RURALS: Review of Undergraduate Research in Agricultural and Life Sciences

Volume 15 | Issue 1 Article 1

April 2022

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Recommended Citation

Bielski, Rustin (2022) "Spotted Knapweed (Centaurea stoebe) Pollen Use-Availability by Bumble Bees in Western Montana," *RURALS: Review of Undergraduate Research in Agricultural and Life Sciences*: Vol. 15: Iss. 1, Article 1.
Available at: https://digitalcommons.unl.edu/rurals/vol15/iss1/1

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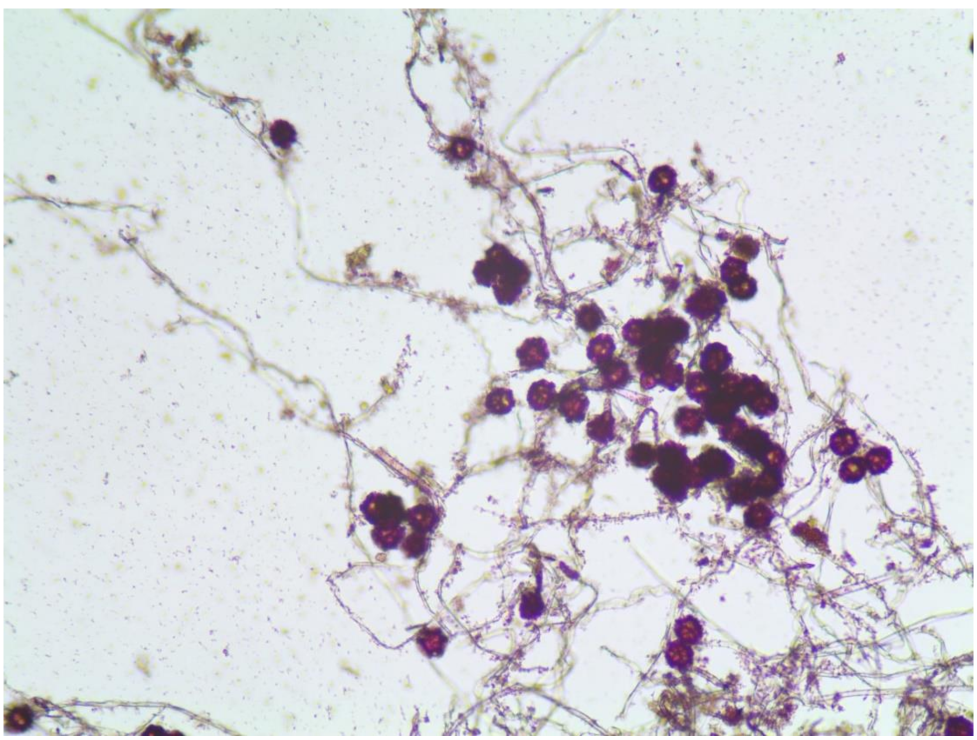
Cover Page Footnote

I would like to thank the Montana Space Grant Consortium for its generous funding for this research. I would also like to thank the Life Sciences Department and Wildlife and Fisheries Department at Salish Kootenai College for their support and for allowing me to use their facilities.

Introduction

Spotted Knapweed (*Centaurea stoebe*) arrived in North America around 1883 in Victora, B.C. It made its debut via contaminated livestock feed or soil from ballasts of trade ships (Mabberley, 1987). Due to its superbly adapted abilities of affective seed dispersal, it soon found its way across the United States, and eventually every county of the state of Montana. Currently, in Montana, Spotted Knapweed covers 4.5 million acres and costs ranchers more than $40 million annually in crop loss and herbicides (Mabberley, 1987). There, Spotted Knapweed grows in open forest, shrubs, and grassland, particularly in sites disturbed by recreation, agriculture, and logging (Mabberley, 1987). Originally from the region spanning central Europe to central Russia, Spotted Knapweed has become most prolific in the Western part of Montana, likely due to the climate similarities. To put into perspective, the abundance of knapweed in western Montana, elk have been observed changing their migration patterns to avoid it (Alper, 2004). Management of this wayward weed is especially difficult because, like most invasive plant species which prosper in disturbed soils, knapweed also thrives in undisturbed ecosystems (Duncan, 2017). This could be due to the phenology of its bloom, later in the season (mid-summer to early fall), when the weather is hot and most of the native flowers have finished blooming. Also, knapweed is successful because of its distinguished ability to release toxins through its roots, which stunt the growth of nearby plants (USDA, 2015). Knapweed has done such a great job at displacing other flowers that pollinator species may now be dependent on them as a food resource. Honeybees, which are major livestock animals in the U.S., utilize knapweed tremendously due to the flower being a copious nectar producer (Runk, 2010). Some beekeepers fear biological control methods of the invasive plant because of their bees' dependence on it (Runk, 2010). Mass conversion of native prairie land in Montana to non-native-dominated agricultural and rangeland, the implication of non-native grasses and non-native forbs leave only invasive non-native forbs to successfully compete with these species because native plants have little chance of recovery. This makes one wonder if native pollinating species that once relied on abundant native flowers must now rely on invasive forbs for sustenance. Food crops in the U.S. confide in insect pollination and have been valued as a 200 billion dollars a year asset, equally important as the water, soil, and sun which the plants grow in (Randall, 2020). The issue with food crops for pollinators is the temporal dynamics of the nectar and pollen resources they provide. Food crops largely grow as monocultures throughout entire regions of the U.S., and flowers bloom in coinciding ephemeral cycles, providing only one large glut of food for pollinators once during the season. Similarly, when an invasive plant takes over an ecosystem, it creates a new resource dynamic for pollinators by displacing the native flowers on which they once relied. However, additional factors such as climate change can also degrade the diversity and abundance of native plants, while invasive plants like knapweed remain unscathed. Where native pollinators get their food throughout the entire phenology of the season lacks understanding, and the pervasiveness of invasive plants like knapweed may serve as a refuge for pollinators when the availability of native plants is lacking within an ecosystem.



Bees are arguably the most important native pollinator because they spend most of their life seeking pollen and transferring pollen from flower to flower. Bees are the only species that intentionally collect pollen, which also aids in the plant pollination process (MSU Extension, 2021). Pollen is the most important resource for bees because they need it to meet nutritional requirements especially due to its high protein content (Ellis et al., 2020). Nectar alone gives bees energy but is not sufficient for their growth and development. Bumble bees (*Bombus spp.)* were chosen as a vector for understanding pollen use representing native pollinators as a whole because throughout the U.S. multiple bumble bee species are in decline due to lack of habitat, pesticide use, climate change, and introduced species (Graves et al., 2020). A combination of these factors has also led to the decline of plant diversity worldwide, and it is little understood how this affects the nutritional intake of animals on the next trophic level. The fecundity and survival rates of these animals can be affected by the loss of plant diversity, especially when homogenized by exotic plants (Bishop et al., 2009). A statistical estimation of the availability of a resource in the wild compared to the use of that resource shows the preference of, or against that resource (Harmon-Threatt et al., 2016). If the relative abundance of the spotted knapweed population in the field is similar to the proportion of spotted knapweed pollen collected by bumble bees, then likely there is no preference for or against spotted knapweed. However, it can be assumed that due to the nature of bumble bees and their evolved symbiotic relationship with native wildflowers, that they will have a negative preference towards spotted knapweed. This study gains perspective on bumble bee selectivity of pollen, comparing preferences of pollen sources in a habitat containing spotted knapweed.

Methods

Study Site

The location for this research was chosen in the town of Kicking Horse, Montana, on the north side of the Kicking Horse Reservoir. This area is owned and operated by the Flathead Indian Reservation, more specifically under the Tribal Trust Lands of the Confederated Salish and Kootenai Tribes. Recreation within the surrounding area is managed by the tribal natural resource department in collaboration with the Montana Department of Fish, Wildlife, and Parks. The wilderness area is most popular for recreation during the fall waterfowl hunting season but is also frequently used by anglers and bird watchers year-round. The location for this research was selected because of its known presence of *Bombus* species and its abundance of native as well as non-native forbs within a connected similar habitat type. There are two main ecosystem types within the study area: Rocky mountain lower montane foothill valley grassland (otherwise known as a bunchgrass prairie), and Northern Rocky Mountain West valley freshwater emergent marsh (in the form of pothole wetlands) (MNHP, 2019). Additionally, some area was agricultural/pastoral use in the past but has since been managed and relatively undisturbed. Also, a portion of the sample area was recently subjected to a prescribed burn (over 2 years ago). The main focus area, primarily the Northern end of the recreation area, was chosen because of its ease of access, however, for this study sample plots were not chosen to be within any boundaries other than the limitations of surrounding private land/ urban areas. Coordinates for a centralized spot of the study area are 47.479117, -114.075389.

Plant Diversity Survey

Plant diversity/ relative abundance surveys were collected on 35 days from mid-spring to late summer over two seasons. Surveys were taken from May 9th to August 19th. On each day of sample collection, a relative abundance survey was performed based on Gillespie, S., Bayley, J., & Elle, E. (Gillespie et al., 2017). Randomly selected within the site, four 50-meter quadrants were chosen, each spaced 50 meters apart. Along each transect line, 10 quadrants were placed five meters apart. Each quadrant measured .25 x 2 meters. Within each quadrant, flowering stems were counted for each species present, and only species containing flowers large enough for bumble bees to utilize were counted. All flowers surveyed were upland forbs. Plant species identification was done using Kershaw, Mackinnon, and Pojar's "Plants of the Rocky Mountains." (Kershaw et al., 2017). The proportion of knapweed flowers to the overall flower community was calculated for each day of sampling. The plant diversity survey was done at least once a week for each week there were flowering plants in bloom over the season.

Bumble Bee Pollen Collection

For each day a plant diversity survey took place, bumblebee pollen collection took place, however not every sample day yielded successful capture of bumblebees with adequate pollen. Pollen samples collected from bumble bees were done based on Harmon-Threatt, A.N., and Kremen, C. (Harmon-Threatt & Kremen, 2015). A 30-minute sweep net capture along fixed transect lines was performed. Three 500-meter-long transect lines were randomly chosen, running parallel, 100 meters apart. One person walked along transect lines at a moderate speed making observations. When a bumblebee was recognized, it was captured, and the target flower was recorded. Captured bumblebees were placed in vials, and then into a cooler to sedate. Once cooled, bumblebees were photographed, identified to species, and pollen that was agglutinated to the corbiculae (pollen basket) was collected and placed back into the cooler. Bumblebees were unharmed during this process and set free after.

Mounting Slides

A reference collection of flower pollen from all forbs in the greater Kicking Horse area was created. Flowers were collected throughout the season and frozen to preserve grain quality. In total 45 different species of flower samples were collected. To isolate pollen from the flowers, pollen anthers were removed and placed in vials, and 2 ml of 91% alcohol was added. Vials were vortexed and then alcohol/pollen solution was siphoned out.

Pollen dehydration technique was based on Moore and Webb (Moore et al., 1999). To dehydrate pollen grains, 91% alcohol pollen solution was centrifuged at 22^{o} C, 16,000 G's, for 4 minutes. Centrifuge settings based on McArt et al (McArt et al., 2017). The alcohol was then decanted, and 1 ml of 96% alcohol was added. Vials were then vortexed, centrifuged on the same settings, and then alcohol decanted again. The recommendation for the grain staining process was an added step based on Faegri and Juerson (Faegri & Iversen, 1989). Fuchine dye was added to the last alcohol bath, from a mixture of 30 ml 99% alcohol to 0.75 g of fuchsine dye. 1 ml of this alcohol dye was added to each vial, vortexed, and centrifuged on the same settings, then decanted. For the final step of dehydration, 1 ml of benzene was added to each vial and vortexed, then vials were left open and covered in a hood for 24 hours.

The ideal mounting medium used is based on Faegri and Juerson (Faegri & Iversen, 1989) of silicone oil with a viscosity between 2000 to 40,000 centistokes. Silicone oil makes for better long-term preservation due to its inability to swell the grains, unlike glycerin solutions. 0.5 ml of 5000 centistoke silicone oil was added to vials and vortexed. A small amount of this final solution was then pipetted to slides and sealed with UV curing epoxy as suggested by Kapp, Davis, and King (Kapp et al., 2000). Throughout the 2019 season, 27 bumblebees were captured and throughout the 2020 season 49 bumblebees were captured, for a total of 76 pollen samples for analysis. The same dehydration and mounting process were used for the pollen samples collected from bumblebees.

Pollen Analysis

Pollen analysis and sample pollen composition were done using methods based on Gillespie, Bayley, and Elle (Gillespie et al., 2017). Grains were analyzed using light microscopy, scanning across a single transect line and counting grain types within the frame. Pollen was either identified as Spotted Knapweed (*Centaurea stoebe*) or "other". Grains were identified by comparing them to reference slide collection. For each bumblebee pollen sample slide that contained pollen on the transect line, a proportion was created regarding spotted knapweed grains to other grains. The mean of these proportions was then calculated and compared to the mean of the proportions calculated from the relative abundance stem count surveys. This was done using a two-sample independent mean T-test. Tests were done to find the probability that bumble bees collected less pollen than there was available throughout the season when compared to a hypothetical random collection of pollen. The alpha level stated before running the test was 0.05. The same test was run again which only included the days in which knapweed was in bloom, comparing the proportions from the relative abundance surveys during these dates, to the bumble bee pollen samples collected on the same dates (July 15th through August 19th of 2019 and 2020. There was a total of 16 sample days, and 49 pollen samples within the window of knapweed bloom time. Some knapweed pollen was seen within pollen samples outside of the study sites observed bloom time of knapweed. However, to calculate preference, knapweed needs to be available and quantifiable, which is why this window of time was selected for analysis along with total season comparisons. The null hypothesis was that proportionately, bumble bees collected the same amount of pollen as there was knapweed available. Alternatively, if it was not proportional, then there would be less knapweed collected by bumble bees than available on the landscape.

Results

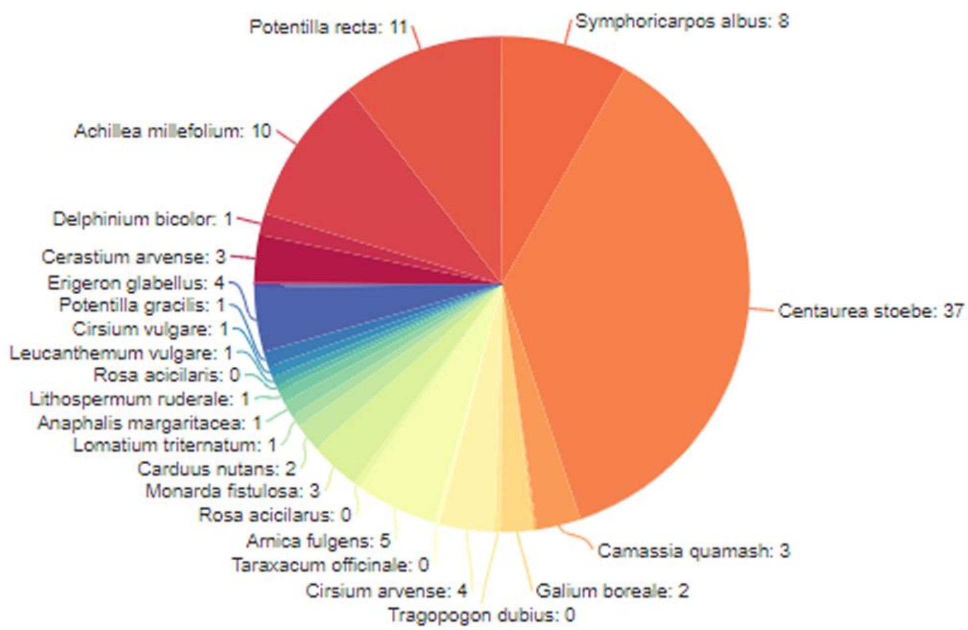


Figure 1: May 9th through August 19th. Overall flower density throughout the season based on stem counts.

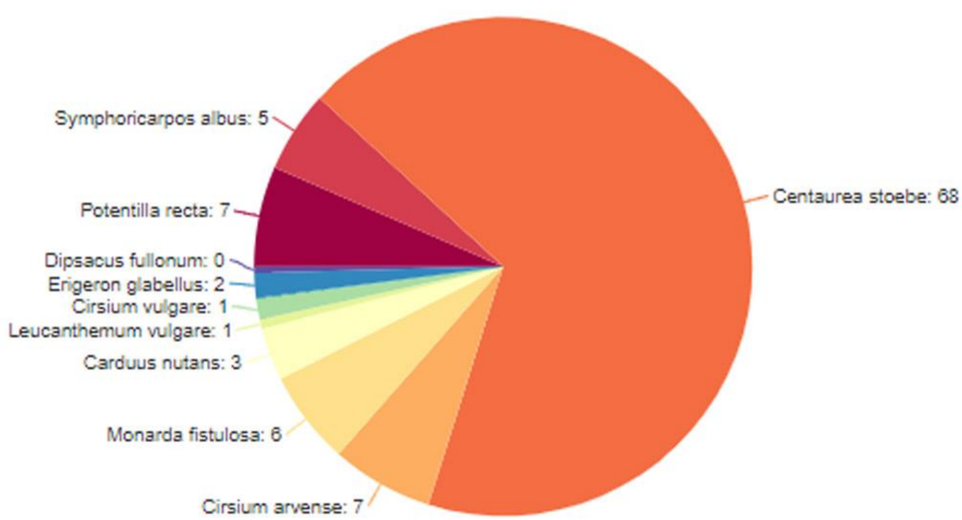


Figure 2: July 15th through August 9th. Flower density throughout the bloom time of spotted knapweed based on stem counts.

Similar results were found in both the test for all sample days throughout the season and the test for just sample days where spotted knapweed was available. For the test containing all sample days, the mean proportion of spotted knapweed density was 0.32 plus or minus 0.13 at a 95% confidence interval. The mean proportion of pollen density was 0.18 plus or minus 0.1 at a 95% confidence interval. The T-value for the two means was 1.789, which yields probability in a random sample to be 0.0388. This probability is less than the stated alpha of 0.05, meaning the null hypothesis is rejected.

In the second test, where only the sample days were analyzed during the blooming time of knapweed at the study site, the mean density of knapweed was calculated to be 0.66 plus or minus 0.14 at a 95% confidence interval. During the same timeline, the proportional mean of knapweed pollen collected by bumble bees was 0.15 plus or minus 0.12 at a 95% confidence interval. The T-value for the two means was 5.477, which yields a probability of 9.25×10^{-7}, far lower than the stated alpha of 0.05. Therefore, the null hypothesis is rejected.

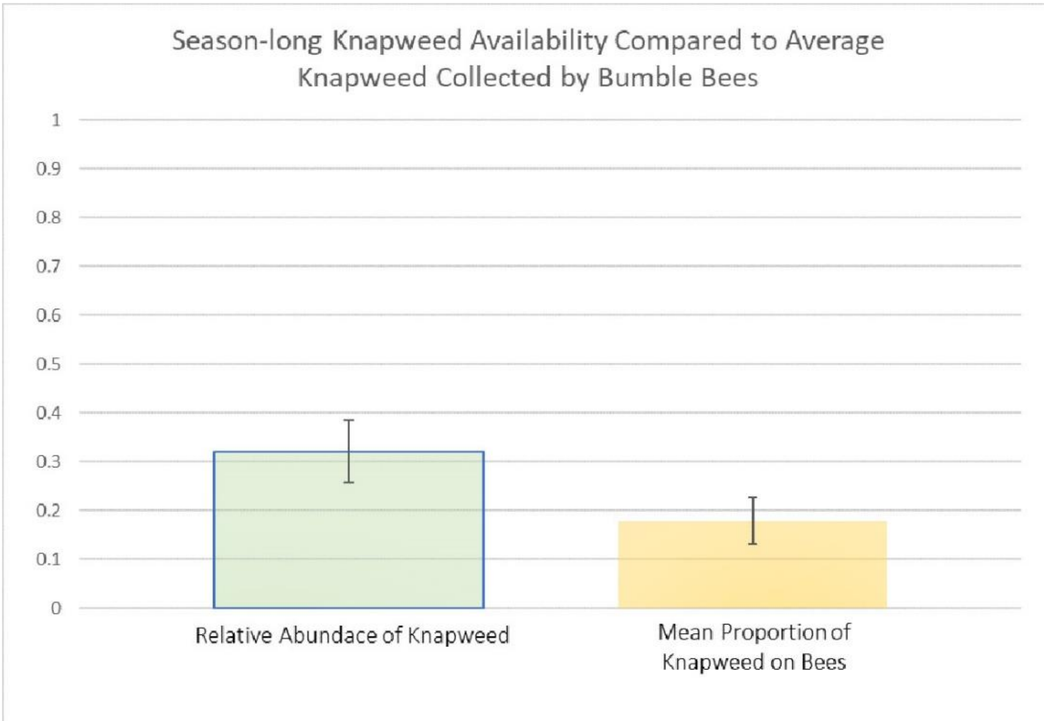


Figure 3: May 9th to August 19th. The green bar gives an approximation of the percentage of knapweed flowers out of total flowers of all plants throughout the season (roughly 3.5 months). Zero represents no knapweed throughout the season while a one represents 100% knapweed flowers at the site throughout the season. The yellow bar represents the percentage of knapweed pollen found on bumble bees out of the total pollen collected by them throughout the season.

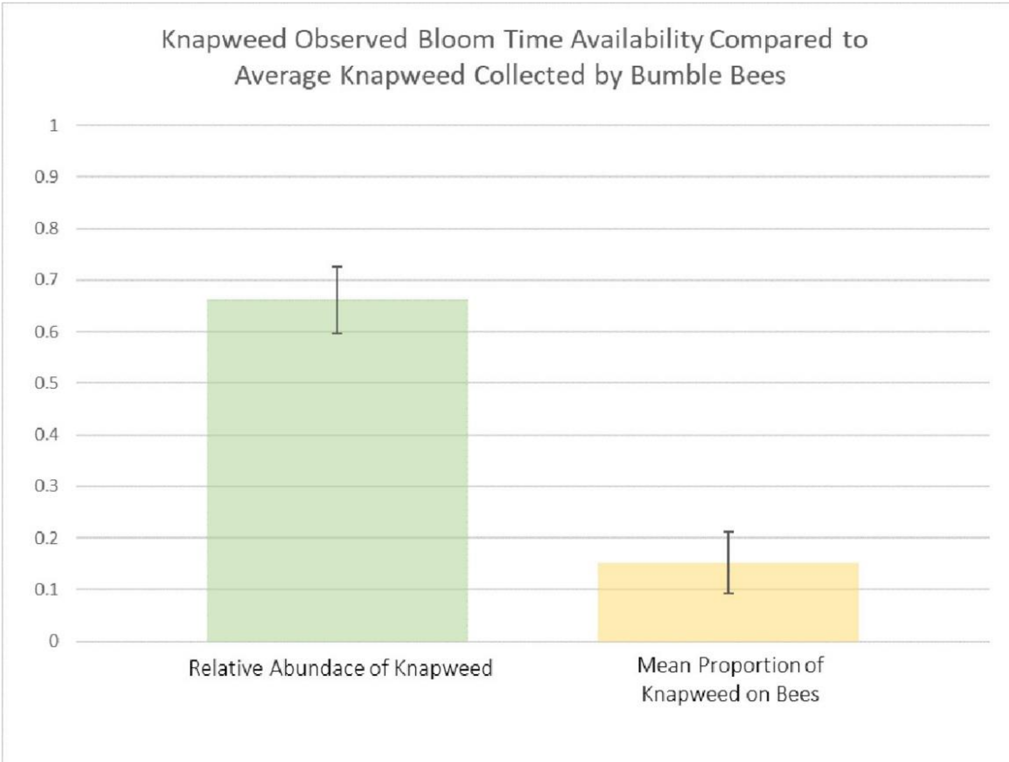


Figure 4: July 15th to August 19th. The green bar represents an approximation of the percentage of knapweed to other flowers blooming within the blooming time of knapweed (roughly one month) at the study site. Zero represents no knapweed throughout the season while a one represents 100% knapweed flowers at the site. The yellow bar represents the percentage of knapweed pollen found on bumble bees out of the total pollen collected by them within the knapweed bloom time.

Discussion

The question put forth when creating this research model was asking whether or not bumble bees selectively avoid knapweed pollen given an ecosystem with relative knapweed availability. Given the data from the pollen samples collected from bumble bees, and the plant diversity stem counts, it is hard to conclude whether or not the data confidently says that bumble bees selectively avoid knapweed. Both versions of the test rejected the null hypothesis, leading to the hypothesis that when spotted knapweed is available on a landscape alongside other flowers, bumble bees selectively avoid collecting knapweed pollen. However, the study site was very small, and not many samples were collected. The main issue was the size of the study site and how the plant diversity survey was taken. Due to the patchy nature of which plants grow, habitat just outside of the study site could have had an abundance of flowers other than knapweed for bumble bees to utilize, causing pollen sacks to be more full of non-knapweed pollen. When the stem count is performed, it would seem like there is more of a percent abundance of knapweed rather that the true abundance of the extended habitat. Near the study site was a large forest area and bumble bee queens are known to create their nests along forest boundaries (Svensson et al. 2000). Since the study site was a disturbed site with relatively no shade or canopy trees, it may have been a poor nesting site for a large proportion of bumble bee species. The flower community in the forested area adjacent to the study site would contain a different flower community and most likely less knapweed based on personal observations. If bumble bees were to use optimal foraging theory when gathering pollen, they would gather the pollen of flowers closest to their nest first, then end up further from the nest and into the study site where they were then captured.

Ideally, if repeated, this study would only use bumble bees from known nest sites, and a relative abundance of available flowers would be calculated based on the potential range those bees could travel to find flowers. Additionally, more than one site would be used, over a larger landscape and many more pollen samples would be taken. The results obtained from this study may give good insight into what is happening at this specific study site in the Kicking Horse Wildlife Mitigation Area, but that is the only place it holds any truth. Flower communities change vastly from one area to another, as well as the insect and other animal populations, all of which may have an effect on the behavior of the bumble bees locally.

Many similar studies look at flower visitation, which does not tell you if the bee is visiting the flower for nectar or pollen. Since pollen is the primary source of nutrition for bees, the determination of their diet is based on flower selection, and bees are known to be more selective of pollen resources than nectar resources (Praz et al. 2008b). A study looking at the nutritious value of pollen pointed out that bumble bees were observed selecting flowers with higher nutritious value and pollen collection was non-random behavior (Harmon-Threatt and Kremen 2015). This could suggest that knapweed pollen is less nutritious than some of the other flowering counterparts of the community and that knapweed relies more on the collection of its pollen incidentally by pollinators. While this may provide evidence that invasive plants are less desirable to bumble bees, that may not be true. A similar study estimating resource preferences of bumble bees using pollen loads showed two invasive plants were found to be highly preferred (Harmon-Threatt et al. 2016 Sep). A study evaluating flower preference by looking at visitations in a heavily knapweed-infested site found that native pollinators preferred native flowers but honey bees preferred the knapweed for both pollen and nectar, especially late in the season (Urbanowicz et al. 2020). So while some non-native flowers may have nutritious value to pollinators, spotted knapweed is likely not one of them. The corolla lengths (distance of the petals) of spotted knapweed are also fairly short, like most flowers in the Asteraceae family, and therefore is a nectar provider to more generalist species. One of the major reasons for the decline of bumble bees is the limited number of host species some bumble bees can use based on the length of their tongue. Species of bumble bees with shorter tongues capable of reaching nectar deep in flowers are in a higher rate of decline than species with longer tongues which can gather nectar from a higher variety of flowers. Lucky for these shorter tongued bumble bees, knapweed is accessible to them as a refuge when species loss of native flowers takes place.

In many of the samples, knapweed pollen was present and sometimes in abundance. Though, on the sample dates knapweed pollen was abundantly collected, almost nothing but knapweed was available within the study site. While pollen is arguably the most important resource for bumble bees, nectar is also needed to keep bumble bees functioning, and knapweed is known to be a copious nectar producer (Runk, 2010). Late in the season, after new queens and drones have emerged from the nest, pollen is no longer needed for the production of royal jelly

(Bradford, 2017). These emerging queens then fatten themselves up on nectar to help them overwinter. Given that spotted knapweed blooms later in the season, it could have adapted this niche of providing nectar for late-season pollinators. However, at the study site, common snowberry (*Symphoricarpos albus*) was also present late in the season and based on observations, is also a copious nectar producer. The relationship between the two plants is unknown, but unless managed, the competitive exclusion will likely occur over time in favor of knapweed. Knapweeds' current abundance at the study site and its ability to displace other plants and change soil chemistry may eventually limit snowberry to a few patches or even none at all. This is the nature of invasive plants in disturbed ecosystems.

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