Pollinator declines and climate mismatches are important in understanding the capacity for alpine plants to respond to possible future scenarios with reduced pollen loads. Using the cushion plant *S. acaulis*, we tested two predictions associated with the reproductive assurance hypothesis and more broadly that pollination stress may influence plant-plant interactions. All predictions were supported. Females were more sensitive than hermaphrodites to reduced pollen loads resulting in reproductive output dropping to below twice that of hermaphrodites. As the pollen supply conditions became more stressful (i.e. reduced), beneficiary plant species on these cushions positively related to percent fruit-set of the cushions. Hence, the reproductive assurance hypothesis and use of pollen reduction experiments can be important tools for ecological experiments on the responsiveness of alpine plant-pollinator systems to future changes in pollen availability.

Pollinator declines and plant-pollinator mismatches are important potential drivers of broad plant-community dynamics in the alpine

Table 3. Summary of GLM results testing the effect of gender on measures of reproductive success under the **reduced** pollination regime with covariate measures of *S. acaulis* surface area (SA), *S. acaulis* floral density, and beneficiary abundance indicated by *. Reproductive measures for hermaphrodites are doubled. Significance is considered at p<0.05 and indicated in bold.

		% fruit-set		seeds/fruit		fruit weight		seed weight		% germination	
Factor	**DF**	**ChiSq**	**p>ChiSq**	**ChiSq**	**p>ChiSq**	**ChiSq**	**p>ChiSq**	**ChiSq**	**p>ChiSq**	**ChiSq**	**p>ChiSq**
Gender (F and 2H)	1	54.79	**<.0001**	41.64	**<.0001**	15.03	**0.0001**	2.29	0.1298	3.45	0.0632
S. acaulis SA*	1	6.66	**0.0099**	3.30	0.0694	1.68	0.1945	2.78	0.0957	298.72	**<.0001**
S. acaulis floral density*	1	68.02	**<.0001**	24.68	**<.0001**	18.14	**<.0001**	3.74	0.053	71.77	**<.0001**
Ben. abundance*	1	61.84	**<.0001**	22.57	**<.0001**	8.62	**0.0033**	0.43	0.511	2.97	0.0847

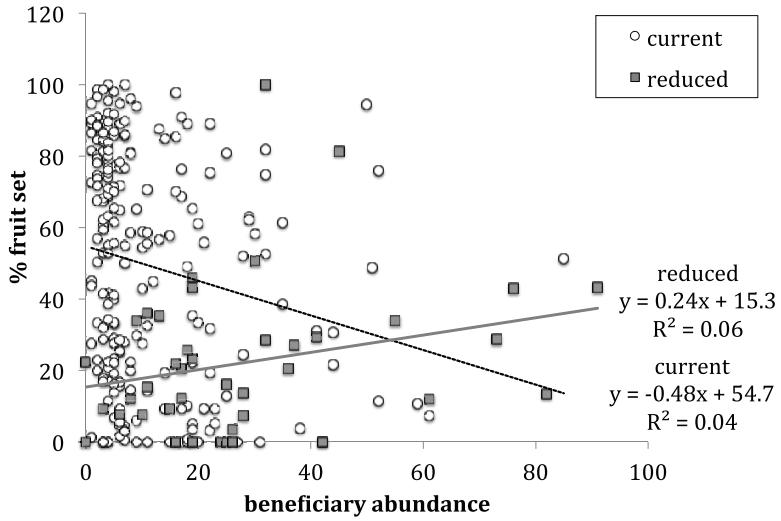

Figure 3. Percent fruit set and beneficiary abundance on individual *S. acaulis* plants under current pollination regime (white circles) and reduced pollination regime (grey squares). The linear best-fit line for plants under current pollination regime (black dashed line) has a negative slope of -0.48 and R^2 value of 0.04. The linear best-fit line for plants under reduced pollination regime (grey line) has a positive slope of 0.24 and R^2 value of 0.06. The slopes of these lines significantly differ (Chi^2 161.25, p-value <0.0001).

if dominant cushion plant species are impacted because they often function as keystone plant species (Arroyo *et al.*, 2003; Bertness & Callaway, 1994; Callaway & Walker, 1997; Cavieres *et al.*, 2006). Current trends of decreasing native pollinator populations are a pressing concern globally (Memmott *et al.*, 2007; Potts *et al.*, 2010; Bartomeus & Winfree, 2013). In these alpine environments bumblebees in particular are suggested to be critical because they are the most effective alpine pollinator (Bingham & Orthner, 1998; Chittka *et al.*, 1999; Gegear & Laverty, 1998) and because some bumblebee populations in BC are in decline (Colla & Ratti, 2010). The future scenario that pollinators emerge before flowers are in bloom due to a warming climate has also been proposed (Hegland *et al.*, 2009) and shown in the alpine of Japan (Kudo, 2013). Although it may not mimic the exact future pollen deposition rates, the experimental design tested herein begins to explore how alpine plants may respond to reduced-pollen loads whilst concomitantly testing the reproductive assurance hypothesis. Differences in reproduction between alpine plant genders are thus a critical avenue of research.

Female cushion plants became less viable under reduced pollen loads indicated by the decrease in percent fruit-set. Although the other measures of reproductive success were not equally decreased under reduced pollination, the decrease in percent fruit-set more than compensates for female plants reproductive disadvantage over hermaphrodite plants. Hence, a reasonable proxy or single measure to consider in similar future studies using dominant cushion plants is percent fruit-set only.

Interestingly, our results also supported the application of the stress gradient hypothesis to pollen limitation in addition to its original formulation for environmental stress or consumer pressure. That beneficiary abundance explains little variation in percent fruit-set (low correlation coefficients), suggests that other variables are of greater importance in determining percent fruit-set. Yet, the relationship between beneficiary abundance and percent fruit-set shifted from negative to positive as the pollination rates were reduced. This supports previous findings that under current pollination to the plant community, beneficiary plants living on cushions generally have a cost associated with the cushion plants reproductive fitness (Cranston *et al.*, 2012; Schöb *et al.*, 2013). The findings here however also further suggest that under reduced pollen loads this cost of facilitation can be diminished likely because competition between cushions and the other species is significantly reduced. Clearly, additional research is needed to identify the causal mechanisms between plant-plant interactions and plant-pollinator interactions with dominant plant species that host other species.

Data availability

F1000Research: Dataset 1. The reproductive effects of reduced pollen deposition via exclosures and hand pollination on the cushion plant *Silene acaulis*, 10.5256/f1000research.4382.d29313 (Reid *et al.*, 2014).

Author contributions

AR and CJL wrote the paper and analyzed the data. AR, RH and OM performed the experiments and collected the data. All authors read and approved the final content of the manuscript.

Competing interests

No competing interests were disclosed.

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Version 2

Reviewer Report 14 August 2014

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✔ **Fabio Bulleri**
Dipartimento di Biologia, Università di Pisa, Pisa, Italy

I think this new version reads well and no further changes are required. This study will hopefully inspire further research on the mechanisms regulating the impacts of reduced pollination rates on plant communities.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 04 July 2014

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✔ **Ernesto Badano**
Division of Environmental Sciences, Potosinian Institute of Scientific and Technological Research (IPICYT), San Luis Potosi, Mexico

This is an interesting study dealing with the effects that facilitated plants may have on the performance of the reproductive success of the nurse species. As far as I am aware, this is the first study reporting such

effects. The article is well written and is easy to read. The arguments provided in the introduction section support the hypotheses raised by the authors, and the methods used to test these hypotheses are sound. I believe that this paper makes an important contribution to the conceptual framework of positive interactions among plants along environmental gradients, mainly because it helps one to understand the outcome of these interactions in future climate change scenarios. I just have a few comments and queries about the methods used by the authors. The other sections of the article are clear and would not require major improvement.

Materials and Methods: Specific comments

1. In the subsection “Study species”, several sentences begin with the abbreviated scientific name of species (*S. acaulis*). Nevertheless, it is not appropriate to use the abbreviated name of the species for beginning a sentence. In this case, the full name of the species should be provided (*Silene acaulis*) while its abbreviated form (*S. acaulis*) must be used when the species is mentioned in the middle of a sentence.

2. In the second paragraph of the subsection “Study species” the authors indicate that, besides *S. acaulis*, several other plant species are relatively abundant. My question on this issue is: Are all these other species beneficiary of *S. acaulis* or they are just sharing the habitat with this cushion plant? This should be clarified to understand the aim of testing the Stress Gradient Hypothesis.

3. In the first paragraph of the subsection “Treatments” the authors indicate that they choose 20 female and male plants for the experiment. This caught my attention because in the previous sections of the article the authors stated that they used a gynodioecious population of *S. acaulis* and, therefore, male plants should not exist. I think that there is a mistake in this sentence, where instead of “*20 female and male plants*” the authors should say “20 female plants and plants with hermaphroditic flowers”.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 07 Jul 2014

Christopher Lortie, Department of Biology, York University, Toronto, Canada

Our response to Dr Badano’s comments:

1. Completed. Thank you.

2. These species were present both on and beside *S. acaulis* cushions. This is now clarified.

3. Yes, you are correct. This is now fixed.

Competing Interests: No competing interests were disclosed.

Reviewer Report 02 July 2014

https://doi.org/10.5256/f1000research.4691.r5192

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He Qiang
School of Environment, State Key Laboratory of Water Environment Simulation, Beijing Normal University, Beijing, China

This is a well conducted experiment and well written manuscript. It is the first experimental test of the stress-gradient hypothesis under pollen limitation stress, showing how we can further our understanding of plant-plant interactions with a new perspective. In my view, this is a neat paper, though I have some concerns that I hope the author can address in a revision.

The main limitation of this paper is that plant-plant interactions are inferred by association, not experimental removal of neighbours. Although a number of studies from alpine habitats suggest that facilitation can be indicated by positive association relationships, this limitation I feel should be clarified in the paper.

It is also a bit confusing to me why the authors did hand pollination for reduced-pollination treatment plants - was this because reduced-pollination treatment plants did not receive pollination at all without hand pollination? If so, the question would be how the authors controlled the magnitude of hand pollination as compared to natural pollination.

A third issue is that the pollen reduction treatments lacked procedural control. For example, one cannot tell the effect of mesh on the data. Also surely, the authors need to provide some details about the cloth mesh they used, such as mesh size etc.

It is unclear what can be the potential mechanisms for facilitation under pollen limitation. Facilitation in physically stressful habitats is produced by neighbours' relief of abiotic stress, and in biologically stressful habitats by associational defence. It is not a must for the authors to experimentally demonstrate this in the paper but I think they should at least discuss some of the possibilities. The authors may also want to include descriptions to encourage future research that experimentally tests for these possibilities.

I have several more specific comments:

1. "*Before bud-burst ...*" Clarify the start and end dates of your experimental treatments. What is the total duration?

2. "*The first 20 female and male plants found were ...*" Does the male here mean "hermaphrodite"? Use terms consistently.

3. For treatments, how many plants in current pollination treatments are female and male, respectively?

4. "*Reproductive output measures were collected from the 40 ...*" 40 hand pollinated, 231 naturally pollinated, this is 271 in total. So this number is inconsistent with the numbers described above: 273 plants were measured. There are three plants that were excluded?

5. Clarify when the beneficiary abundance was quantified and how this was done. It is the total number of plants living within the cushion. Shouldn't it be density per unit area?

6. How many beneficiary species occurred within your 273 study cushions? Would it be possible that beneficiary diversity rather than abundance is more likely to affect cushion plant reproductive success?

7. Actually, the beneficiary increased cushion plant fruit set under reduced pollen limitation. This means the beneficiary was actually a benefactor. This is a bit confusing; might it use neighbours instead?

8. "*we used a generalized linear model (GLM) with Poisson distribution ...*" Poisson distribution is often used for positive integer response variables. How about over-dispersion? Over-dispersion can substantially affect significance tests.

9. "*Under the current pollination rates, percent fruit-set and beneficiary abundance are negatively related ...*" Did pollination reduction affect the relationship between other reproductive variables and beneficiary abundance?

Overall, this is a great paper. The publication of this paper can be a stimulator of a new direction of positive species interactions with environmental stress.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reader Comment 02 Jul 2014

Christopher Lortie, Department of Biology, York University, Toronto, Canada

Fantastic feedback. Thank you. We will address all these comments and revise appropriately.

Competing Interests: No competing interests were disclosed.

Author Response 07 Jul 2014

Christopher Lortie, Department of Biology, York University, Toronto, Canada

Our response to Dr He's comments:

- *The main limitation of this paper is that plant-plant interactions are inferred by association, not experimental removal of neighbors. Although a number of studies from alpine habitats suggest that facilitation can be indicated by positive association relationships, this limitation*

I feel should be clarified in the paper.

The following sentence was added to the 'Data analysis' section, "As there was no experimental removal of beneficiaries, interactions are inferred by association".

- *...how the authors controlled the magnitude of hand pollination as compared to natural pollination.*

  The following sentences were added to the 'Treatment' section, "To experimentally reduce plant pollination, plants were covered with mesh and hand pollinated three times over the duration of the snow-free season. It is assumed that plants that are covered and hand pollinated three times receive less pollen than the plant open to natural pollination during the 33 snow-free days."

- *A third issue is that the pollen reduction treatments lacked procedural control. ...Also surely, the authors need to provide some details about the cloth mesh they used, such as mesh size etc.*

  It is not really possible to put mesh on without influencing pollinators so this was not conducted. We used fine cloth mesh purchased from a fabric store. It was flexible and durable. This was added to the methods section. See reference Donnelly et al. 1998.

- *It is unclear what can be the potential mechanisms for facilitation under pollen limitation. ...It's not a must for the authors to experimentally demonstrate this in the paper. But I think at least they should discuss some of the possibilities. The authors may also want to include descriptions to encourage future research that experimentally tests for these possibilities.*

  Good point. I think it may be too premature to assert possible reasons for this response. We do highlight the need for further research on this topic in the sentence, "Clearly, additional research is needed to identify the causal mechanisms between plant-plant interactions and plant-pollinator interactions with dominant plant species that host other species."

Responses to specific comments:

1. Added from June 26th to August 29th, 2010.
2. Yes, great point. Revised.
3. Added; 124 were female and 107 were hermaphrodites.
4. A total of 271 *S. acaulis* plants were measured.
5. Yes, but we do not have % area covered unfortunately. We took measures of beneficiary abundance and richness.
6. We checked beneficiary diversity and found that it was not strongly related, but that beneficiary abundance was a more informative measure. There were 16 other plant species growing on the cushions.
7. I think introducing a new term would cause confusion not add clarity. They are the same plants they just appear to switch roles (competitor to facilitator) depending on pollen supply.
8. The over-dispersion tests in the GLM did change some of the p-values of the factors but not near the alpha of $p < 0.05$.

9. This pattern is consistent in the measure of seeds/fruit but not the other measures of reproductive output.

Competing Interests: No competing interests were disclosed.

Reviewer Report 02 July 2014

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?

Fabio Bulleri
Dipartimento di Biologia, Università di Pisa, Pisa, Italy

This is an interesting article reporting on a study investigating how reduced pollen supply influences reproduction of self-compatible versus self-incompatible plants of an alpine cushion species and its interaction with associated species. The main strength of the study is that of providing hints on the potential role that factors other than environmental conditions can play in modulating species interactions. In a relatively recent work by Maestre *et al.* (2009), stress has been divided into two major types, resource and non-resource. I am not sure whether pollen availability can be seen as a resource, but I would not have major issues with viewing pollinator availability as a quantifiable, limiting resource. Maybe, these concepts could be touched upon somewhere in the Introduction.

The main weakness of the study is the lack of quantification of pollen delivery in different treatments: I think the authors should try to give the reader, at the very least, a gross idea of the order of magnitude of differences between natural and artificial pollen supply. The second point is that there is not a formal gradient of pollen supply, as this implies more than two levels. This does not impinge on the robustness of the results, but limits the insight the study offers into the Stress Gradient Hypothesis.

Finally, both in the Abstract and M&M I did not get clearly who the benefactors and beneficiary were. Actually, only from the Results did I understand that you were analyzing the effects of species hosted by the cushion on the cushion-forming species. I sense that referring to the cushion and associated species might be a better solution.

SPECIFIC COMMENTS

Introduction

- It is not clear what grounds the alternative model for pollinator abundance (increasing with elevation) in alpine environments builds upon.

- In the Introduction, you refer to the stress source sometimes as 'pollination supply' and sometimes as 'reduced pollen' or 'less pollen'. It would be useful to specify if the limiting resource is pollen supply (which implies reduced production by plants) or pollinator abundance (I guess the second, from the model you propose in the Introduction).

- Is there any evidence of competition between hermaphrodite and female plants when pollen is not limiting?

Material & Methods

- You need to be consistent in the way you refer to hermaphrodite plants.

- Some plants could receive pollen from insects while others were covered with mesh and hand-pollinated. It is not clear how insect versus hand pollination generated a gradient of pollen supply. Do you assume that delivery of pollen by insect was much higher? If so, which order of magnitude?

- "*Instead of testing the effect of beneficiaries on measures of reproductive success over a range of environmental gradients (Bertness & Callaway, 1994) we tested whether variation in the current and experimentally reduced pollen supply can be viewed as potential stressor*." Again, it is not clear here who is expected to be facilitated and by whom (see my general comments). Likely, it would be useful to be more specific in your predictions at the end of the Introduction. Also, switch beneficiary and % fruit set in the last sentence of the Abstract (the way it is, it suggests that you are mainly looking at effect of fruit set on beneficiary abundance).

Results

- Estimates of effects could be reported in Tables, so as to give an idea of their direction.

- The relationship with beneficiary abundance explains little variation in % fruit set (Fig. 3), suggesting that other factors are of overwhelming importance. This point should be made clear in the Discussion.

Discussion

- It would be interesting if the authors could offer a possible explanation for a positive relationship between beneficiary abundance and % fruit set under reduced pollen supply. What are the potential mechanisms?

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reader Comment 02 Jul 2014

Christopher Lortie, Department of Biology, York University, Toronto, Canada

Thank you for the suggestions. We will address all of them and provide a revised version.

Competing Interests: No competing interests were disclosed.

Author Response 08 Jul 2014

Christopher Lortie, Department of Biology, York University, Toronto, Canada

Our response to Dr Bulleri's comments:

- *I am not sure whether pollen availability can be seen as a resource, but I would not have major issues with viewing pollinator availability as a quantifiable, limiting resource. Maybe, these concepts could be touched upon somewhere in the Introduction.*

  This is a good point. This is now added in the Abstract and made clearer in the introduction.

- *I think the authors should try to give the reader, at the very least, a gross idea of the order of magnitude of differences between natural and artificial pollen supply.*

  Unfortunately this is very difficult as there is no estimation of current pollen deposition rates. This limitation has been more clearly stated now in the following sentence, "It is assumed that plants that are covered and hand pollinated three times receive less pollen than the plant open to natural pollination during the 33 snow-free days".

- *The second point is that there is not a formal gradient of pollen supply, as this implies more than two levels. This does not impinge on the robustness of the results, but limits the insight the study offers into the Stress Gradient Hypothesis.*

  Agreed. This is clearly stated in the following sentence, "Instead of testing the effect of beneficiaries on measures of reproductive success over a range of environmental gradients (Bertness and Callaway 1994) we test this effect under two pollen regimes: current and reduced".

- *It is not clear what grounds the alternative model for pollinator abundance (increasing with elevation) in alpine environments builds upon.*

  Good question. Unfortunately, there is really no model for pollinator abundance in the alpine/on elevation gradients. We would love to develop one though but likely not in the context of this outcrossing experiment as it is focused on botany and ecology. We hope that the proposal that pollen limitation is a potential stressor can lead to the expansion of this set of theory. Nice opportunity for the future.

- *In the Introduction, you refer to the stress source sometimes as 'pollination supply' and sometimes as 'reduced pollen' or 'less pollen'. It would be useful to specify if the limiting resource is pollen supply (which implies reduced production by plants) or pollinator abundance (I guess the second, from the model you propose in the Introduction).*

  Yes, great. Thank you.

- *Is there any evidence of competition between hermaphrodite and female plants when pollen is not limiting?*

  Yes. See Figure 3 and the following sentence in the results, "Under the current pollination rates, percent fruit-set and beneficiary abundance are negatively related (slope = -0.48, R^2 = 0.04, Figure 3)."

Material & Methods

- *You need to be consistent in the way you refer to hermaphrodite plants.*

  Revised.

- *Some plants could receive pollen from insects while others were covered with mesh and hand-pollinated. It is not clear how insect versus hand pollination generated a gradient of pollen supply. Do you assume that delivery of pollen by insect was much higher? If so, which order of magnitude?*

  Yes, the assumption is that the plants that had pollen delivered by insects received more pollen because they could receive pollen every day compared to the three hand-pollination events. The order of magnitude of this difference would be purely a guess. This is a very important assumption and limitation of this study.

- *Again, it is not clear here who is expected to be facilitated and by whom (see my general comments). Likely, it would be useful to be more specific in your predictions at the end of the Introduction.*

  Revised, thank you. "Specifically, we predict facilitation between cushions and beneficiaries to increase when pollen supply is reduced."

- *Also, switch beneficiary and % fruit set in the last sentence of the Abstract (the way it is, it suggests that you are mainly looking at effect of fruit set on beneficiary abundance).*

  Revised and clearer now.

Results

- *Estimates of effects could be reported in Tables, so as to give an idea of their direction.*

  Good idea Fabio. We chose to do stats on the primary dataset and not calculate derived data such as effect size measures (Rii) for two reasons. We were not confident that Rii, now common in facilitation studies, was appropriate to the treatments here and secondly that we elected to visualize percent change in Figure 1. We feel that figure one does a good job of showing the strength of evidence, i.e. effects, and that a table was thus not needed. Let us know if you think we should also include the data from this figure in a table but we think the current figure gives a good overview of the evidence/biological significance.

- *The relationship with beneficiary abundance explains little variation in % fruit set (Fig. 3), suggesting that other factors are of overwhelming importance. This point should be made clear in the Discussion.*

The following sentence has been added to the discussion, "That beneficiary abundance explains little variation in percent fruit-set (low correlation coefficients), suggests that other variables are of greater importance in determining percent fruit-set."

Discussion

- *It would be interesting if the authors could offer a possible explanation for a positive relationship between beneficiary abundance and % fruit set under reduced pollen supply. What are the potential mechanisms?*

  Good point. I think it may be too premature to assert possible reasons for this response. We do highlight the need for further research on this topic in the sentence, "Clearly, additional research is needed to identify the causal mechanisms between plant-plant interactions and plant-pollinator interactions with dominant plant species that host other species."

Competing Interests: No competing interests were disclosed.

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