



Comparison of Hand-Pollinated and Naturally-Pollinated Puget Balsamroot (*Balsamorhiza deltoidea* Nutt.) to Determine Pollinator Limitations on South Puget Sound Lowland Prairies

Authors: Fazzino, Lisa, Elizabeth Kirkpatrick, H., and Fimbel, Cheryl

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Lisa Fazzino¹ and H. Elizabeth Kirkpatrick, University of Puget Sound, 1500 N. Warner, Tacoma, Washington 98416
and

Cheryl Fimbel, The Nature Conservancy of Washington, 120 E. Union #215, Olympia, Washington 98501

Comparison of Hand-pollinated and Naturally-pollinated Puget Balsamroot (*Balsamorhiza deltoidea* Nutt.) to Determine Pollinator Limitations on South Puget Sound Lowland Prairies

Abstract

South Puget Sound prairies are fragmented and degraded, which has a profound effect on plant populations, especially those that are already species of concern, such as Puget balsamroot (*Balsamorhiza deltoidea* Nutt.). Small Puget balsamroot populations may be caused by inadequate pollination via insufficient pollinator services or by low quality pollen. Comparing potential germinants of hand- and naturally-pollinated inflorescences, which takes into account seed set and germination rates, illustrates the extent of pollination limitation on three South Puget Sound prairies on Joint Base Lewis-McChord (7S, Upper Weir, and Johnson). Our results demonstrated that Puget balsamroot is self-incompatible. On all three prairies, hand-pollinated inflorescences produced more potential germinants than naturally-pollinated inflorescences ($P < 0.001$), indicating that Puget balsamroot is pollen limited. In addition, 7S had a significantly greater number of floral and soil resources than either Johnson or Upper Weir ($P < 0.05$). However, there were not proportionally more bees found on 7S as compared to Johnson or Upper Weir. Therefore, Puget balsamroot on 7S may be pollen limited because pollinator populations are too small. Determining pollinator population sizes on the south Puget Sound lowland prairies could prove to be useful in determining the relative effects of pollinator limitation and low pollen quality. We suggest that fragmented ecosystems are more susceptible to pollinator limitations than congruent systems because ranges of pollinators no longer adequately overlap to facilitate travel between the ecosystem fragments.

Introduction

It has been estimated that >90% of the southern Puget lowland prairies have been lost to urban development, forest invasion or conversion, and agricultural uses (Crawford and Hall 1997). To compound the problem of degradation, the south Puget lowland prairies are also becoming fragmented and isolated from each other (Figure 1). Fragmentation and isolation can profoundly impact an ecosystem. It decreases the success of sexual reproduction for many flowering plants by reducing the abundance and diversity of pollinators that are required for successful pollination (Steffan-Dewenter and Tscharntke 1999, Wagenius 2004, Aguilar et al. 2006). In addition, habitat fragmentation often results in a change in the foraging behavior of pollinators (Steffan-Dewenter and Tscharntke 1999, Aguilar et al. 2006). For example, habitat fragmentation can incite “perceived” barriers in some bee species that are not crossed and therefore other patches are not visited by those bee pollinators (Osborne and Williams 2001).

An increase in habitat fragmentation has been linked to reduced seed set in mustard (*Sinapis arvensis*) and radish (*Raphanus sativus*), a pattern which may apply to other small and isolated plant populations (Steffan-Dewenter and Tscharntke 1999). Pollinator limitation can be detrimental to plant populations, especially those already endangered (COSEWIC 2009, WSDNR 2010).

Pollinators are central to the survival of some plant populations. However, there is some evidence that invertebrate pollinators such as bumblebee and honeybee populations have declined across North America (Cameron et al. 2011 and Ghazoul 2005, respectively). For this study, we were interested in exploring questions related to potentially declining pollinators in Puget lowland prairies, and considered various plants as possible candidates. Puget balsamroot (*Balsamorhiza deltoidea* Nutt.) emerged as a promising subject. Bees are probable pollinators for this species, as they are integral pollinators for its close relative, the arrowleaf balsamroot (*Balsamorhiza sagittata* (Pursh) Nutt.) (Cane 2005). In addition, Puget balsamroot is known to be a nectar source for other insects (Chappel and Crawford 1997) and is dependent upon these unidenti-

¹Author to whom correspondence should be addressed. E-mail: lfazzino@pugetsound.edu

fied species for pollination. We suspect that increasing fragmentation of the south Puget lowland prairies may be impeding pollinator movement among prairie fragments, potentially resulting in pollinator limitations for some species. With the Puget balsamroot currently listed as endangered in British Columbia and classified an R2 species of concern in the state of Washington (COSEWIC 2009, WSDNR 2010), patches of this plant are becoming more rare and more fragmented. Anecdotally, we have observed that Puget balsamroot does not produce plump seeds, a condition that has been suggested with other species as possibly indicating inadequate pollination (Wagenius 2004, Wist and Davis 2005). Therefore, we hypothesize that populations of this species may be subject to pollen limitation (i.e., insufficient transfer of high-quality pollen) due to either pollinator limitation or low quality pollen and that such pollen limitation is a factor contributing to the small populations sizes.

To determine potential pollination limitation, we compared seed set from naturally-pollinated plants with seed set of hand-pollinated plants on three different prairies. Hand-pollinated inflorescences presumably reach a maximum potential for seed production in the given environment, and can be used as a control with which to compare naturally-pollinated flowers for pollination effectiveness (Cane 2005). In addition, we evaluated Puget balsamroot's ability to self-pollinate, as this capability would affect its dependence on external pollination. If Puget balsamroot were determined to be self-incompatible and hand-pollinated inflorescences had greater reproductive potential than naturally-pollinated inflorescences, then it is likely that pollinator limitation is implicated in the low population levels found in the study area. However, as soil nutrient availability can also impact plant population levels (Bloom et al. 1985, Jensen et al. 2001, Epstein and Bloom 2005), we also analyzed soil nutrients (nitrate, ammonium, organic matter, phosphorus, potassium, calcium, magnesium, and manganese) for each prairie to investigate potential resource limitation in addition to pollinator limitation.

Methods

Puget Balsamroot

Puget balsamroot is found from southeast British Columbia (Vancouver Island) through the western part of the Sierra Nevada mountains in California (Douglas and Ryan 2001). Generally, the species grows in dry, shallow soils (Douglas and Ryan 2001). This perennial plant sprouts in the early spring and has generally

completed flowering by early summer, at which point seeds have formed and have been released (Douglas and Ryan 2001). There are 15 reported populations in Washington, although five have likely been extirpated (Joseph Arnett, Washington Natural Heritage Program, personal communication); there is little information regarding the size and stability of the 10 remaining populations.

Prairie Sites

We located three prairies on Joint Base Lewis-McChord near Rainier, WA, that had Puget balsamroot populations large enough for our study (> 45 plants). Sites included Johnson Prairie (N 46.92724, W 122.73322), Upper Weir (N 46.91526, W 122.70686), and 7S (N 47.10583, W 122.50431) (Figure 1). We used 15 - 19 pairs of Puget balsamroot on each prairie, depending on population sizes. The prairie soils were generally shallow, acidic, sandy to gravelly, and well-drained, with a thin (2.5 cm) layer of decomposed organic-matter on the surface. Due to a pronounced Mediterranean climate, drought conditions can be common in the summer (Crawford and Hall 1997).

Experimental Design and Data Collection

Pairs of Puget balsamroot plants were chosen based on similar size and close proximity. On one plant of each pair, we chose two inflorescences haphazardly with immature styles. One of these was hand-pollinated and the other remained naturally-pollinated as a control. On the other plant of each pair, we chose a single inflorescence, also showing no mature styles, to remain naturally-pollinated to serve as a control for differences in resource allocation of the first plant. A separate subset of plants was chosen on each prairie to test for self-pollination capabilities; between one and three inflorescences on each of these plants were tested for self-pollination (Cane 2005).

All treated inflorescences were bagged in mid-May while still immature to prevent uncontrolled pollination. These pollination bags were approximately 16 cm by 25 cm and made from fine mesh netting. Once mature, one of three treatments was applied to each inflorescence. Naturally-pollinated inflorescence bags were removed and inflorescences were pollinated through natural means only (insects and wind or self pollination) while hand-pollinated inflorescences received supplemental pollination. To hand pollinate inflorescences, mature anthers showing available pollen were taken from a separate individual and rubbed on each mature style of the test inflorescences (Cane 2005). Hand-pollination

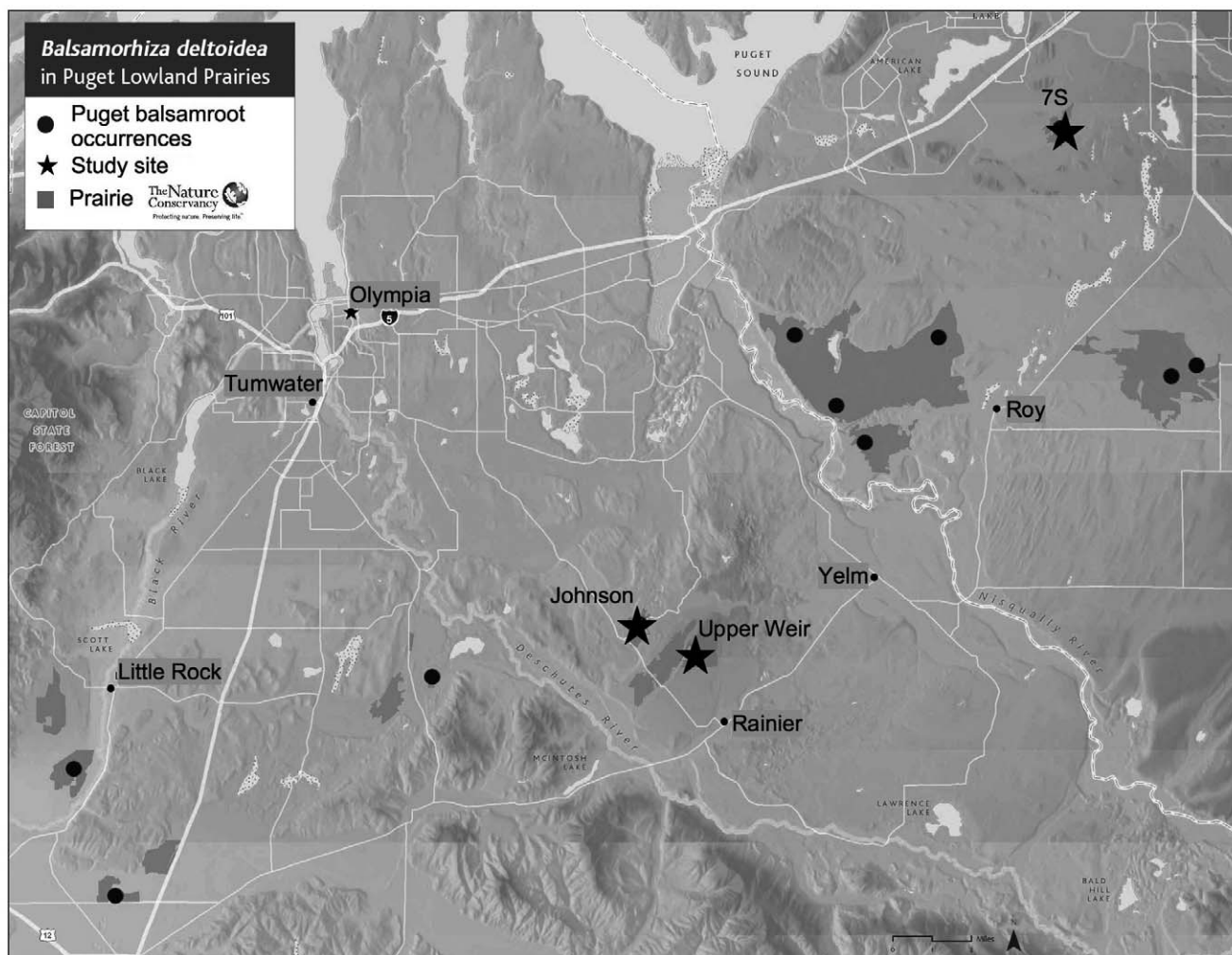


Figure 1. South Puget Sound prairies and study sites. Joint Base Lewis-McChord prairies are separated by distances of 0.5 km to 25 km, and vary in area from approximately 708 to 52 ha. Some sites are becoming particularly isolated, such as the 7S study site. (Reproduced with permission from the South Sound Nature Conservancy).

treatments continued throughout the flowering of inflorescences with hand-pollination occurring every other day until all styles were shriveled, approximately 16 days from first flowering between the end of May and first week of June, 2009 (Wagenius 2004). Self-pollinated inflorescences remained bagged until all styles were shriveled. After all styles had shriveled on an inflorescence, the bag was replaced with a coarser mesh predation bag to prevent seed loss and predation (Cane 2005). Once plants began dropping mature seeds in the second bags, the inflorescences were collected. They were transferred to brown paper bags and kept in a drying oven at 55 °C until analyzed.

After a minimum of seven days in the drying oven, the inflorescences were dissected for seed collection. Seeds were separated from scales and other detritus by hand. Seeds of each inflorescence were separated

into four categories blind to treatment (shriveled, less shriveled, less plump, and plump) based on physical attributes and the extent of shriveling (Figure 2). Shriveled seeds were completely withered and often broken or discolored brown rather than dark gray. Less shriveled seeds were mostly withered, but usually remained dark gray and often had a square shape at one end and distinct side ridges. Less plump seeds were only somewhat withered, and always dark gray, but not fully expanded. Side ridges were still pronounced, but less so. Plump seeds were also dark gray and fully expanded. The square top was completely gone and was instead rounded. Side ridges were only slightly pronounced. Seeds were then counted per inflorescence and weighed collectively. Seeds were kept in airtight plastic cups and frozen to preserve them for germination tests.



Figure 2. Seed morphological categories. Harvested seeds were separated into four categories according to physical attributes delineating probable viability: shriveled, less shriveled, less plump, and plump. Shriveling was the main characteristic taken into account, along with coloration and the shape of the ends of the seed coat. Shriveled seeds were often discolored brown, while other categories were dark gray. Furthermore, the plumper seeds were, the rounder and more full they appeared.

Germination Rates

Seeds were chosen randomly from each seed and treatment category and soaked for 24 hours in water before being placed between moist paper towels and kept at 0 °C for 8 - 10 weeks. Seeds were then returned to room temperature and allowed to germinate. After two weeks, sprouting seeds were counted and germination rates were determined. Germination rates were not determined for naturally-pollinated plump seeds because of inadequate sample size, and thus were assumed to be equal to naturally-pollinated less plump germination rates, as hand-pollinated germination rates of plump and less plump seeds were very similar (Table 1).

Initial Bee Populations and Floral Abundance

Bees were caught and counted in 30 pan traps per prairie on 23 May, 2009 to give an indication of the bee populations during the Puget balsamroot bloom (Droege 2008, Westphal et al. 2008). A line of 30 pan

traps of alternating colors (white, fluorescent blue, and fluorescent yellow) of 29.6 ml plastic Solo bowls containing soapy water were spaced at 5 m intervals on bare ground or flattened vegetation to catch bees (Droege 2008).

Floral surveys were conducted on 23 May, 2009 on each prairie and consisted of a single 1 m by 141 m transect in which all flowering heads intersecting the transect were counted. Inflorescences were counted as single flowering heads and were identified to species. These floral surveys represent an initial assessment of the potential for interspecific competition for pollination services for the Puget balsamroot. Species were weighted to standardize for flower size differences as determined by relative flower disc size.

Soil Analyses

On March 19, 2010, 12 replicate soil samples were taken at each of the three prairies in a 3 x 4 m grid covering the Puget balsamroot population to determine nutrient levels. Each sample was a composite of 6 - 8, 10 cm cores, 2 cm in diameter. Soil samples were analyzed for nitrate, pH, organic matter, ammonium, phosphorus, potassium, calcium, magnesium, and manganese by USA Analytical Services, Inc. (Pasco, WA) giving a relative indication of the nutrient pool available to Puget balsamroot in the three sites. Nitrate and pH were measured with ion-selective electrodes, organic matter was measured by back-titration, ammonium and phosphorus were measured colorimetrically, and

TABLE 1. Percent germination rates of Puget balsamroot.

Treatment	Seed Categories (%)			
	Shriveled	Less Shriveled	Less Plump	Plump
Hand-pollinated	0.0	13.0	30.7	31.7
Naturally-pollinated	0.0	4.0	10.4	10.4*
Selfed	0.0	0.0	0.0	0.0

*Plump naturally-pollinated seed germination rates were not measured directly.

potassium, calcium, magnesium and manganese were measured using atomic absorption spectrophotometry. All of these measured soil resources are necessary for successful plant growth and photosynthesis (Evans and Wildes 1971, Matoh and Kobayashi 1998, Stitt et al. 2002, Epstein and Bloom 2005).

Data Analysis

To combine the numbers of seeds in each category (shriveled, less shriveled, less plump, and plump) with the corresponding germination rates, “potential germinants per inflorescence” was calculated by multiplying the number of seeds in a specific category by the corresponding germination rate for each inflorescence. Pollination treatments within a prairie were compared using a one way ANOVA test. Pooled pollination treatments across prairies (all hand-pollinated and all naturally-pollinated) were transformed using square root of the common log to achieve homogeneous variances and compared using a t-test. Bee population data, floral abundance data, and flowers per bee data were compared among prairies using chi-square tests. Soil nutrient data were compared using a one-way ANOVA with a post-hoc comparison. All statistical analyses were carried out using SPSS 13.0 for Windows. Statistically significant differences were defined as $P < 0.05$.

Results

Germination Rates

No self-pollinated (selfed) inflorescences produced any viable seeds in any seed category. In addition, no seeds from the shriveled category germinated. However, less shriveled seeds did have limited germination in both hand-pollinated and naturally-pollinated inflorescences, 13.0% and 4.0% respectively. Furthermore, less plump and plump seeds proved to have comparable germination rates, 30.7% and 31.7% in hand-pollinated and 10.4% and 10.4% in naturally-pollinated inflorescences (Table 1).

Pollination Treatment Effects

Hand-pollinated inflorescences produced more than twice the number of potential germinants per inflorescence on all three prairies in comparison to naturally-pollinated inflorescences ($P < 0.001$). However, hand-pollinated inflorescences did not differ in the number of potential germinants among prairies. In comparison, naturally-pollinated inflorescences on Upper Weir produced significantly more potential germinants per inflorescence than those on 7S ($P < 0.020$), but those found on Johnson prairie did not differ in the number

of potential germinants per inflorescence from either Upper Weir or 7S ($P > 0.050$, Figure 3).

Bee Populations and Floral Abundance

Pan trapped bees provided a survey of bee populations on the three prairies (Table 2). Upper Weir and 7S, respectively had 62.3% and 37.3% more bees found in the pan traps on 23 May, 2009 than those found in Johnson ($P = 0.001$ and $P = 0.042$, respectively). However, there was no significant difference between the number of bees found in pan traps on Upper Weir and 7S ($P = 0.237$).

Floral transects provided a survey of the floral populations on the three different prairies and estimated the potential for competition for pollination services for the Puget balsamroot (Table 2). 7S had the most flowers with 171.5% more than Johnson and 740.8% more than Upper Weir ($P = 0.001$ both comparisons). Furthermore, Upper Weir had 209.7% more flowers than Johnson ($P = 0.001$).

To integrate the bee population and floral transect data, we also calculated the number of flowers requiring pollination services per bee observed on the prairies. 7S had significantly more flowers requiring pollination services per bee with 7.7 flowers per bee compared to both Johnson (4.6 flowers per bee) and Upper Weir (1.1 flowers per prairie) ($P = 0.02$) (Table 2). Johnson also had significantly more flowers per bee than Upper Weir ($P = 0.02$).

Soil Nutrients

Background soil nutrient data provided an estimate of nutrient pools found on the different prairies at the time Puget balsamroot is blooming (Table 3). 7S prairie had significantly more nitrate, organic matter, potassium, calcium, and magnesium than either Johnson or Upper Weir prairies ($P < 0.05$). Furthermore, both Upper Weir and 7S prairies had significantly more ammonium and manganese than Johnson prairie ($P < 0.05$). Phosphorus levels did not differ significantly among the three prairies and was uniformly low. Although these values give initial indications of soil nutrient levels, they only show a snapshot of nutrient levels as opposed to annual levels, and nutrient levels often change throughout the year depending on ecological circumstances and microbial loop activity (Jensen et al. 2001).

Discussion

We hypothesized that the small populations of Puget balsamroot found in the south Puget lowland prairies were the result of insufficient production of viable

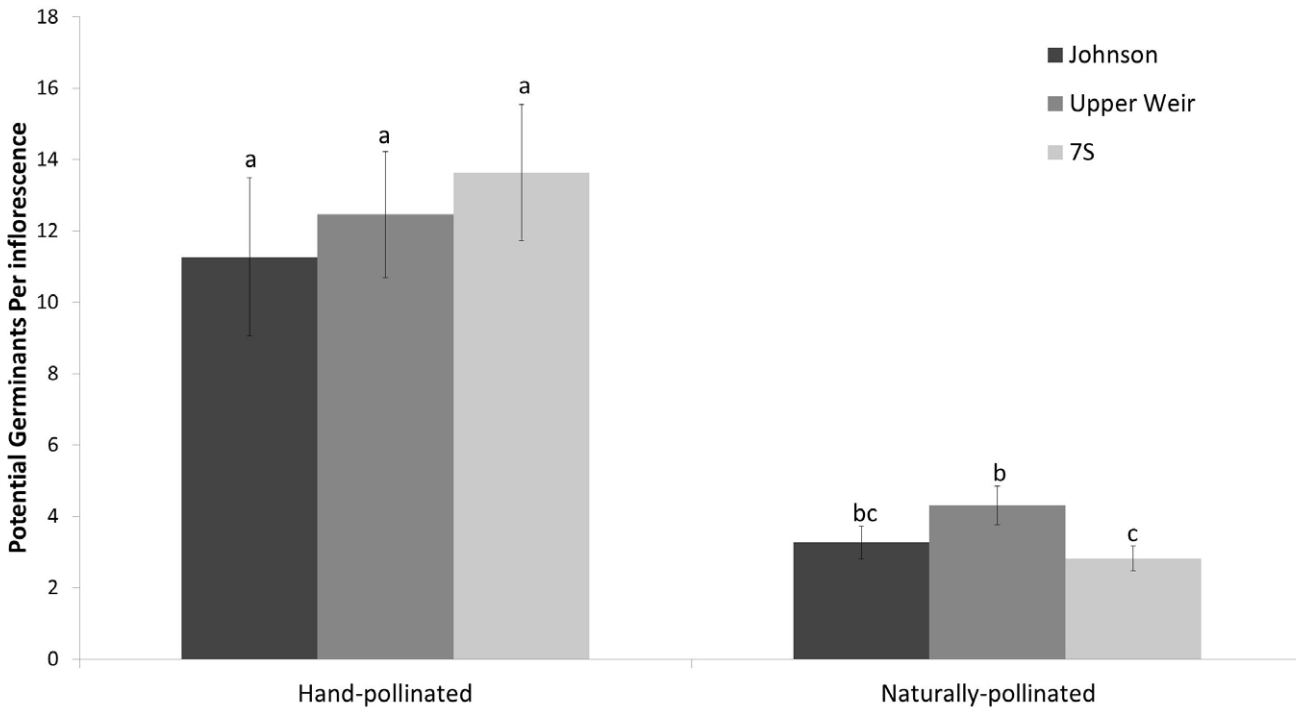


Figure 3. Number of potential germinants per inflorescence in hand-pollinated and naturally-pollinated inflorescences on three prairies. Selfed inflorescences showed no potential germinants in all prairies (not shown) ($n=9$ Johnson, 6 Upper Weir, 6 7S). Hand-pollinated inflorescences produced significantly more potential germinants than naturally-pollinated inflorescences on all prairies ($P < 0.001$). Upper Weir naturally-pollinated inflorescences had significantly more potential germinants than those on 7S, but naturally-pollinated inflorescences on Johnson did not differ from those on either Upper Weir or 7S ($P < 0.02$). Error bars represent standard deviation and bars that share letters are not statistically different at $\alpha = 0.05$ ($n=13, 13, 17, 27, 25, 35$).

TABLE 2. Survey of bee populations and floral abundance. Across each row, values that share a letter are not significantly different at $\alpha = 0.05$.

	Prairie		
	Johnson	Upper Weir	7S
Initial Prairie Survey			
Total Bees Observed (May)	69 ^a	95 ^b	112 ^b
Floral Abundance (Flowers)	319 ^a	103 ^b	866 ^c
Flowers per Bee	4.4 ^a	1.1 ^b	7.7 ^c

seeds via poor pollination or low nutrient availability. We estimated seed viability using potential germinants per inflorescence, an approach that combines morphological features of seeds indicative of viability and germination rates.

Self-pollination Potential

No seeds from any selfed inflorescences germinated, indicating that Puget balsamroot is not capable of self-pollination (Table 1). This simplifies the analysis of hand and naturally-pollinated effects on seed set since

TABLE 3. Average (\pm SD) soil nutrient levels of three prairie study sites. Across each row, values that share a letter are not significantly different at $\alpha = 0.05$.

Soil Nutrients (ppm)	Prairie		
	Johnson	Upper Weir	7S
Nitrate	2.8 \pm 1.1 ^a	2.5 \pm 0.6 ^a	4.9 \pm 0.9 ^b
Ammonium	43.2 \pm 10.8 ^a	59.4 \pm 16.4 ^b	58.8 \pm 17.2 ^b
Organic Matter	21.0 \pm 4.5 ^a	24.7 \pm 4.6 ^a	29.5 \pm 8.9 ^b
Phosphorus	2.6 \pm 0.7 ^a	3.6 \pm 3.1 ^a	5.5 \pm 6.1 ^a
Potassium	161.2 \pm 48.6 ^a	168.3 \pm 33.7 ^a	216.6 \pm 47.0 ^b
Calcium	5.6 \pm 2.5 ^a	5.5 \pm 1.5 ^a	12.1 \pm 5.3 ^b
Magnesium	1.1 \pm 0.5 ^a	1.1 \pm 0.3 ^a	2.1 \pm 0.8 ^b
Manganese	18.9 \pm 6.3 ^a	23.1 \pm 10.5 ^b	32.2 \pm 18.4 ^b
pH	5.6 \pm 0.2 ^a	5.6 \pm 0.4 ^a	5.9 \pm 0.2 ^b

viable seeds cannot be produced via self-pollination. However, it is possible that attempted but unsuccessful self-pollination can interfere with natural-pollination by clogging receptive stigmas with low-quality pollen (i.e., nutritionally low or genetically incompatible),

thereby preventing viable pollen tubes from reaching an ovule, and preventing the creation of a viable seed (Ramsey and Vaughton 2000). Although we did not test this hypothesis, stigma clogging could drastically influence Puget balsamroot pollination success and should be investigated in the future.

Seed Morphology Categories

Seed morphology categories provide an indication of pollination success, since plump seeds indicate high seed viability (Wagenius 2004, Wist and Davis 2005), which is possible only with successful pollination using high quality pollen. Our results showed that germination rates were consistently greater for plump and less plump seeds than for the less shriveled seeds, regardless of whether they were hand pollinated or naturally pollinated (Table 1). Therefore, high germination rates indicate a higher pollen quality in Puget balsamroot, although available resources undoubtedly play a role in germination rates as well in a field setting. Just as plump seeds can be explained by pollination with high quality pollen, the presence of shriveled seeds can be explained by pollination with low quality pollen.

Pollination Limitation

Hand-pollination treatments should indicate the true potential of inflorescences to produce viable seeds with their available resources, whereas natural-pollination should indicate the realized ability to produce seeds (Cane 2005). Hand-pollinated inflorescences produced significantly more potential germinants than naturally-pollinated inflorescences on all three prairies (Figure 3), indicating that naturally-pollinated inflorescences are not producing the maximum seed set of which inflorescences are capable given the available resources. These results support the hypothesis that pollination limitation exists in Puget balsamroot on all three prairies.

Pollination failure (i.e., unsuccessful pollination) can be caused by multiple factors. Wilcock and Neiland (2002) describe three types of pollination failure: pre-dispersal, dispersal, and post-dispersal. We cannot reliably separate these three types of pollination failure because all three stages of pollination failure contribute to our measured seed set characteristics. However, we focused on possible dispersal pollination failure, defined as the failure of pollen to be transferred from anthers to stigmas (Wilcock and Neiland 2002) by examining pollinator populations and plant populations flowering simultaneously with the Puget balsamroot. Dispersal pollination failure includes the possibility of 1) having too few pollinators compared to the number of flowers

on a site and 2) flowers being relatively unattractive to potential pollinators, when compared to other flowers on site.

The number of potential pollinators for Puget balsamroot can be affected by both the number of pollinators on the prairie and the number of flowers to be pollinated. Upper Weir prairie had the highest number of flowers per pollinator (Table 2), which may explain why it also had the highest number of germinants per inflorescence (Figure 3).

However, almost equal numbers of bees observed and the number of flowers requiring pollination on Upper Weir does not imply that equal pollination of all flowering species took place. Many factors can contribute to the successful pollination of a flower including, but not limited to, floral display-number and size of flowers (Conner and Rush 1996), flower morphology (e.g., Aigner 2004), flowering time (Hirao et al. 2006), and nutritional quality of pollen (Robertson et al. 1999). Interactions of these factors affecting pollination could be especially important on the 7S prairie where there may be more competition for pollinator services compared to that of Johnson and Upper Weir prairies illustrated by the high number of flowers per bees found on 7S. However, we did not conduct any preference tests that directly compared the attractiveness of Puget balsamroot with other prairie flowers and therefore cannot accurately discuss these interactions. Such a study would certainly provide useful insight into pollinator preferences and allocation of pollination services in an ecosystem.

Pollination limitation may also be affected by nutrient availability to the plant. An increase in nutrients available for growth and photosynthesis allows for an optimal allocation of energy to other needs (Bloom et al. 1985, Tilman 1988) such as seed maturation. 7S had significantly greater quantities of many soil nutrients (nitrate, organic matter, potassium, calcium, and magnesium) than either Johnson or Upper Weir ($P < 0.05$), and Upper Weir had significantly more of two soil nutrients (ammonium and manganese) than Johnson ($P < 0.05$) (Table 3). These results suggest significant differences exist in baseline resource availability on the three prairies. However, the highest potential germinants in naturally-pollinated Puget balsamroot were observed on Upper Weir, not 7S. This result suggests that the 7S prairie was the most severely pollinator-limited of the three prairies.

Our data support the hypothesis that pollination limitation of the Puget balsamroot is occurring on the south Puget lowland prairies, which could partially explain

the small population sizes of the Puget balsamroot. As soil nutrients were unable to explain patterns of pollination limitation in our three prairies, we conclude that this limitation is likely caused by inadequate pollinator populations. Other investigators have suggested that as fragmentation increases, pollinators are unable to commute to all sections within a formerly contiguous site, increasing limitations on local flora (Steffan-Dewenter and Tscharntke 1999, Osborne and Williams 2001, Aguilar et al. 2006, Dauber et al. 2010). Determining individual ranges of the major pollinators in the south Puget Sound prairies would be helpful in determining

whether these prairies are becoming too fragmented to support pollinator populations.

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