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Reproductive system of a mixed-mating plant responds to climate perturbation by increased selfing

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How plants respond to climatic perturbations, which are forecasted to increase in frequency and intensity, is difficult to predict because of the buffering effects of plasticity. Compensatory adjustments may maintain fecundity and recruitment, or delay negative changes that are inevitable but not immediately evident. We imposed a climate perturbation of warming and drought on a mixed-mating perennial violet, testing for adjustments in growth, reproduction and mortality. We observed several plasticity-based buffering responses, such that the climatic perturbation did not alter population structure. The most substantial reproductive adjustments, however, involved selfing, with a 45% increase in self-pollination by chasmogamous flowers, a 61% increase in the number of cleistogamous flowers that produced at least one fruit and an overall 15% increase in fruit production from selfed cleistogamous flowers. Reproductive assurance thus compensated for environmental change, including low pollinator visitation that occurred independently of our climate treatment. There was also no immediate evidence for inbreeding depression. Our work indicates that plants with vegetative and reproductive flexibility may not be immediately and negatively affected by a climatic perturbation. The stabilizing effects of these reproductive responses in the long term, however, may depend on the implications of significantly elevated levels of selfing.

1. Introduction

The long-term impacts of climate change on plant populations remain unclear [1–3]. Many significant responses to climate change have been described, including earlier flowering time and seed maturation, changes in distribution along latitudinal or elevation gradients, and increased vegetative growth [4–6]. Other studies, however, have shown modest or inconsistent responses to shifts in temperature and precipitation, under both experimentally imposed conditions and longer-term field observation [2,7–9]. These inconsistencies limit our ability to forecast future impacts of climate change [10].

Several factors may underlie the inconsistent distributional and demographic responses to climate change. First, climate change can be buffered by a range of growth and reproductive responses that maintain demographic performance despite substantial environmental change [11]. These may include plastic-based adjustments such as earlier flowering or higher selfing [11]. They may also include density-related responses, where less frequent species favoured by the new conditions capitalize on the climate shifts, thereby increasing population growth [12]. The implication is that changes in distribution or phenology driven by climate may be easily observed, but may not affect persistence, because compensatory mechanisms maintain population vital rates.

Second, the effects of climate change may unfold indirectly by altering trophic or competitive interactions [13–15]. Persistent herbivory, for example, may prevent rapid changes in community composition by suppressing species that might otherwise thrive in the new climate [7]. Pollen limitation, whether associated with climate change or non-climatic factors such as habitat loss, can potentially hamper how plants respond to perturbations by limiting pollen flow [7,16–18]. Climate perturbations, or the short-term effects of climate

change, may also widen the competitive differences between dominant exotic and rarer native species [19].

Finally, there may be response lags in some plant populations, such that the direct effects of climate change are not immediately apparent [7,9]. For example, various forms of compensatory buffering may provide short-term resiliency in growth and reproduction, but have longer-term costs through the draining of carbohydrate reserves or increased inbreeding depression from selfing [11,20].

Here, we test the effects of an abrupt climate perturbation on a mixed-mating perennial species, examining its influence on growth, reproduction and mortality, and how these responses are mediated indirectly through factors including pollen limitation. Uncertainty over climate responses is especially acute for mixed-mating species, which represent approximately 42% of the world's flora [21] and, in the most extreme case, produce both outcrossed and obligately selfed flowers (dimorphic cleistogamy). The production of two flower types can minimize negative responses to environmental duress by allocation flexibility and reproductive assurance [22,23]. Alternatively, the production of two sets of flowers can lead to greater consumption of maternal resources and potential risks of inbreeding if there is an over-reliance on reproductive selfing [21]. We combine field-based demographic surveys with experimental work manipulating temperature and drought, pollinator visitation, and pollen limitation to test the direct impacts of a climatic perturbation on plant performance. We also test the potential longer-term demographic consequences of the climatic perturbation by quantifying its impacts on the reproductive output of outcrossed and selfed flowers, seedling establishment, and the importance of maternal effects.

2. Material and methods

We worked in a 10.3 ha oak savannah ecosystem on Vancouver Island, British Columbia, Canada [24–26]. The regional climate is sub-Mediterranean, with cool, wet winters (mean 4°C) and warm, moisture-limited summers (mean 19°C). The growing season extends from April to June, after which soil moisture becomes limiting. Pollinator activity typically peaks from early to mid-May [24]. Pollen limitation appears to be less prevalent in this system compared with elsewhere, at least for several relatively abundant forb species, with 220 pollinator taxa reported for the system regionally [24]. The savannah is presently dominated by perennial non-native pasture grasses [25] (electronic supplementary material, figure S1a). Our study species, *Viola praemorsa* spp. *praemorsa*, is endemic to the Pacific Northwest of North America [26]. Lifespan can exceed 10 years, with reproduction beginning at 2–5 years depending on habitat conditions. *Viola praemorsa* is non-stoloniferous with a mixed-breeding system. Showy chasmogamous (CH) flowers, which are facultatively outcrossing, are mostly produced in late April to June. Production of obligately selfed cleistogamous (CL) flowers occurs from May to late June, when plants begin to senesce. The violet is the earliest-flowering native forb in the study area by approximately two weeks.

In 2007, a demographic survey counted approximately 3500 individuals in the study area [25], from which 38 permanent 1 m² plots with 250 adult violets were established. In 2009, we selected 20 of these plots based on similarity in violet density (mean = 7 plants m²; $t = 0.19$, $p = 0.66$), and randomly assigned 10 climate and 10 control treatments. The relative abundance of plant functional groups was similar in all 1 m² plots, with exotic grass cover from 55 to 75%. The remaining species were mostly flowering perennial forbs (see the electronic supplementary material, figure S1a), including exotic legumes (e.g. *Vicia* spp.)

and native spring ephemerals similar in life history to the violet [25]. Cover of the violet ranged from 1 to 3% per plot.

(a) Climate treatment

We constructed roofed circular chambers to increase temperature and prevent rainfall, installing them prior to CH flowering in early May 2009 (see the electronic supplementary material for more detail). To quantify violet performance, we weekly measured seedling abundance per plot and, for each juvenile and adult plant, the number of leaves, CH flowers, CL flowers, seed pods and mortality from May to July 2009. We classified three age classes: seedlings (cotyledons present), juveniles (flowers absent) and adults (one or more flowers present). Plants were permanently staked and numbered. Flower types were identified based on morphology. CH flowers are zygomorphic with five yellow petals on a pedicel, which originates from the plant base. CL flowers are apetalous, with short pedicels that arise secondarily from stems higher on the plant. Flower performance was quantified for both CH and CL flowers, on all individuals in the 20 experimental plots. Individual flowers were marked with loosely wrapped coloured thread so progress could be followed throughout the season (see the electronic supplementary material, figure S1c). As plants matured, we quantified fertilization success (the presence of a swollen ovary) and fruit success (capsules forming into a dry seed capsule).

(b) Pollen limitation and reproductive ecology

We tested for pollen limitation, the extent of self-fertilization in CH flowers in control versus climate plots and the effects of the climate treatment on pollinator visitation to determine whether the chambers act as barriers to pollination. We used three pollination treatments: pollen supplementation (PS), pollinator exclusion (PE) and no manipulation (control), with the supplemented pollen coming from 10 randomly selected plants from an on-site nursery (see electronic supplementary material for more detail). For the plants with flowers brushed with pollen or bagged to prevent pollinator visits, these treatments were applied to every CH flower on the plant. We applied the pollen immediately to ensure viability, by brushing it onto the stigmas of open flowers. Most flowers were visited two days later for a second pollen application. When fruits began to form (approx. two weeks later), we encased the capsules with fine mesh given that the seed capsules are explosive and the seeds difficult to collect. Once dehisced, we counted seeds including the proportion that were viable (per capsule per plant, averaging these values for each plant). Seed viability was determined by assessing seed hardness (hollow seeds were considered unviable). The mean proportion of flowers producing fruit was the number of flowers with fruit divided by the total number of flowers, averaged for each plant.

(c) Pollinator observation

To measure the pollinator visitation, we captured 30 h of video with a digital camcorder over a two-week period on sunny days when pollinators were observed to be actively foraging. Each trial used three adult violet plants in pots (flower number 6–20 per plant) placed among co-flowering forb species that are frequently visited by pollinators [24]. A visit was counted when an insect probed a flower. Trial duration varied from 1 to 4 h daily, depending on weather conditions.

(d) Performance differences between chasmogamous versus cleistogamous offspring

Given the potential for increased selfing in response to climatic perturbation and for negative effects of selfing on seed viability [11], we tested for performance differences between seedlings

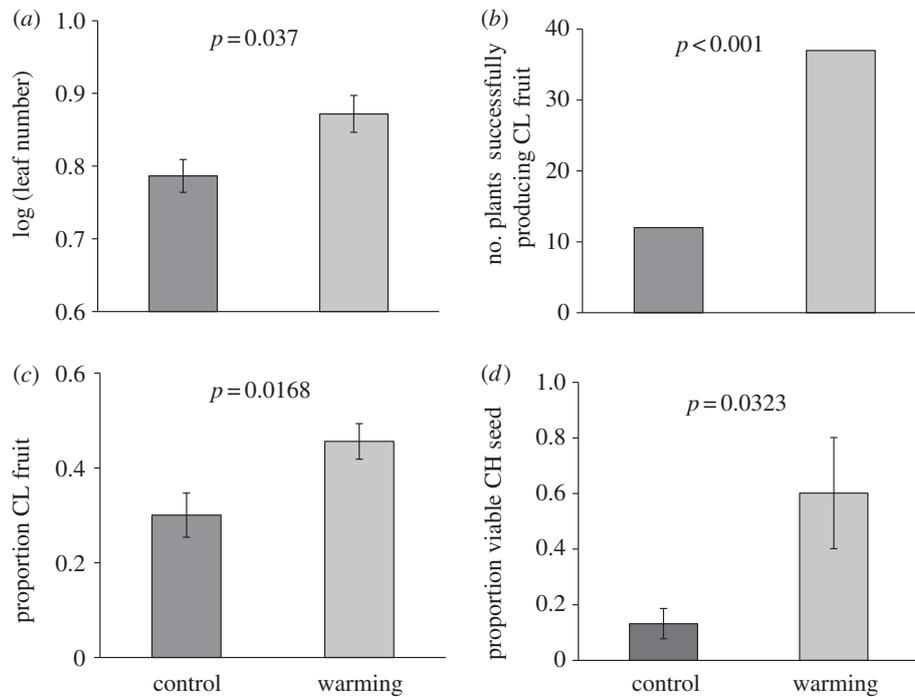


Figure 1. Impacts of experimental warming on measures of plant performance: (a) plant size (measured as leaf number), (b) the number of plants that successfully produced at least one cleistogamous fruit, (c) the proportion of cleistogamous flowers successfully producing fruit and (d) the proportion of CH seeds that were viable in the PE (autogamy) treatment.

from CH versus CL flowers, including whether selfing negatively affected reproduction or recruitment. We determined whether CL seed had reduced seed mass and viability, and whether selfed progeny performed better near their maternal plants (see electronic supplementary material for more detail).

(e) The potential for non-climatic influences

We accounted for three non-climatic factors that could influence the experiment: (i) whether the chambers excluded herbivores and pollinators, thereby affecting plant size and reproductive performance; (ii) whether neighbouring exotic grasses had positive responses to the climatic perturbation that could indirectly shape violet performance; and (iii) whether underlying variation in soil depth influenced response.

We compared herbivore damage in control and chamber plots by counting the number of leaves per plant that were damaged or removed by insects and deer. We tested for pollinator visitation indirectly, by comparing outcrossing success of unbagged and bagged flowers inside versus outside the chambers. Possible access points into the chambers by pollinators can be seen in the electronic supplementary material, figure S1b. We contrasted biomass between control and chambers by clipping above-ground biomass from randomly selected 10 cm² areas in each of the 20 plots after the forbs had started to senesce in early July. The vegetation was sorted by functional groups (grasses, all forbs), oven-dried for 48 h at 68°C and weighed. To test for a possible influence of soil depth, we measured soil depth in each plot using a 0.85 m steel rod driven to bedrock.

(f) Statistical analysis

All statistical analyses were conducted using R [27]. To test for performance differences between treatment and control plots, we compared the different performance measures using generalized linear mixed models (GLMMs). We summed the individual-level reproductive responses collected throughout the growing season (flower production, fruit production) for each individual and used the leaf production at the time of the last

survey in the analysis. Flower production, fruit production, seed viability, mortality, leaf production and above-ground biomass were classified as response variables and analysed at the plot level. We tested for autonomous self-fertilization and pollen limitation in the warming and control treatments by comparing the difference in mean seed production per individual using an additional GLMM, which is appropriate for unbalanced datasets [28]. To test for differences in CH and CL seedling establishment between maternal and novel environments, we conducted an additional GLMM analysis. Here, the fixed effects were flower type, environment (maternal, novel or pot) and plant size (see electronic supplementary material for more detail).

3. Results

(a) Responses to climatic perturbation

The climate treatment caused significant performance changes compared with control plots (figure 1). Plant size, based on number of leaves per plant, increased significantly ($t = 2.1$, $p = 0.037$; figure 1a). By contrast, there was no significant change in biomass of neighbouring exotic grasses within the chambers ($t = 0.704$, $p = 0.492$; electronic supplementary material, table S1). The warming treatment impacted fruit production. The number of plants that successfully produced at least one CL fruit increased by 61% ($t = 6.836$, $p < 0.001$; figure 1b), the proportion of CL fruit per plant increased by 15% ($t = 2.448$, $p = 0.0168$; figure 1c) and there was a 45% increase in the proportion of selfed viable CH seed produced (indicated by the PE × warming treatment interaction term in the electronic supplementary material, table S2; $t = 2.198$, $p = 0.0323$; figure 1d). These plants, which could only set seed through autogamous selfing, produced on average 2.10 seeds (s.d. = 2.6) in the warming treatment and 0.80 seeds (s.d. = 1.6) in the control plots. There was a significant interaction between the warming treatment and log (leaf number) ($t = -2.131$, $p = 0.0365$; electronic supplementary material, table S3), where CL fruit responses to

climate were amplified in larger plants. There were no changes in plant mortality ($Z = -0.004$, $p = 0.996$), numbers of naturally recruiting seedlings, numbers of CH flowers per plant and numbers of CL flowers per plant (despite increases in CL fruit production; see the electronic supplementary material, tables S1 and S3).

(b) Pollen limitation and reproductive ecology

Across all pollination treatments, CH seed number ranged from 1 to 17 per capsule. We detected significant pollen limitation, but this was not influenced by the warming treatment (indicated by the PS \times warming treatment interaction term in the electronic supplementary material, table S2; $t = -0.387$, $p = 0.6994$), with the brushing of pollen on CH flowers almost doubling seed-set ($t = 2.487$, $p = 0.0138$; electronic supplementary material, table S2) compared with open pollinated controls. Pollen supplementation produced an average of 4.50 seeds per capsule (s.d. = 4.4), compared with 2.10 seeds per capsule (s.d. = 2.9) in the control treatment. The climate chambers did not appear to significantly affect pollinator visitation, as seed-set in control CH flowers did not decline in the warming chambers ($t = -0.007$, $p = 0.99$).

(c) Linkages between chasmogamous and cleistogamous flower performance

We tested for trade-offs between CH flower or fruit production and subsequent CL production, including whether treating CH flowers with pollen addition (brushing) or PE (bagging) affected CL production or reproductive performance later in the growing season. This did not occur. There was no relationship between CH success and CL success ($t = 0.9$, $p = 0.38$), but plant size is positively related to CH and CL flower production (electronic supplementary material, figure S2).

(d) Video surveys

Pollen limitation appeared to be at least partly explained by low flower visitation. There was only one floral visitor, a bee fly (*Bombylius major*), during 30 h of pollinator observation. There was no evidence for pollinator facilitation (i.e. neighbouring species attracting pollinators, thereby increasing visits to the violet), as 16 unidentified pollinators visited co-occurring *Vicia sativa* flowers (a non-native plant species) during the same observation period but ignored the violets.

(e) Non-climatic factors

We observed no differences in insect herbivore damage between control and chamber plots ($t = -0.706$, $p = 0.4822$; electronic supplementary material, table S1), and we did not detect deer browsing in the control plots (i.e. larger plant size in the chambers was not explained by protection from browsers). Neither the biomass of neighbouring exotic grasses nor soil depth altered climate responses (see the electronic supplementary material, figure S3).

We observed no short-term negative impacts of selfing in the CL seeds. We observed no difference in germination success compared with seed from outcrossed flowers ($t = -0.13$, $p = 0.9$). Seedling establishment was higher for both seedling types in the non-maternal plots ($t = 2.5$, $p = 0.02$; figure 2, 'novel'). We detected no evidence for maternal effects, as

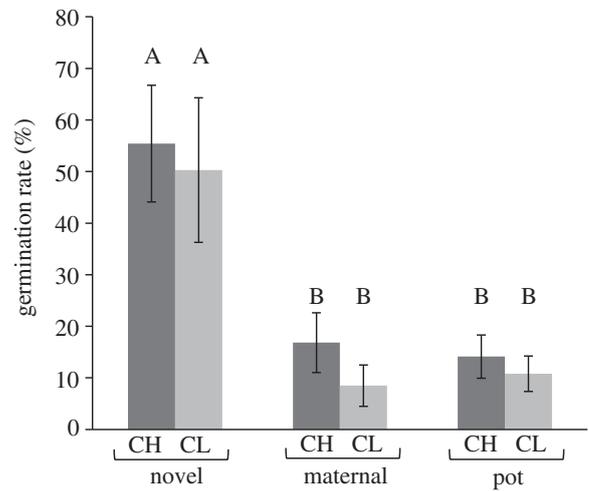


Figure 2. Germination success of chasmogamous (CH) and cleistogamous (CL) seeds from *Viola praemorsa*. Seeds were collected in the summer of 2008, planted the same autumn and surveyed in the summer of 2009. Planting locations were within 20 cm of their parent (maternal), in a novel environment greater than 100 m from their parent (novel) and in pots in the on-site nursery (control; pot). Means with the same letter are not significantly different.

parent size (based on leaf number) did not affect recruitment success for either seed type ($F_{2,64} = 0.4$, $p = 0.55$).

4. Discussion

Many studies have shown that climate change can alter the growth, phenology and reproductive dynamics of plants [29–31]. The longer-term implications, however, remain unclear given that these alterations may involve compensatory responses that maintain population performance [3]. We examined the reproductive responses of a mixed-mating violet to an extended pulse of warming and drought. We tested whether shifts in growth and allocation between putatively outcrossed and obligately selfed flowers buffered the impacts of the climate perturbation on reproductive performance. Our observations revealed strong compensatory dynamics, with the climate treatment triggering increases in leaf production and reproductive effort that maintained pre-treatment levels of recruitment. The plants thus demonstrated dramatic changes in performance, but with no apparent short-term consequences for population persistence. The most substantial reproductive responses, however, involved increased selfing. Elevated temperature and drought increased CL fruit production and elevated the viability of autogamous CH seed production, resulting in an overall increase in the proportion of selfed progeny. The potential negative consequences of increased selfing in mixed-mating plants remain debated [11,21,23]; it is thus unclear whether a reliance on selfing with climatic perturbation increases the longer-term risks of inbreeding depression. In the short term, at least, we observed no evidence that selfing caused difficulties in seed production, seed viability or the performance of seedlings in the field. Because we did not determine the outcrossing rate directly, we cannot rule out the influence of geitonogamy on the pollination of control plants, which were open to outcrossing for the duration of the experiment. However, the low pollinator visitation and small difference in seed-set between the autogamy and control treatment suggests that the outcrossing rate is likely to be low.

The buffering of environmental change by selfing reveals the importance of reproductive assurance for maintaining seed production in changing environments [23]. The responses derived from both flower types, with warming driving higher CH selfing and CL fruit production. We observed pollen limitation, with pollen addition increasing seed-set in CH flowers by 45%, but this occurred independently of the climate treatment. This result, where selfing compensates for pollen limitation, may be not be surprising for *Viola*, given that low visitation rates have been reported previously for this genus [32] and for rare species in general [11]. As well, *V. praemorsa* is the earliest forb to flower in this system, potentially reducing visitation rates even though pollinator diversity is high [24]. More generally, cleistogamy can be an evolved response to pollen limitation so it may not be surprising that the species has mechanisms to compensate for low pollinator visitation. Given that at least 85% of the world's flora have the capacity to self [31], our results suggest that climatic perturbations have the potential to increase selfing. Increased self-fertilization following anthropogenic perturbation has been predicted, but mostly in association with the reduction of pollen flow with habitat fragmentation [11].

We predicted the possibility of allocation-based trade-offs between CH and CL flowers, given that our focal species exhibits sequential flower production with CH flowers developing first. We observed no allocation linkages between the flower types, which is consistent with previous work on another *V. praemorsa* subspecies [33]. Adding pollen to CH flowers and increasing seed-set produced no change in CL flower production, CL capsules per plant or CL seeds per capsule. Similarly, when we bagged the CH flowers and reduced seed-set, there was no change in CL reproductive effort. The only CL responses we observed were mediated by external factors relating to the climate manipulations. It is unclear what explains the absence of linkages between flower types. Trade-off advantages are assumed to relate to some form of bet-hedging, increasing the chances of producing seed but minimizing the over-production of flowers [32]. We observed a presumably more metabolically expensive strategy, because two sets of flowers were produced regardless of circumstance. This may again relate to reproductive assurance, as only 25 per cent of CH flowers produced fruit, whereas CL had 70% success under the same conditions.

This above result differs from recent work on another *V. praemorsa* subspecies that did not demonstrate reproductive assurance [33]; however, we accounted for possible alternative explanations for our observed responses. In the field, climate chambers can potentially act as herbivore exclosures for deer or insects [34], but we saw no difference in herbivory. Climate

chambers can also exclude pollinators, and undoubtedly visitation was constrained, but we detected no difference in pollen limitation. Site factors can also mediate climate responses given their influence on plant size and fecundity [26], but our climate responses occurred independently of soil depth. Finally, we predicted that dominant grasses could capitalize on the warming pulse, possibly widening differences in growth or reproduction, as seen elsewhere in Mediterranean coastal grasslands of western North America [19]. There was no change in standing above-ground biomass in the grass community, in contrast to the violet, which increased leaf production.

We confirmed that human-mediated environmental change can increase reproductive selfing [11]. What remain unclear, however, are the risks of increased selfing [20]. Persistent selfing in mixed-mating plants has been associated with a range of potentially negative consequences, including inbreeding depression, pollen and seed discounting, and the concentration of juveniles near maternal plants given that selfed seeds tend to be dispersal-limited [11,26]. However, we observed no immediate negative consequences. There were no changes in seed mass and seed production per capsule in either flower type, and no functional differences between CL and CH progeny in size even when accounting for maternal effects—larger parents did not result in more successful offspring. There was also no evidence for the importance of the maternal environment for germination success of CL or CH seed. Reproductive assurance thus maintained seed output, which has been predicted, but also the number of recruiting individuals in the population.

Our results reveal a resiliency to climatic perturbation by a mixed-mating species, determined by compensatory responses of growth and reproduction. Future work is needed to test whether other mixed-breeding species, especially those with hermaphroditic monomorphic flowers, respond similarly with higher selfing, and whether they are similarly resistant to short-term negative consequences. The presence of short-term resiliency could mask potential demographic difficulties in the future if selfing continues at high levels, but without longer-term studies this may not become evident until negative effects start to become expressed in the population.

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