Pollinators limit seed production in an early blooming rare plant, Barneby's clover (*Trifolium barnebyi*), in central Wyoming

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ABSTRACT

The reproductive ecology of rare plants is often not known, yet the persistence of plant populations depends on successful mutualisms with pollinators and favorable environmental conditions. As atmospheric temperatures rise, phenology of plants and pollinators may become mismatched, and vegetative reproduction may be reduced due to higher temperatures and less moisture during the growing season. We investigated the reproduction of Barneby's clover, a mat-forming perennial endemic to central Wyoming that grows in the crevices of sandstone bedrock. Our objectives were to evaluate a method for monitoring changes in vegetative growth as well as assess the pollination and seed-set of Barneby's clover. We established five monitoring locations and used a divided frame to estimate percent cover along each transect. We conducted seed-set experiments in three locations to measure self-pollination and if pollinating insects limited seed production. We used vane traps and bee bowls to capture pollinators and examined pollen carried on bees. Percent cover along transects was closely associated with spring moisture and the previous year's results. The percent cover of Barneby's clover decreased over the four years we monitored. Barneby's clover did not self-pollinate, but the plant appeared to be highly reliant on pollinators. The number and mass of viable seeds per flower, and number of bees captured increased as the season progressed indicating that more and larger seeds were made when more pollinators were present. Pollen from Barneby's clover was primarily carried by *Andrena* bees, although we found the pollen on seven other bee genera. A mismatch in timing between blooming and pollinating insect emergence could limit seed production in the future if Barneby's clover blooms earlier over time; however, bloom timing for Barneby's clover ranged between April and June depending on the microhabitat the plant lived in and we observed much higher seed production in later blooming plants. Continued monitoring of mat cover and the timing of blooming will provide information about the condition of this rare plant.

INTRODUCTION

Rising air temperatures are causing a mismatch in the phenology of plants and their pollinators (Rafferty et al. 2015). As spring continues to begin earlier each year, the plant-pollinator relationships may be altered by a disparity between the flowering season and the emergence of pollinators (Parmesan and Yohe 2003; Memmot et al. 2007). Warmer spring temperatures can cause plants to bloom earlier (Parmesan and Yohe 2003; Kameyama and Kudo 2015), which may (Bartomeus et al. 2011) or may not (Memmot et al. 2007; Kudo 2014) correspond with earlier pollinator emergence. Rising temperatures may change more than the timing of emergence. For example, Memmot et al. (2007) estimate that up to 50% of pollinator species will undergo increasing restrictions to the quantity of available food due to the effects of increasing temperatures on flower phenologies. Therefore, rising air temperatures have the potential to negatively impact pollination and these effects may be even more pronounced for

rare plants. Rare plants occur in small areas or at low densities and often have specialized pollinators (Rasmussen et al. 2020) suggesting that a mis-match between their phenologies could have dire effects for both pollinators and their host plant (Memmot et al. 2007; Kudo 2014).

In addition to the emergence timing of pollinators potentially changing, native pollinators are also declining globally (Abrol 2012; Wagner 2020; Potts et al. 2010). Bees and butterflies are among the groups reported with the highest declines, and these insects are the most prolific pollinators (Goulson and Nicholls 2016; Rhodes 2018). The combination of fewer pollinating insects and the potential for mismatched timing with blooming flowers indicates these mutualisms may be at risk. Fewer pollinators could mean less genetic diversity in plant populations and fewer viable seeds produced (Aigner 2004; Ramos-Jiliberto et al. 2020). Without the genetic variation provided by sexual selection, a species may have reduced biological fitness to confront changes in the environment (Agrawal 2001). Rare plants may be especially vulnerable to declining pollinators, because a higher proportion of such species use specialist pollinators (Rasmussen et al. 2020). For example, the mat-forming Alpine clover (*Trifolium dasyphyllum*) was highly reliant on bumblebees for pollination (Bauer 1983). The decline of insect pollination services has been associated with such declines in several insect-pollinated plants (Biesmeijer et al. 2006).

Rare plant species provide services to the habitats and ecosystem in which they live (Xu et al. 2020), but there is often little known about their ecology (Burmeier and Jensen 2008; Miller et al. 2019). Rare plants can be the dominant species in the habitats where they occur and in others can have low densities (Rabinowitz 1981). Regardless of their abundance, information about the ecology of a rare species is essential for making informed management decisions (USDI 2001, 2010). Knowledge of a plant's reproduction is critical to understand a species current trajectory and their ability to persist. Plants can reproduce through two main ways; vegetative reproduction occurs by producing ramets, which are genetically identical to the parent plant through rhizomes or similar structures, and sexual reproduction occurs through seed production where pollen is usually transferred from another plant resulting in increased genetic diversity. Plants that can reproduce both vegetatively and sexually may be more likely to be self-incompatible (pollen and ovule from same plant cannot produce seeds) in order to maintain genetic diversity (Aigner 2004). Nearly 88% of plants globally rely on animals to transport pollen among plants to maintain genetic diversity (Ollerton et al. 2011). Most insects visit flowers to drink the nectar and pollen sticks to their bodies while they drink. In addition to drinking nectar, bees actively collect pollen to feed their young, signifying that bees are the most prolific pollinators in most ecosystems (Larson et al. 2018). When we observe a bee visiting a flower, that bee may be getting a drink, collecting pollen or both. In order to understand which plants bees actively collect pollen at, the pollen loads on bees must be examined. Examining the pollen on pollinators collected in the vicinity of a specific plant is a valuable method of estimating which insects transport pollen.

We studied the reproduction of a rare, mat-forming plant endemic to central Wyoming to estimate the degree mats changed and seed production was limited in an early blooming plant. Our objective was to evaluate a method for monitoring changes in vegetative growth and estimate the pollination and seed-set. Our study plant was Barneby's clover (*Trifolium barnebyi*), which lives in crevices of sandstone bedrock and is only known from a small area. Barneby's clover blooms early in the spring before most other plants in its habitat, which may make this rare species particularly vulnerable to pollinator declines. Our specific questions were: 1) Does the cover of Barneby's clover mats vary among years?, 2) Can Barneby's clover self-pollinate? 3) To what degree do pollinators limit viable seed production? and 4) What pollinators carry the pollen from Barneby's clover? Results will provide information to managers about what may limit the reproduction of this rare plant.

METHODS

Study Species

Barneby's clover is a mat-forming perennial forb (≤5 cm tall; 2 in) that is a Bureau of Land Management (BLM) Wyoming Sensitive Species (USDI 2001, 2010). The first known collection was by H. Dwight, D. Ripley and Rupert C. Barneby in 1947. Duane Isely, a specialist in the Fabaceae (Leguminosae), described this collection as the type for *Trifolium haydenii* var. *barnebyi* (Isely 1980). In 1981, Robert Dorn and Robert Lichvar elevated the taxon to a species, *Trifolium barnebyi*, based on their own collections (Dorn and Lichvar 1981). Although surveys and additional collections have added significantly to the known extent of Barneby's clover since its description (Marriott 1986, Fertig 1995, Scott and Sato 1998), little is known about population trends, life history, or pollination ecology of the species. Photo-monitoring on The Nature Conservatory (TNC) was established in 1995 and repeated in 2017, suggesting that the cover of mats reduced for Barneby's clover over that 22 year time period (Anderson 1995, Coffman 2017).

Study Area

The five known populations of Barneby's clover are in the southeastern foothills of the Wind River Range in Fremont County, Wyoming (Figure 1). Barneby's clover grows in the seams and crevices of Nugget Sandstone and the Frontier Formation in the southeastern foothills of the Wind River Range (Marriott 1986, Fertig et al. 1994). Eighty percent of Barneby's clover populations partially or wholly occur on lands managed by the BLM Lander Field Office. Most Barneby's clover grows on sparsely vegetated rock outcrops along the rim of Red Canyon. Other areas occupied by Barneby's clover are between large flat rocks on slopes in the open or among dry conifers (*Pinus flexilis*, *Juniperus scopulorum*) and sagebrush (*Artemisia tridentata*). The area receives an average of 33.3 cm (13.1 in) of precipitation annually (Lander airport weather station; Western Regional Climate Center, wrcc@dri.edu).
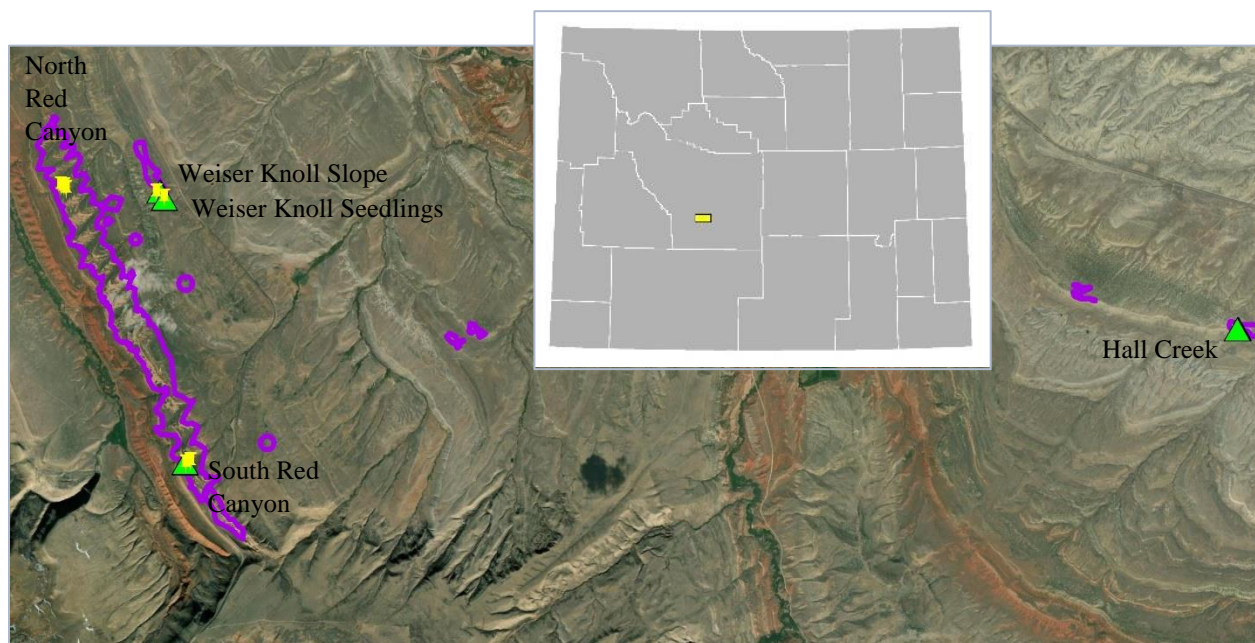

Figure 1. The location of monitoring transects (green triangles), and seed-set experiments and pollinator sampling stations (yellow pins) in the Barneby's clover populations (purple outlines). The inset map shows the location of our study area in Wyoming (ESRI 2011).

Monitoring

To assess the trends and vegetative reproduction of Barneby's clover on BLM lands, we established five monitoring transects and estimated cover annually in July between 2018 and 2021. We placed two transects at Weiser Knoll and called them Weiser Slope and Weiser Seedlings. Hall Creek was divided into Hall 1 and Hall 2. One site was monitored at South Red Canyon. Transect sites were chosen to reflect a range of management (i.e. grazing, recreation), ecological conditions, and substrates (Figure 2; Appendix A). Each transect consisted of a belt between 6.9 and 18 m (22.6 and 59 ft) in length where a plot frame (30 x 30 cm) with sixteen 7.5 x 7.5 cm cells (Figure 3) was placed on the ground along the belt to assess cover. At South Red

Canyon and Weiser Knoll Seedling transects, the frame was placed both above and below the belt because the transects were relatively short (15 m and 6.9 m, respectively). We placed the plot frame on one side of the belt at the other sites. Specific information on the location and placement of the transects is in Appendix A (Transect Maps and Coordinates). We placed the plot frame at each 30 cm intervals along the belt and counted the number of cells with Barneby's clover present. We used a plot frame to monitor Barneby's clover because the compact and extensive mats of this species makes distinguishing individuals very difficult without destructive sampling. Using the line-point intercept method could result in a wide range of results due to the patchiness of the mats, especially at Hall Creek where Barneby's clover mats only grow in narrow rock crevices. In order to get repeatable and quantifiable sampling, we used a plot frame to collect information on cover. We took photographs of each frame and examined them for the presence of Barneby's clover in each cell. Cells were scored as present (1) or absent (0) to calculate the percent of cells in which live Barneby's clover were present (Table 1).
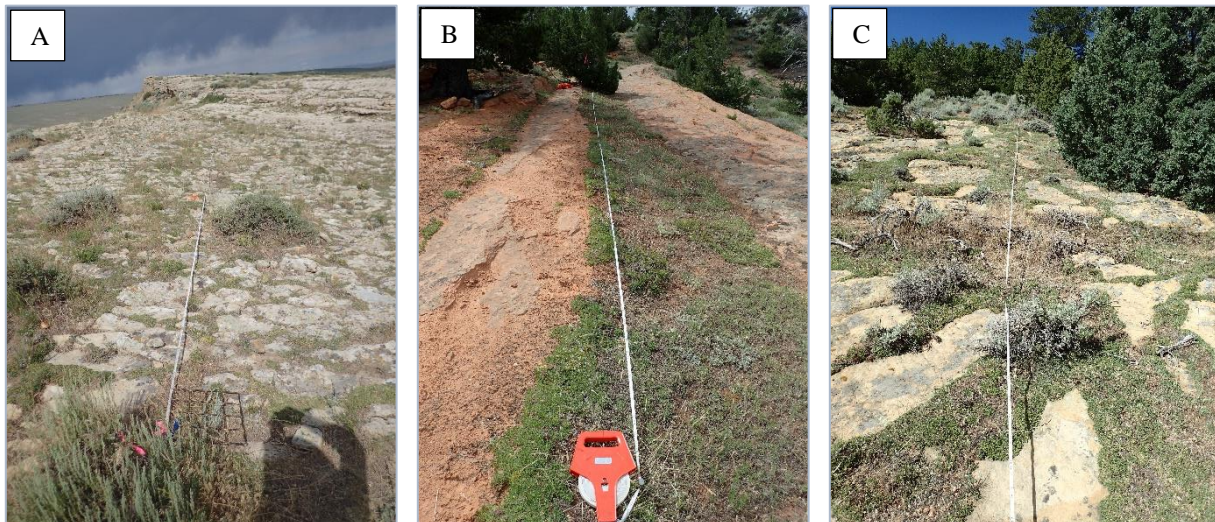

Figure 2. We established five monitoring transects for Barneby's clover populations at three sites. A.) Hall Creek, B.) South Red Canyon and C.) Weiser Knoll.

Figure 3. The frame used to monitor Barneby's clover is 30 x 30 cm with 16 - 7.5 x 7.5 cm cells. Barneby's clover was counted as present in a cell if any part of the plant appears within a cell. For example, 12 of the cells in the photograph contain live Barneby's clover. The frame was moved along a belt for a pre-determined distance.

Seed-Set Experiments

We measured the seed production of Barneby's clover to estimate the degree to which pollinators may limit sexual reproduction. We selected three areas along the rim of Red Canyon where Barneby's clover is most abundant (North Red Canyon, South Red Canyon and Weiser Knoll) to collect pollinators and measure seed-set (Figure 4). We measured seed-set of bagged, open, and hand-pollinated flowers to estimate the relative importance of self- and cross-pollination. We selected 20 Barneby's clover plants to estimate the degree to which these pollen limited seed production. Plants were selected before flowers bloomed from 15 April through 16 May 2019 and each plant cluster received the three treatments. Bagged treatments restricted pollinator access and measured the degree to which flowers can self-pollinate. Open treatments left blooms accessible to local pollinators to visit and measured ambient levels of seed-set. The hand-pollinated treatment added excess pollen in addition to local pollinators to measure seed production when pollen was not limiting. Pollen came from plants at least 50 m away and we delicately brushed collected anthers on the stigma of the treatment bloom. Blooms were bagged with mesh bags before (bagged treatment) or after (open and hand-pollinated treatments) flowers bloomed to contain the developing seeds and were held in place with fishing line weighted with color-coded eye bolts (Figure 5). For the hand-pollinated treatment, we recorded the number of flowers pollinated, and marked and recorded the flowers not ready for pollination in each flowerhead. We recorded the date that each flower in the hand pollinated treatment bloomed and was hand-pollinated. We monitored treatments and collected fruits when flowerheads were ripe,

from 4 June through 17 July 2019. Flowerheads were placed in paper bags, returned to the laboratory and dried.
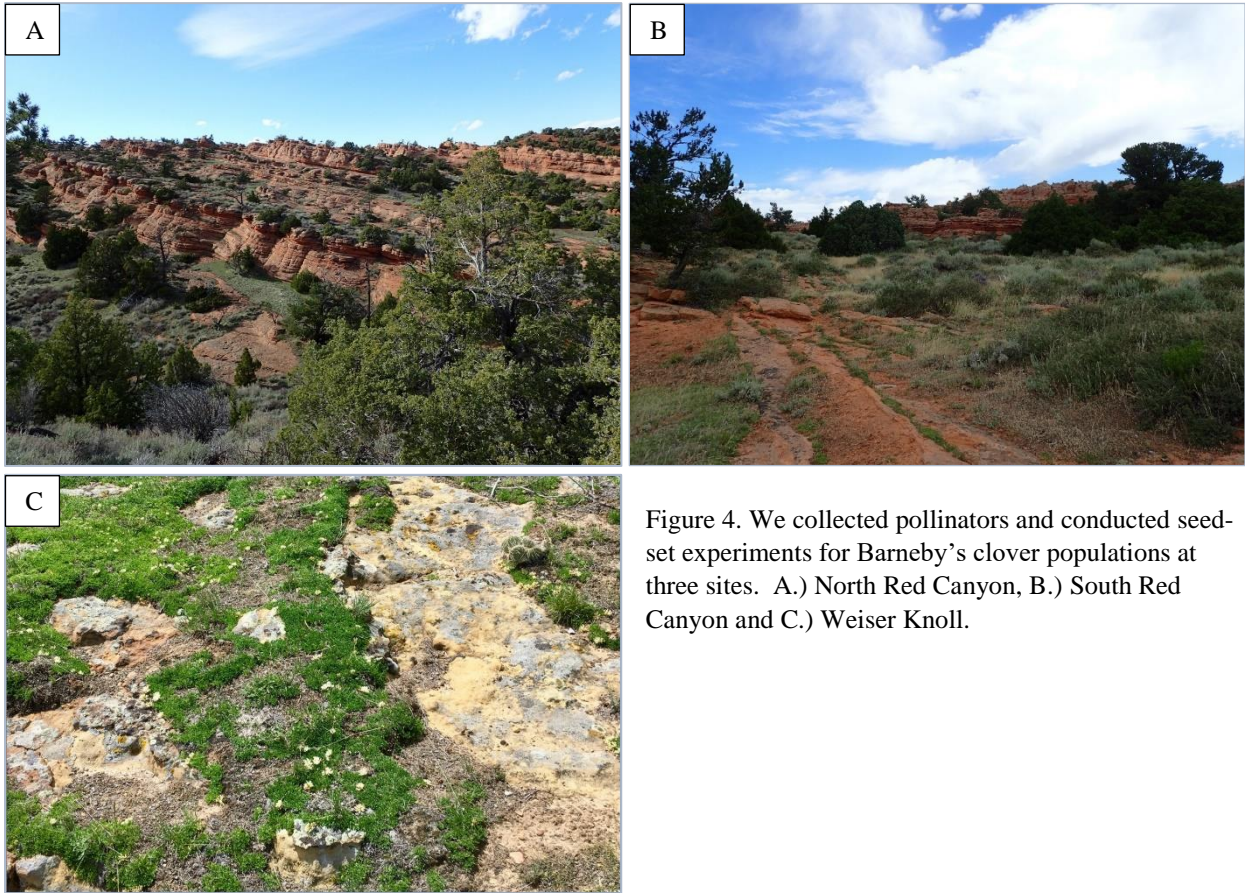
Figure 4. We collected pollinators and conducted seed-set experiments for Barneby's clover populations at three sites. A.) North Red Canyon, B.) South Red Canyon and C.) Weiser Knoll.

Each Barneby's clover flower produced only one legume seed pod, and there were multiple flowers per flowerhead. We cleaned, counted, and weighed seed pods and seeds to estimate the degree to which Barneby's clover self-pollinated or depended on pollinators. We counted the number of flowers per flowerhead and the number of ovules per flower. We examined the pods under a dissecting microscope to count the ovules. We weighed the pods and seeds together, because the unfertilized ovules were too small to remove from the pods without damaging. We noted seeds that appeared viable by appearance (size and mass) counted and weighed them for each plant. We tested viability of the seeds that appeared fertilized with Tetrazolium staining. Tetrazolium has been used to measure the germinative potential of seeds since at least 1918 (Lindenbain 1964). We placed the seeds between moistened paper towels for 24 hours, cut them to expose the endosperm, and immersed them in tetrazolium solution for 24 hours. The endosperm of viable seeds turned pink or red, while the endosperm of non-viable seeds remained white.
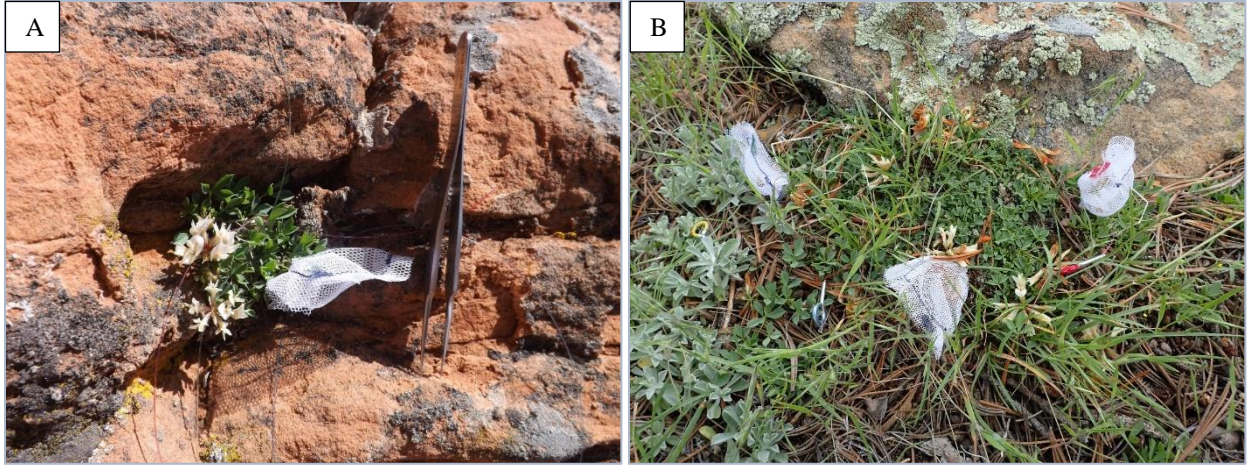
Figure 5. A.) A Barneby's clover plant with a mesh bag over the pollinator restricted treatment, and the hand-pollinated and open treatments marked with yellow and red fishing line, respectively. B.) A Barneby's clover plant with a mesh bag over each treatment, after pollination.

Pollinators

We collected pollinators at the same sites as the seed-set experiments to estimate which insects are pollinating this rare plant. We deployed seven pollinator stations across sites for 24-48 hours 10 times between 15 April and 21 June 2019. Pollinator stations consisted of one blue vane trap (vane trap hereafter) and three bee bowls (yellow, blue and white) filled with soapy water (Figure 6). We recorded the location, dates and times we deployed and retrieved pollinator samples, weather, and other notes for each sampling event. Pollinator stations were used to estimate the abundance and diversity of pollinators within the Barneby's clover population, and to assess which pollinators collect Barneby's clover pollen.
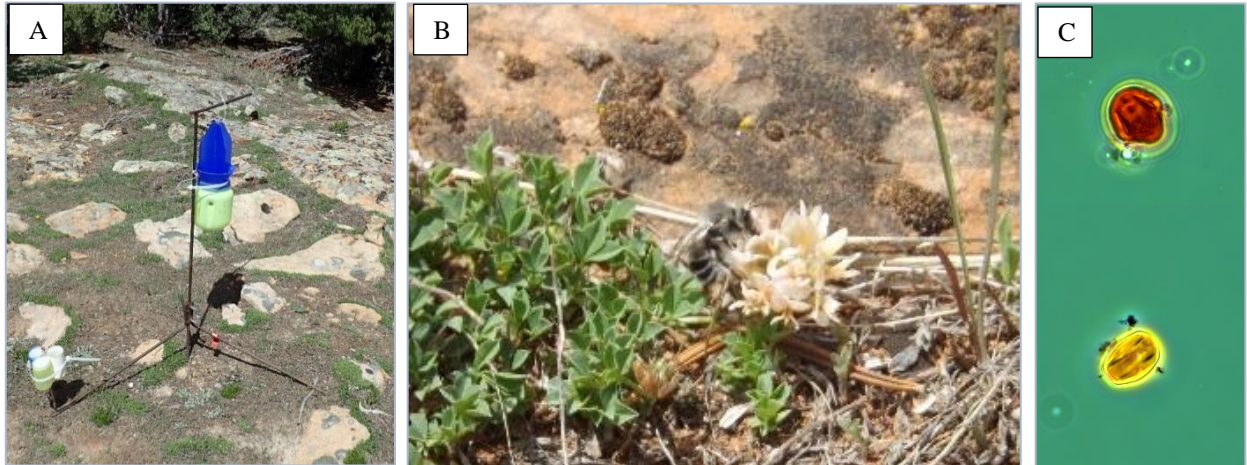

Figure 6. A.) We collected pollinators using pollinator sampling stations with vane traps (top) and bee bowls (bottom). B.) *Anthophora* visiting a Barneby's clover bloom and a C.) stained Barneby's clover pollen grains.

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Pollen

We identified pollen carried on bees to estimate who pollinated Barneby's clover. We collected and identified (Dorn 2001) plant species that were blooming at the same time as Barneby's clover and we made a pollen library from the collected flowers. We prepared pollen from the scopa of individual bees (one hind leg for all bees except those with scopa on abdomen). We performed acetolysis and stained pollen with Safranin O from flowers and bees to make features on pollen grains clearer before slide mounting (Jones 2014). We counted and identifying pollen grains from all female bees with scopa (non-cleptoparasites) under a compound microscope at 200x.

Analyses

We used random effects models (lmer) and linear regression (lm) to estimate differences among variables. Differences in the number, mass and viability of seeds were assessed using random effects models where plant cluster was the random effect, and treatment and site were fixed effects using lme4 package (Bates et al. 2015). When a fixed effect was significant ($\alpha = 0.05$), we used the emmeans function in the emmeans package (Lenth 2021) with a Bonferroni adjustment to estimate which treatments or sites differed. We used linear regression to estimate how the number of viable seeds changed with the number of pollinators captured and Julian day. We transformed the number and mass of seeds because they were not normally distributed or had non-constant variance using $\ln(x+1)$. All analyses were done in Program R (R Core Team 2017).

RESULTS

Climate

Spring precipitation varied among years and months. The year 2020 was drier than 2018, 2019 and 2021 ($p < 0.0001$). Precipitation differed among all months ($p \leq 0.009$) except April and May ($p = 0.98$). In general, June was typically the driest month, and April and May were the wettest months (Figure 7). Mean maximum and mean temperatures were higher in June 2021 compared to previous years and May 2019 were cooler than the other years.
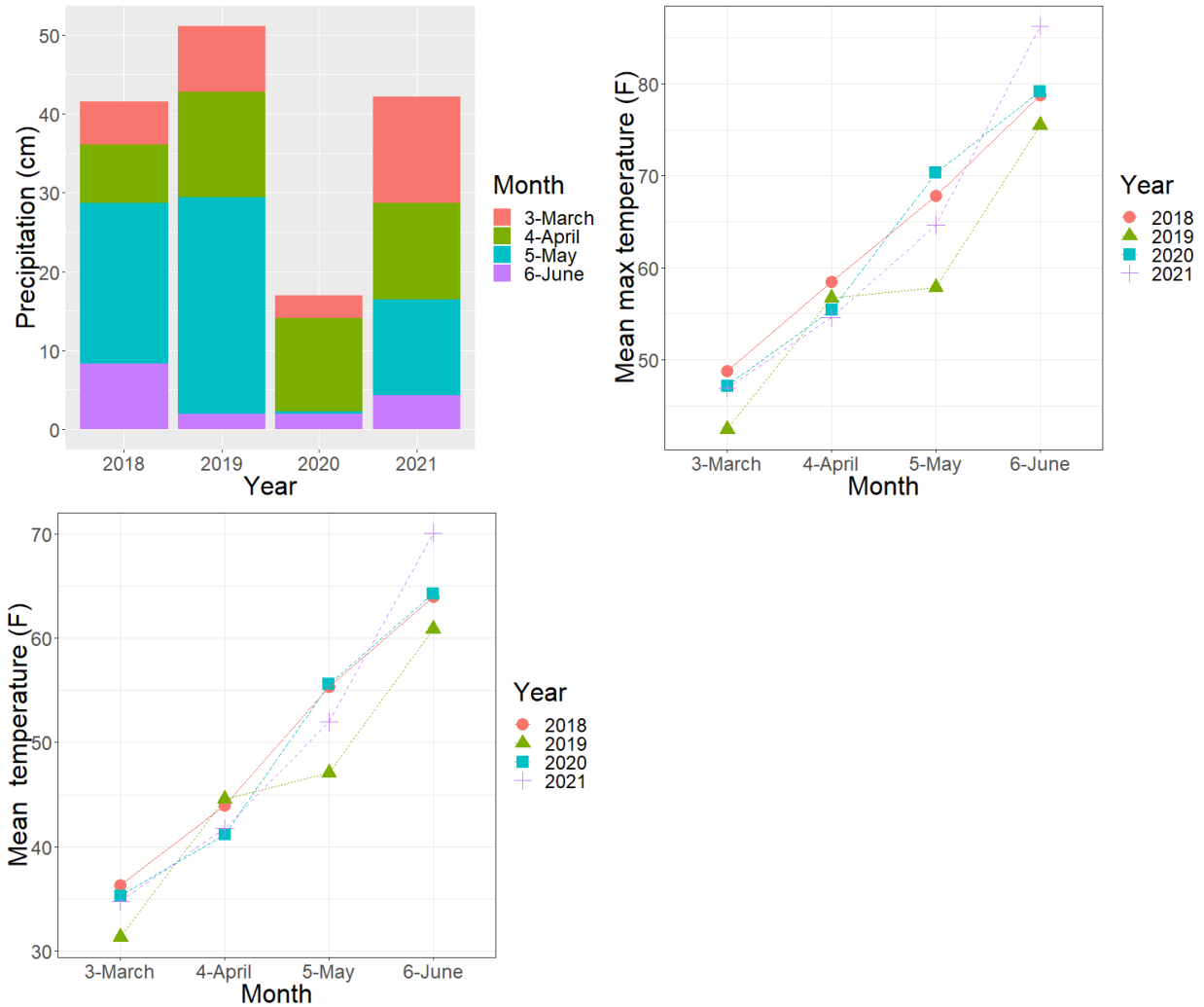
Figure 7. Precipitation varied among years and months that we studied Barneby's clover. The driest year was 2020, and April and May were the wettest months.

Monitoring

The percent cover of Barneby's clover decreased over the four years of monitoring, which is likely due to lower precipitation. Cover of Barneby's clover decreased among years (slope = -0.056, t = -13.3, p < 0.0001). Barneby's clover declined in 2020, coinciding with lower precipitation in March and May of that year (Table 1, Figure 8). Less precipitation generally decreased the cover of Barneby's clover among the four years of measurement (t = 2.8, p = 0.005); however, neither mean temperature (t = -0.5, p = 0.62) nor mean max temperature (t = -0.74, p = 0.46) altered cover. Cover of Barneby's clover was similar in 2020 and 2021 despite higher spring precipitation in 2021. The cover of Barneby's clover varied among sites (p < 0.0001); the Hall sites had the highest percent cover and Weiser Seedlings had the lowest (Figure 8).

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Table 1. Percent cover of Barneby's clover at five sites in central Wyoming measured annually in July between 2018 and 2021

Site	2018	2019	2020	2021
Hall 1	64.5	65.6	59.1	60.2
Hall 2	59.4	59.9	53.1	53.4
Red Canyon	40.4	40.1	33.8	34.7
Weiser Knoll Seedlings	27.2	24.5	22	21.3
Weiser Knoll Slope	47.8	49.7	44.5	44.5

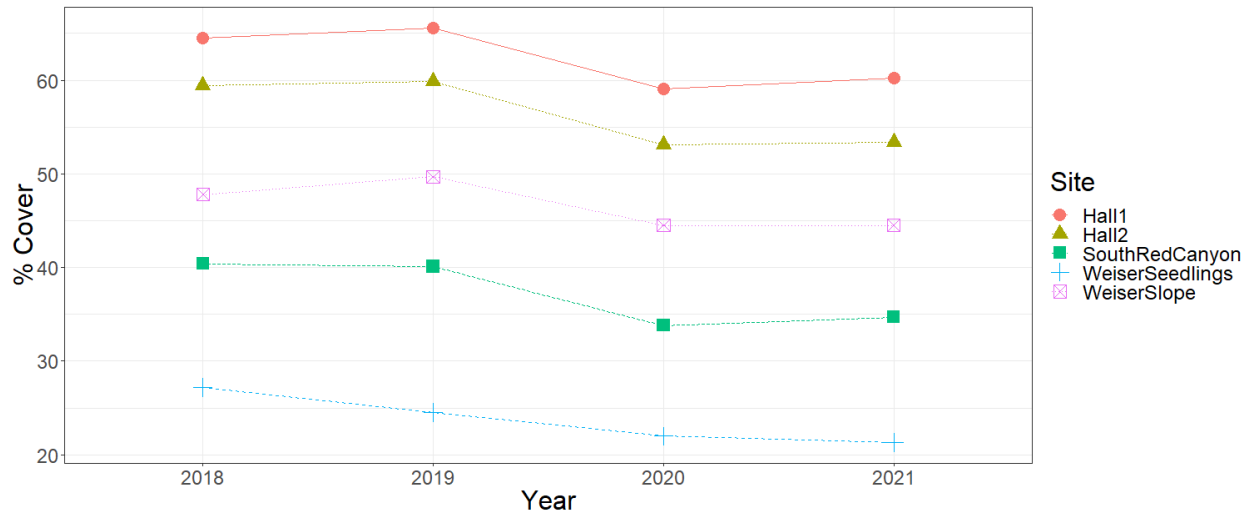
Figure 8. Percent cover of Barneby's clover between 2018 and 2021 at five sites in central Wyoming between 2018 and 2021.

Seed-set Experiments

Barneby's clover required insect pollinators to produce viable seeds. The number of ovules per flower did not differ among the three treatments (lmer; $F = 0.06$, $p = 0.94$; Table 2; Figure 9a) or among the sites (lmer, $F = 0.43$, $p = 0.66$); however, the mass of ovules in a pod differed by treatment (lmer; $F = 8.8$, $p = 0.0007$) and site (lmer; $F = 6.1$, $p = 0.01$). The bagged treatment (Bonferroni, $p = 0.0005$) produced lighter ovules compared to the open and hand-pollinated treatments (Bonferroni, $p > 0.07$). The Weiser Knoll site produced the heaviest ovules (Bonferroni, $p = 0.01$) compared to the other two sites (Bonferroni, $p > 0.13$).

The number of viable seeds per flower estimated using Tetrazolium staining differed by treatment (lmer; $F = 11.3$, $p = 0.0002$; Figure 9b) and site (lmer; $F = 7.0$, $p = 0.007$; Figure 9c). The bagged treatment (Bonferroni, $p = 0.0001$) produced zero viable seeds, and the open (Bonferroni, $p = 0.07$) and hand-pollinated treatment (Bonferroni, $p = 0.09$) produced viable seeds. The Weiser Knoll site produced the most viable seeds (Bonferroni, $p = 0.006$) and the South Red Canyon site produced the fewest (Bonferroni, $p = 0.05$).

The mass of viable seeds per flower differed by treatment (lmer; $F = 16.3$, $p < 0.0001$; Figure 9d) and site (lmer; $F = 3.2$, $p = 0.07$). The lightest seeds were made in the bagged treatment (Bonferroni, $p = 0.0001$) and the heaviest seeds were produced in the hand-pollinated treatment (Bonferroni, $p = 0.005$). The open and hand pollinated treatments did not differ indicating that pollen was not limiting in 2019 (Bonferroni, $p = 0.14$; Table 2; Figure 9). Plants at Weiser Knoll (Bonferroni, $p = 0.07$) produced the heaviest seeds and South Red Canyon made the lightest seeds (Bonferroni, $p = 0.32$). Fertility appeared to vary among individual plants. Plants that produced viable seeds in the hand pollinated treatment were more likely to also produce viable seeds in the open treatment; however, the random effect term identifying individual plants was never significant ($p > 0.13$).

Table 2. Barneby's clover did not produced seeds when pollinators were excluded (bagged treatment); however, more, heavier and viable seeds were produced when insects pollinated blooms (open treatment) and when excess pollen was available (hand-pollinated treatment). Mean values and standard errors for each variable and treatment.

Variable	Bagged	Hand Pollinated	Open
Number of ovules per flower	2.1±0.1	2.1±0.1	2.1±0.1
Mass per ovule in pods (µg)	40.1±2.4	449.9±89.3	478.9±125.3
Mass of viable seeds (µg)	0.0±0.0	1849.7±361.7	1543.7±356.5
Number of viable seeds per flower	0.0±0.0	0.2±0.04	0.2±0.06
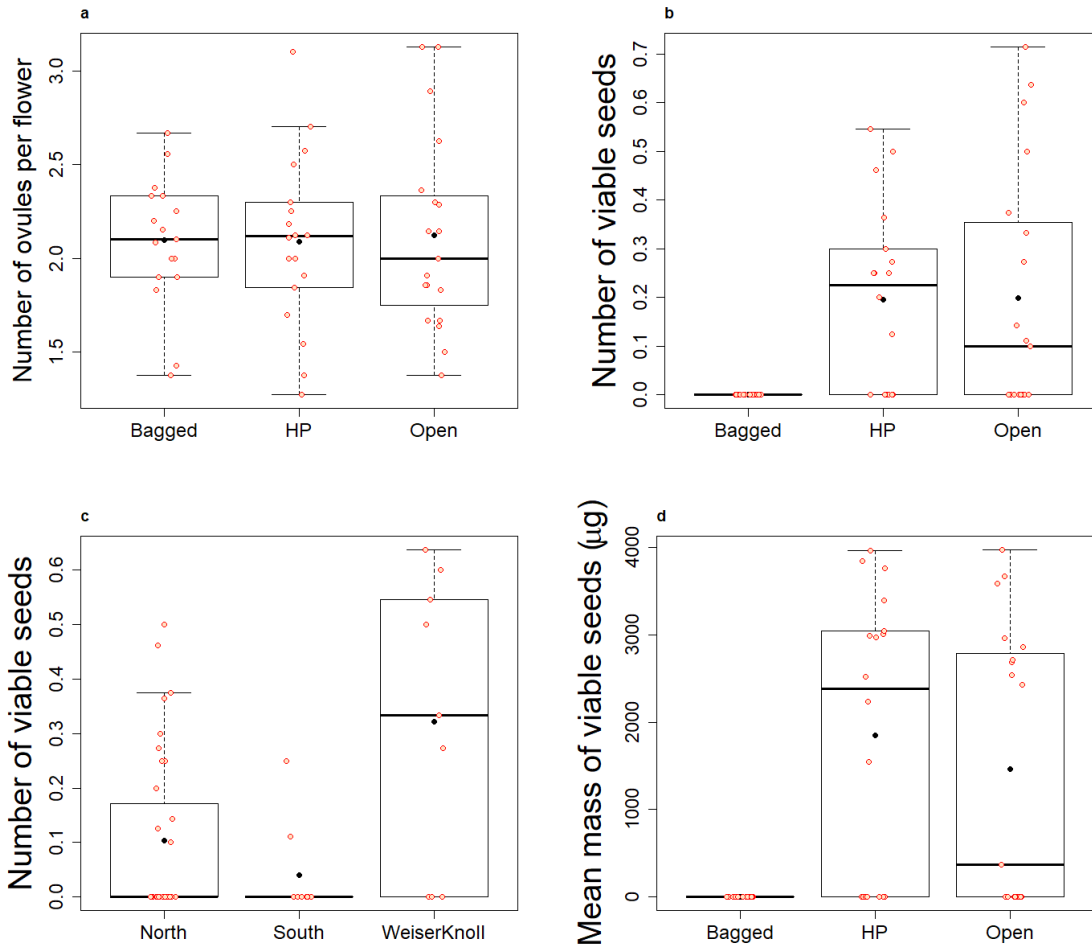
Figures 9. a.) The number of ovules per flower did not differ among treatments indicating that flowers contained a similar number of seeds that could potentially develop. b.) The number of viable seeds per flower differed among treatments and c.) sites. d.) The mass of viable seeds per flower were much lower in the bagged treatment compared to the hand pollinated (HP) and open treatments, indicating that Barneby's clover does not self-pollinate. Circles are average values, the bold line is the median value, the lower and upper limits of the box are the 25th and 75th percentiles, and the whiskers are the upper limits of the data excluding outliers. Red circles represent all data points collected.

Barneby's clover produced more viable seeds as spring advanced in the open and hand pollinated treatments. The number of viable seeds per flower (lm; t = 2.5, p = 0.017; Figure 10a) and the mass (lm; t = 60.4, p = 0.06; Figure 10b) increased with Julian day pollinated (Figure 10b). We captured more bees (bee catch rate; individuals/day) as the season progressed (Figure 10c). Barneby's clover may produce more viable seeds when we captured more bees (t = 0.86, p = 0.39; Figure 10d) but not heavier viable seeds (t = .77, p = 0.44: Figure 10e).
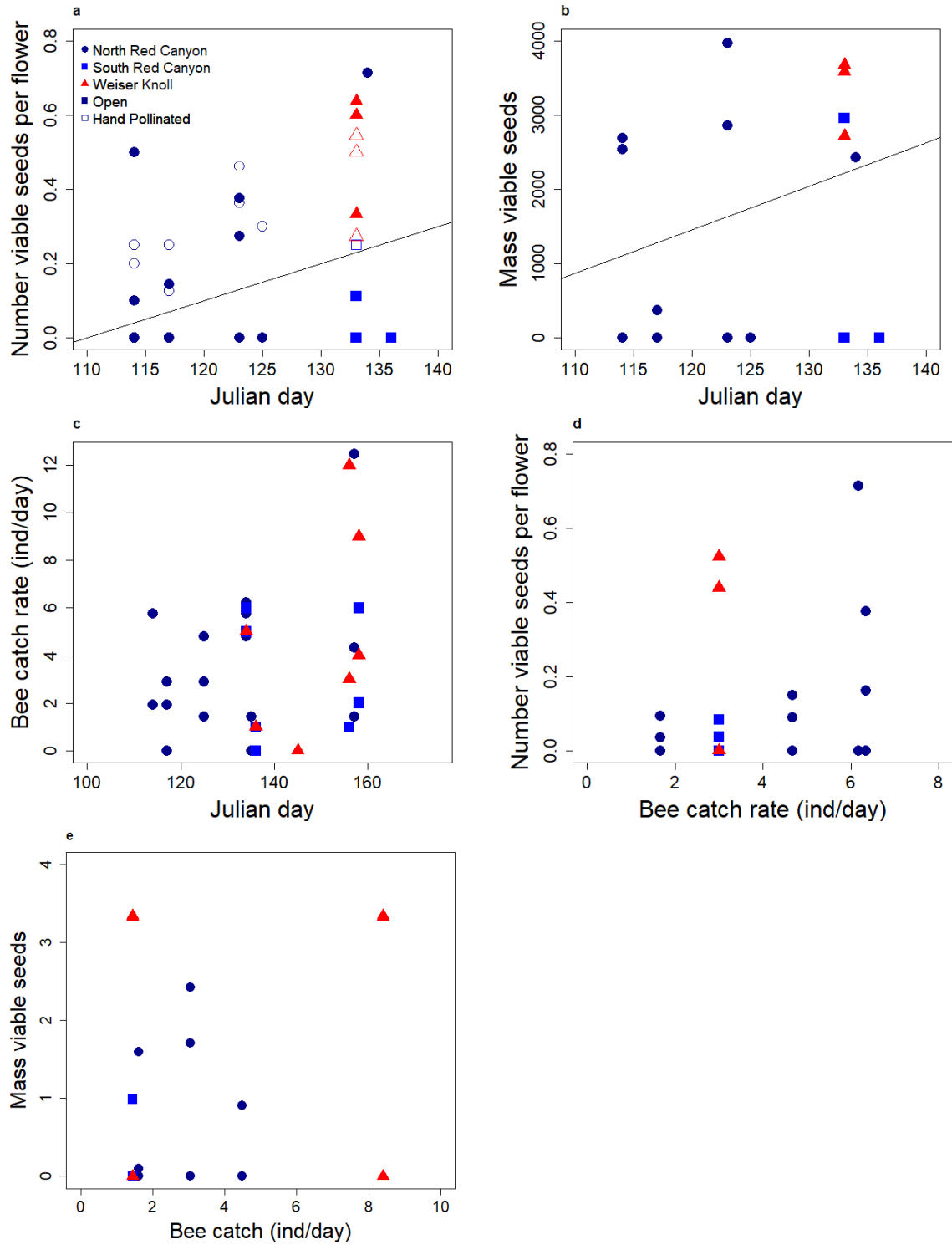
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Figure 10. The a.) mass and b.) number of viable seeds increased as spring progressed in both the open and hand pollinated treatments, likely because the c.) number of bees increased during the bloom period. d.) The number of viable seeds increased with the number of bee captured, e.) but the mass of viable seed did not vary with bee numbers.

Pollinators

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We captured 14 genera of pollinators when Barneby's clover was blooming (Table 3). The bee genera, *Lasioglossum* and *Agapostemon*, were the most abundant followed by *Anthophora*, *Eucera*, *Osmia* and *Bombus*.

Table 3. We captured 15 genera of pollinators when Barneby's clover was blooming. *Anthophora*, *Eucera*, *Agapostemon*, *Lasioglossum* and *Osmia* were the most abundant genera collected. We discovered pollen from Barneby's clover on 8 genera of bees.

Order	Genus	Subgenus	Species	Number	Pollen
Hymenoptera	*Agapostemon*		*angelicus/texanus*	6	
Hymenoptera	*Agapostemon*		*sericeus/obliquus/femoratus*	11	Yes
Hymenoptera	*Agapostemon*		*virescens*	11	Yes
Hymenoptera	*Andrena*			4	Yes
Hymenoptera	*Anthophora*			29	Yes
Hymenoptera	*Apis*		*mellifera*	1	Yes
Hymenoptera	*Bombus*		*bifarius*	2	Yes
Hymenoptera	*Bombus*		*centralis*	6	Yes
Hymenoptera	*Bombus*		*fervidus/californicus*	1	Yes
Hymenoptera	*Bombus*		*huntii*	8	Yes
Hymenoptera	*Eucera*			28	Yes
Hymenoptera	*Halictus*		*farinosus*	8	Yes
Hymenoptera	*Halictus*		*parallelus*	1	
Hymenoptera	*Halictus*		*rubicundus*	2	
Hymenoptera	*Hoplitis*			2	
Hymenoptera	*Lasioglossum*	Dialictus		7	
Hymenoptera	*Lasioglossum*	Evylaeus		24	Yes
Hymenoptera	*Lasioglossum*	Lasioglossum		4	
Hymenoptera	*Melecta*		*pacifica*	9	
Hymenoptera	*Osmia*			23	
Hymenoptera	*Psuedomasaris*		*vespoides*	1	
Hymenoptera	*Sphecodes*			3	
Hymenoptera	*Tetraloniella*			3	
Lepidoptera	*Hyles*		*lineata*	1	

Pollen analysis

The pollen from Barneby's clover is ellipsoid. Each grain has three groves: two groves run along most of the grain's length on one long side and two smaller groves run along the length of the grain on the opposing side. The surface has light texturing. Each pollen grain is 4.9 to 6.6 µm and the width varies between 2.8 and 4.9 µm (Figure 6c).

Eight genera of bees carried pollen grains from Barneby's clover on their legs indicating that they actively collected the pollen (Figure 11a, Table 3). Of all the Barneby's clover pollen we encountered, mining bees (*Andrena*) carried the most pollen (85%; mean per individual) followed by non-native honey bees (*Apis*; 8.5%), sweat bee (*Halictus*; 2%), bumble bees (*Bombus*; 2%), a sweat bee (*Agapostemon*; 1%), a digger bee (*Anthophora*; 1%), long-horned bee (*Eucera*; <1%) and the most common sweat bee in Wyoming (*Lassioglossum*; <1%). Of all the pollen grains identified on individual bees, pollen from Barneby's clover made up 86% of pollen loads for honey bees, 70% for *Andrena*, 68% for *Anthophora*, 60% for *Lasioglossum*, 49% for *Halictus*, 44% for *Bombus*, 25% for *Agapostemon* and 22% for *Eucera*, indicating that most bees were heavily visiting this plant (Figure 11b). The amount of pollen from Barneby's clover carried on bees increased over time from the week of 21 April (week 1) to the week of 10 June (week 8; Figure 12a). We did not find any Barneby's clover pollen on bees after that week. The proportion of individuals in each bee genera that carried Barneby's clover varied between 19% (*Lasioglossum*; n = 26) and 94% (*Bombus*, n = 16). Only one honey bee (*Apis*) was collected and that individual collected pollen from Barneby's clover.
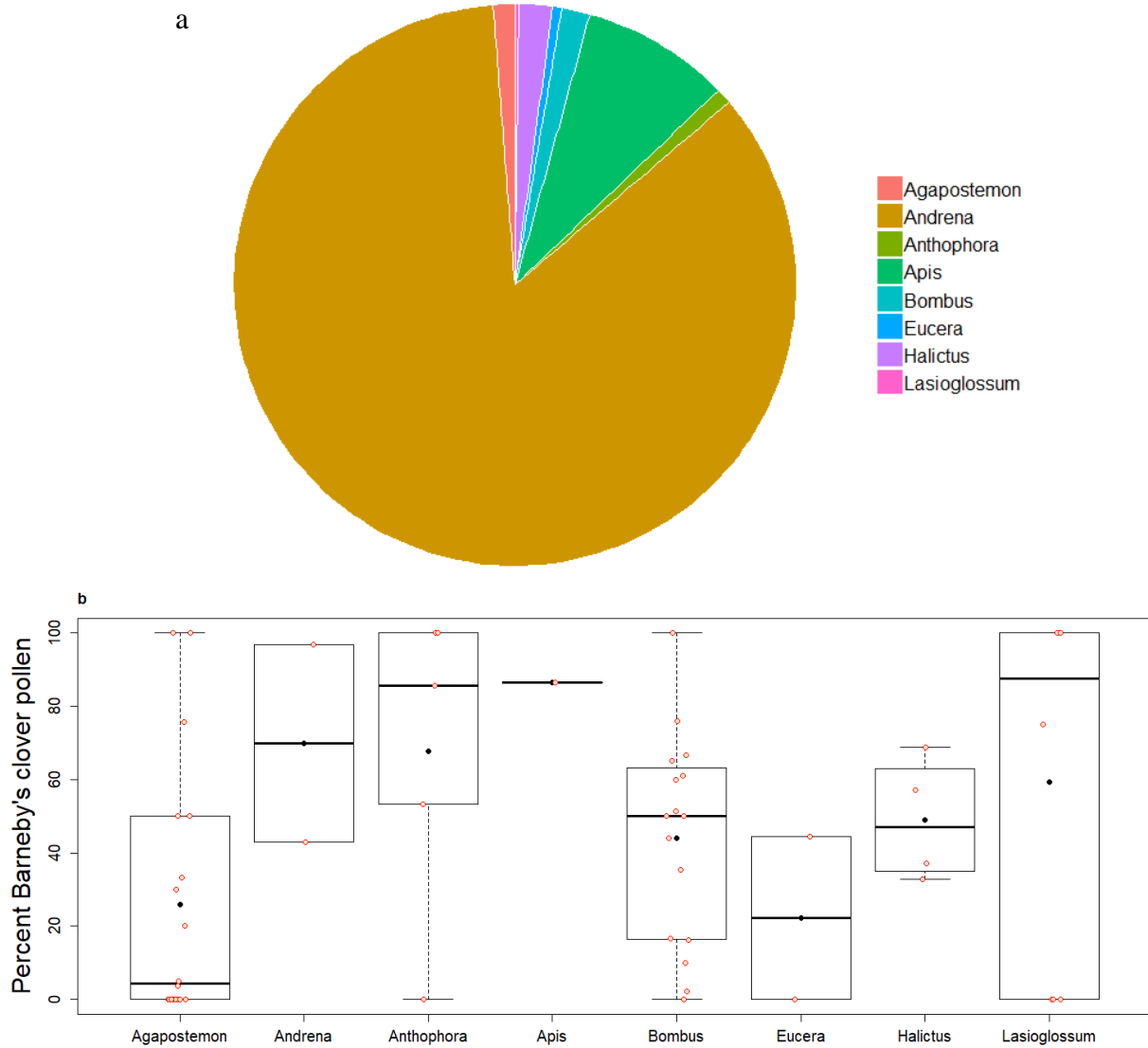
Figure 11. a) Mean percent of pollen grains from Barneby's clover carried by eight bee genera. The mining bee, *Andrena*, carried by far the most Barneby's clover pollen. Barneby's clover is one of the few plants blooming when bees emerge in the spring and is likely a critical pollen and nectar source. b) The percent of pollen grains from Barneby's clover carried on individual bees by genus compared to all pollen grains counted on an individual. Circles are means, lines are medians, lower and upper limits of boxes are the 25th and 75th percentiles and whiskers encompass the minimum and maximum values.
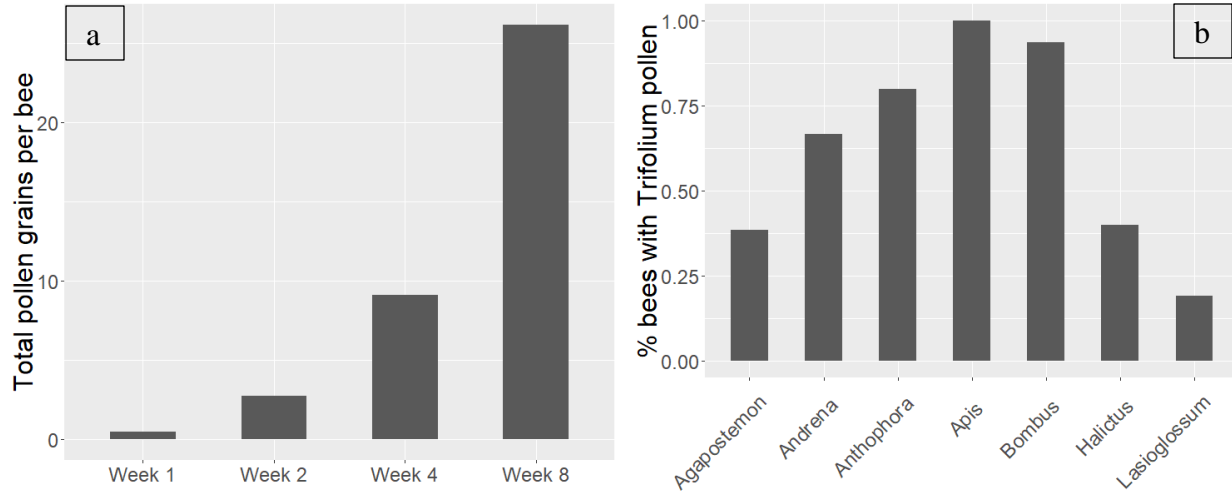
Figure 12. a) The mean number of Barneby's clover pollen grains carried per bee increased throughout the time period and we did not find any pollen on bees after week 8. Week one was 24 April 2019. b) The percent of individuals of each bee genus that carried Barneby's clover pollen.

DISCUSSION

Barneby's clover required insect pollinators for sexual reproduction and more bees resulted in more viable seeds produced. Flowerheads in the bagged treatment produced no viable seeds, which indicated that this species likely does not self-pollinate. Some species in the *Trifolium* genus are capable of self-pollinating while others do not. For example, white clover (*Trifolium repens*) had very low self-compatibility (Atwood 1941, 1942), while the tetraploid red clover (*Trifolium pretense*) readily self-pollinated (Vleugels et al. 2019). The number and mass of viable seeds produced by Barneby's clover increased over the time period and bee abundance also increased over that time, indicating that seed production is likely limited by pollinators. The relationships between bee abundance and seeds was not significant, but this may be due to a small sample size and the data spanning a narrow range. In contrast, Kehrberger and Holzschuh (2019) found that bee visitation per plant and seed-set decreased with bee abundance and Julian Day in another rare, early blooming plant, *Pulsatilla vulgaris* (European pasqueflower), perhaps due to increased competition for pollinators as other species bloomed. Few other plants were blooming at our sites during the blooming period and we expect the competition among blooming plant species was low in our study.

The aspect and exposure at the three sites likely explained differences in flowering time and ultimately viable seed production. We first observed plants blooming in North Red Canyon on 16 April, while plants in a shaded rocky hollow bloomed much later (21 June; E. Freeland, personal communication). The North Red Canyon site likely started blooming the earliest due to earlier snowmelt, more exposure and higher temperatures compared to the other sites. Barneby's clover bloomed at about the same time in the Weiser Knoll and South Red Canyon. The Weiser Knoll area faced east and was in a juniper woodland with relatively deep soils that were cooler

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and resulted in snow melting off the site later and cooler temperatures. South Red Canyon was under snow later than North Red Canyon as well and the cooler conditions likely delayed blooming there till later. This variability in flowering time, despite its restricted distribution, may well contribute to the persistence of this rare plant (Kameyama and Kudo 2015).

The fertility among individuals appeared to differ and may be due to lack of genetic diversity between nearby individuals, the level of polyploidy, or other genetic or microhabitat differences (Dufresne et al. 2014; Aigner 2004). Variations in microhabitats, such as the amount of soil, availability of moisture, sunlight and nutrients may affect individual fertility (Burmeier and Jensen 2008). Earlier blooming individuals may be more likely to receive pollen from their own flowers, or a closely related (nearby) individual, thus reducing their fertility (Kameyama and Kudo 2015). We did discover a mat of red flowered Barneby's clover, which seemed to be entirely infertile. This may be an indicator of mutations within the species that contribute to lower fertility in some individuals. Investigating genetic diversity within Barneby's clover would help us estimate the degree to which this population is suffering from inbreeding depression (Dufresne 2014, Aigner 2004).

Our sites differed in environmental characteristics, which affect the sexual and asexual reproduction of Barneby's clover. Asexual reproduction in Barneby's clover occurred by forming mats and sexual reproduction occurred via pollination and seed production. We hypothesized that asexual reproduction at Weiser Knoll varied little over time and sexual reproduction mainly occurred in years with moist, warm springs. Areas with thin, erodible soil (e.g., Weiser Seedlings) are probably vulnerable to higher temperatures and heavy precipitation events which may reduce their ability to increase mat cover or produce seeds. We expect that windier sites with more exposure (e.g., Hall Creek) may bloom earlier resulting in fewer pollinators to transporting pollen and fewer seeds produced. Asexual reproduction and low competition probably maintains Barneby's clover at such sites. Barneby's clover also grows in areas with little to no soil over erodible sandstone bedrock and vertical surfaces (e.g., North Red Canyon). The powerful forces of water and wind may cause Barneby's clover mats to break up and wash to other microhabitats on the rim. In this portion of its habitat, Barneby's clover is a continuous colonizer, maintained by the eroding rock and soil patches. The mats are dispersed by the erosion and seeds can germinate in new spaces of bare soil. Mats that remain in the same place for many years may become depleted of the nutrients due to the thin soil, which may be the cause of the loss of live mats shown in the photo-point monitoring.

Early blooming plants, such as Barneby's clover, are expected to have a greater probability to be limited by pollinators because of a mismatch in the timing between blooming and pollinator emergence (Memmot et al. 2007; Kudo 2014). Our results show that Barneby's clover produced fewer viable seeds when it began to bloom and we captured few bees at that time. Sexual reproduction at Red Canyon may decrease over time as spring begins earlier there, especially if the emerge timing of pollinating insects does not coincide. Our plots of bee abundance against the number and mass of seeds may not have been significant because of differences in phenologies among sites. Those differences likely affected when bees emerged as well as when

flowers bloomed. If we plotted the time since the first blooming flower at each site, we may have observed a relationship.

Monitoring a mat-forming species that grows on rock outcrops and in crevices is challenging and required non-traditional methods beyond line-point intercept surveys. Individuals of Barneby's clover cannot be differentiated due to the density of the mats. In 1995 and 2017, The Nature Conservancy conducted photo-point monitoring on Barneby's clover on their Red Canyon Ranch Preserve (Anderson 1995, Coffman 2017). This method indicated loss of live mats and encroachment by grasses. Due to the length of time between photos, interpreting the rate of mat loss and encroachment or correlating events with potential explanations is difficult. Also, there was no way to quantitatively examine the data from the photos. To find a rigorous method to monitor Barneby's clover, we researched several ideas using three-dimensionally measurements, including methods for monitoring coral (Rogers et al. 1983, Elzinga et al. 1998) and the mat-forming rare plant Shoshonea (*Shoshonea pulvinata*; Pipp 2016). We experimented with line-point intercept methods along a flexible chain; however, the crevices were often narrow between large slabs of rock (especially at the Hall Creek area) making the line-point intercept method not repeatable. The presence/absence of Barneby's clover in cells of a frame along a belt transect was the most feasible method because the frame was wider, making the exact placement of the line less important. Also, the use of presence/absence much less subjective and relatively fast compared to attempting to evaluate percent cover in each cell or the entire frame.

The monitoring results were comparable among years despite different investigators collecting the data. We feel the frame along the belt transect gave an accurate assessment of the changes, which our research has shown can be related to spring precipitation, as well as the coverage documented the previous year. This method may be used on vertical features and may be easiest with a flexible or jointed frame. Also, monitoring should be conducted every one to five years if possible. Weather data loggers near the monitoring sites would be useful to measure the precipitation and temperatures the plants experience. Climate data would help untangle trends in long-term monitoring of the plant and how their populations may change due to warming conditions.

Barneby's clover has a variety of reproductive traits, which help this plant to persist in a limited and harsh range. The variation in blooming time due to different aspects, exposures and microhabitats allows Barneby's clover to encounter pollinators over two months, although earlier blooming plants were often pollen limited in our study. Earlier blooming Barneby's clover may become more pollen limited over time if a mismatch between pollinator emergence and blooming becomes more pronounced, potentially leading to increased inbreeding. Mats of Barneby's clover on thin soil may deplete the nutrients and become decadent, but mats and soil can move during large precipitation events, which may allow mats to form in new places and soil to become renewed. The monitoring method we established worked well and could be modified to monitor in areas with more ledges and outcrops. Investigating the dynamics of the genetics and demography of this plant will increase the understanding and enhance management capabilities.

ACKNOWLEDGEMENTS

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