

Effects of nectar robbing on the volatile organic compounds and nectar chemistry of intraindividual flowers in *Corydalis caseana* ssp. *brandegeei*

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Keywords: nectar robbing, floral volatiles, nectar chemistry, *Corydalis caseana*, *Bombus mixtus*

Abstract

Plants are able to change volatile organic compounds in response to herbivory, and these chemicals can communicate with other parts of the same plant and with other plants. Nectar robbers like *Bombus mixtus* introduce a new microbial community to a flower's nectar that is different from the one introduced by legitimate pollinators, and this impacts chemical phenotype. This study asks: Does nectar robbing affect the nectar chemistry and volatiles of other flowers on the same inflorescence or the same plant? To answer this question, I compared sugar concentration and volatile organic compound composition of untreated, artificially robbed, and naturally robbed flowers and other flowers on the same *Corydalis caseana* ssp. *brandegeei* plant. Sugar concentration is lower in robbed flowers than in unrobbed flowers since rain can enter the nectary through natural or artificial robbing holes, but variation in our data between naturally robbed, artificially robbed, and control plants' sugar concentrations is more due to confounding variables than a systemic response to robbing. Volatile organic compound composition varies between artificially robbed, naturally robbed, and control plants but does not vary significantly between robbed and unrobbed inflorescences on the same plant.

Introduction

Chemical impacts of herbivory on plants have been studied by the scientific community. Many plants have the ability to produce new volatile organic compounds or change other chemical characteristics when they are damaged, and these volatiles can communicate damage and the need for resistance to other parts of the plant or even nearby plants (Karban, 2014). Studies of leaf herbivory have shown that plants can exhibit a systemic response to herbivore damage (Röse et al., 1996). Scientists have also begun to study impacts of nectar robbing, and we know that nectar robbing has impacts on microbial makeup and chemistry of nectar. However, gaps in both of these areas of research remain regarding the impact of robbing and florivory on nectar chemistry and volatile organic compounds of intraindividual flowers.

Nectar robbing is a form of floral larceny in which short-tongued insects chew through the nectar spur of a long tubular flower in order to access nectar without pollinating the flower. This appears to be an exploitative relationship as opposed to the pollination mutualism typically studied in pollinator science: it takes metabolically expensive nectar without helping the plant to reproduce, and evidence suggests it also deters legitimate pollinators (Varma et al., 2020). This phenotypic change appears to occur at least partially due to differing microbial communities introduced by legitimate pollinators vs. nectar robbers (Morris et al., 2020, Vannette & Fukami, 2016). Nectar microbes can have a variety of effects on floral phenotypes, including lowering total sugar concentration, lowering pH (Vannette et al., 2013), and influencing volatile organic compound composition, which is an important component of floral attractiveness to pollinators (Rering et al., 2018). Bees are able to detect microbial volatiles and are able to select between solutions containing different volatiles (Schaeffer et al., 2019). When flowers rely on pollinators to reproduce, volatiles that attract bees are important for floral fitness.

Nectar can exhibit antimicrobial or defensive secondary metabolites to protect against harmful microbes or herbivory (Alder & Irwin, 2005). It is challenging for plants to defend against nectar robbers, as many chemical defenses against robbing also decrease visitation by legitimate pollinators and lead to reduced reproductive outcomes (Alder & Irwin, 2005). This challenge is unique to floral larceny and requires specific study beyond leaf herbivory. Irwin et al. showed one effective mechanism: *Ipomopsis aggregata* has diluted nectar which reduces robbing without deterring hummingbird pollinators (2004). Other examples of chemical nectar defense mechanisms include toxins (Tiedeken et al., 2014) or high pH (Prÿs-Jones & Willmer, 1992). The former can have varying effects on legitimate pollinators, sometimes not affecting them at all and effectively targeting nectar robbers only (Stephenson, 1982) and sometimes negatively affecting pollinators at a cost to plant reproduction (Adler & Irwin, 2012). The latter can be produced by dissolved ammonia, which seems to deter nectar-robbing ants (Prÿs-Jones & Willmer, 1992). Many plants also emit herbivore-induced plant volatiles in response to attack that can signal damage to other plants and other parts of the same plant (Arimura et al., 2002; Karban et al., 2006), showing that plants are able to alter characteristics in response to attack. Evidence shows that damage from herbivores sometimes prompts a different plant response than artificial damage (Lehtilä & Boalt, 2008). The aforementioned microbial research suggests one reason this difference might be greater in my research than in general herbivory studies.

To fill the gap in research on plant response to nectar robbing, I asked: Does nectar robbing affect the nectar chemistry and volatiles of other flowers on the same inflorescence or the same plant? I studied this question in *Corydalis caseana* subsp. *brandegeei*. If nectar robbing affects the chemical phenotype of flowers on the same inflorescence or individual, I expect to see differing sugar concentration and volatile components in the neighboring flowers of inflorescences that have experienced nectar robbing, artificial robbing, or no treatment. If the effect is stronger within the same inflorescence, I expect to see a larger difference in sugar concentration between untreated and robbing-adjacent flowers than between untreated and robbing-adjacent inflorescences.

Methods

Field methods

I conducted field research in Crested Butte, CO, USA on Ute land promised in the Treaty of 1868 from which the Ute people were forcibly removed by 1881 (*Indian Land Cessions: U.S. Congressional Documents*, n.d.). The site is located near the Washington Gulch Trail #403 (38.969809, -107.045564) on Forest Service Road 811. It contains a large *C. caseana* population, which blooms from mid-June to September and is primarily pollinated by *Bombus appositus* and robbed by *Bombus occidentalis* and *Bombus mixtus*. *C. caseana* plants have up to 20 flowering stems, each with a primary inflorescence that has up to 70 flowers and secondary inflorescences with 5-30 flowers each. After opening, flowers persist for four days.

To prevent microbe introduction by pollination and uncontrolled nectar robbing, I carefully secured organza bags around all unopened inflorescences on 23 *C. caseana* plants. Each subsequent day in the field I continued to bag new unopened inflorescences on the plants.

On day 1, I applied treatments to bagged inflorescences. On each plant I labeled two inflorescences: one unmanipulated and one with one of the 3 treatments. For the manual robbing treatment, I artificially robbed all open flowers one one inflorescence per plant by piercing the nectar spur with a pair of ethanol-sterilized dissecting scissors. Then I again covered the inflorescence with an organza bag. For the natural robbing treatment, I captured a *B. mixtus*

worker and placed it in a bag with an inflorescence, allowing it to primary rob open flowers. Then I released the nectar robber and re-bagged the flower. For the unrobbed treatment, I left all flowers untouched and bagged. On each plant I treated two inflorescences: one unmanipulated and one with one of the 3 treatments.

On day 3, I collected my samples.

First, I collected volatiles from 14 plants using scent traps. I followed the solid-phase extraction scent trap design established in Rering et al. (2020). The traps contained 40 mg Tenax TA[®] (60–80 mesh) and 20 mg Carboxen[®] 1003 (40–60 mesh). The Tenax adsorbent material captures larger plant volatiles and the Carboxen captures smaller, more polar microbial volatiles; the Drierite removes water molecules from the air before it moves through the trap. To collect samples, I enclosed the inflorescence in an oven bag heat sealed to approximately 15 by 30 cm and connected the bag to the preassembled scent trap using Tygon tubing. I used cotton pads and a twist tie to secure the bag without damaging the stem. After allowing the plant volatiles to equilibrate in the bag for 30 minutes, I used an air pump to draw air through the trap for 15 minutes. For ambient air controls, I ran the pumps and scent traps without bagging a flower; for vegetative controls, I bagged a leaf in the same method described above. I avoided to avoid damaging leaves as much as possible to prevent the release of green leaf volatiles.

Second, I harvested the treated flowers from 23 plants by carefully removing them from the stalk using ethanol-sterilized forceps and placing them in a clean Ziploc sandwich bag. I tested the sugar concentration by removing nectar from each flower with a 10- μ L microcapillary tube and dropping it on a refractometer.

Lab methods

I used thermal desorption gas chromatography-mass spectrometry (GC-MS) to analyze volatiles collected via scent traps. Samples were analyzed using the Markes Ultra autoloading system connected to a Markes Unity-xr unit and the Shimadzu GC-MS QP2020 available at RMBL. I loaded the scent traps into the autosampler where they went through a two-stage desorption process at varying temperatures to help separate molecules. I used Shimadzu GCMS Postrun Analysis software to match volatile spectra with spectra from the National Institute of Standards and Technology reference library and, when available, used synthetic samples to further confirm identifications. I also analyzed control and vegetative samples in order to isolate which compounds are specific to floral parts and nectar.

Statistical methods

I used Kruskal-Wallis and Wilcoxon tests to compare sugar concentration between control plants, artificially robbed plants, and naturally robbed plants. I looked at the differences between treated nectar, same-inflorescence nectar, and same-plant nectar in each treated plant. I also looked at pooled data among all treated plants to compare the three treatments' overall effects on treated, same-inflorescence, and same-plant nectar. I visualized the multivariate data using non-metric multidimensional scaling. A redundancy analysis was then used to look at the difference in VOC profiles between treated inflorescences, untreated inflorescences from treated plants, and untreated plants. I also completed filtered redundancy analyses focusing on robbed and unrobbed inflorescences within each plant treatment. To assess the importance of specific compounds, I used Kruskal-Wallis tests. Analyses were done in R (R Core Team, 2021). The VOC analyses were done using the *bouquet* (Powers et al., 2021) and *vegan* (Oksanen et al., 2020) packages to load and organize data and the *capscale* and *anova* functions to run analyses.

Results

Nectar Sugars

There was a statistically significant difference between the sugar concentration of control, artificially robbed, and naturally robbed plants. When comparing untreated flowers on the robbed inflorescence from each group, differences were significant when comparing all three treatments ($p=2.4e-8$) and between each pair of treatments (Figure 1). In flowers on the untreated inflorescence, the groups were different ($p=2.3e-5$), and differences were significant in artificially robbed/control and artificially robbed/naturally robbed (Figure 2). In both of these comparisons, artificially robbed plants had the lowest sugar concentration, control plants had an intermediate concentration, and naturally robbed plants had the highest sugar concentration. When comparing robbed flowers between treatments, the groups were again different ($p=3.4e-8$) and comparisons between each group were significant (Figure 3). Here, artificially robbed flowers had the lowest sugar concentration, naturally robbed flowers were intermediate, and untreated flowers had the highest.

There were also significant differences between flower categories within plant treatment groups. Naturally robbed and artificially robbed plants both saw significant differences ($p=.0068$ and $.00012$, respectively), with robbed flowers having the lowest concentration, unrobbed flowers on robbed inflorescences the intermediate, and flowers on the untreated inflorescence the highest (Figures 4 and 5). The difference between unrobbed and untreated flowers was significant in artificially robbed plants ($p=.033$) but not naturally robbed plants ($p=.61$). There were no significant differences between flower groups in control plants ($p=.28$) (Figure 6).

Volatile Organic Compounds

In an unconstrained ordination, treatment groups appear similar (Figure 7). However, in a constrained redundancy analysis (RDA), there are significant differences between plant groups ($p=.005$) (Figure 8). Within plant groups, there are no significant differences between inflorescence treatments under the same analysis (artificially robbed: $p=.42$, naturally robbed: $p=.753$). Some important explanatory compounds in the RDA are significantly different between groups, including Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-; ethyl acetate; and butanoic acid, 2-methyl-, methyl ester (Figure 9).

Discussion

Nectar Sugars

Differences between plant groups within a flower type are likely explained more by confounding environmental factors within a small sample size ($n=23$) than by the treatments themselves since the two robbing treatments had concentrations in opposite directions when compared to the control treatment when looking at unrobbed or untreated flowers. However, significant differences in robbed flowers are important and can likely be explained by rainwater getting into holes in artificially and naturally robbed flowers but not being able to enter the unrobbed control flowers to the same extent. Artificially robbed flowers had a lower concentration than naturally robbed flowers, potentially suggesting that the artificial holes allowed in more rain than natural holes. If this is the case, an adjustment to the study design would be required. In the future, data could be collected on soil moisture and plant age, or a greenhouse experiment comparing effects of natural and artificial robbing may be justified.

Within plant groups, robbed flowers consistently had lower sugar concentrations due to rain. The difference between unrobbed flowers on robbed inflorescences and flowers on untreated inflorescences is significant in artificially robbed plants, with flowers on treated inflorescences having a lower concentration that is closer to that of the robbed flowers; this could be due to the small sample size, but it could also suggest some sort of systemic response unrelated to rain.

Volatile Organic Compounds

Although there were no differences between VOC content within plant groups (e.g. between robbed and unrobbed inflorescences on naturally robbed plants), there were significant differences between plant groups. As in the nectar sugar data, it is unclear whether this is due to natural variation between plants and a small sample size ($n=14$) or whether there are significant differences based on treatment. If the latter is true, the similarity between inflorescences within plant groups suggests a systemic response to nectar robbing. Interestingly, two of the three significant compounds originate in plants while the third originates in either plants or microbes. Again, this suggests either natural variation between plants or a systemic response within a plant, as opposed to a change in volatiles purely based off of microbes introduced to nectar.

Future Directions

The study of nectar robbing is in early stages when compared to herbivory or pollination. What we know from these areas of research can be applied to nectar robbing in this and future study designs. Repeating this experiment in a controlled environment where plants are the same age and receive the same soil moisture would potentially reduce confounding differences between plant groups. A larger sample size would also help achieve that goal.

Acknowledgements

This research took place on the traditional homeland of the Ute peoples. By 1868, the US Government had already begun taking land away in what is now Colorado, but in the Treaty of 1868 the third of the state west of the 107th meridian was made Ute treaty land. However, by 1881, the government took even that land and forcibly removed the Ute peoples from this region. Today, Ute reservations are mostly in southern Colorado and Utah, and Ute people live all over. The history, present, and future of Indigenous people in the United States is often purposefully and forcefully erased by a settler colonial narrative, including in the field of ecology.

Thank you to Valerie Martin, Dr. Robert Schaeffer, Ryan Stuart, Willow Lovecky, Dr. John Powers, James and Katie Cox, and my family.

Figures

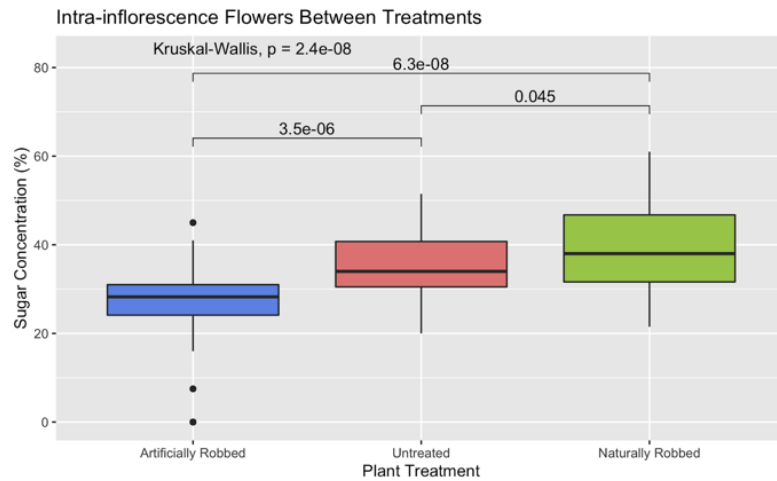


Figure 1: Intra-inflorescence flowers between treatments. This figure compares sugar concentrations between unrobbed flowers on robbed inflorescences in each of the plant treatments. The significant differences in sugar concentration between treatments is likely more due to environmental differences between plants than it is attributable to the treatments applied.

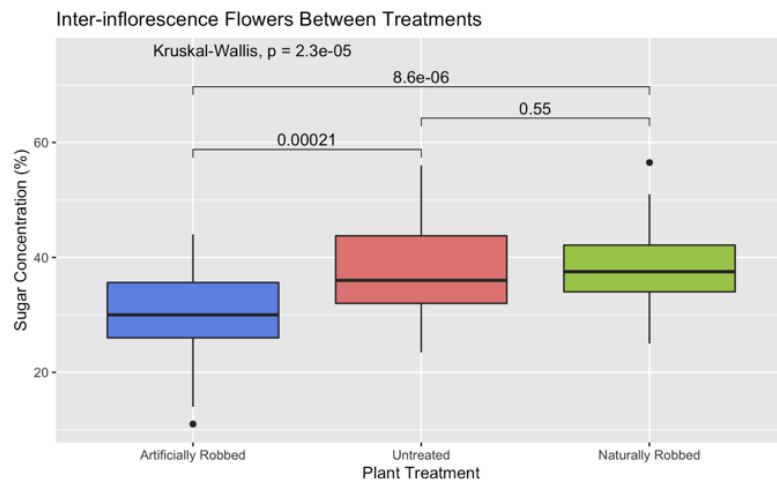


Figure 2: Inter-inflorescence flowers between treatments. This figure compares sugar concentrations between unrobbed flowers on untreated inflorescences in each of the plant treatments. The significant differences in sugar concentration between treatments is likely more due to environmental differences between plants than it is attributable to the treatments applied.

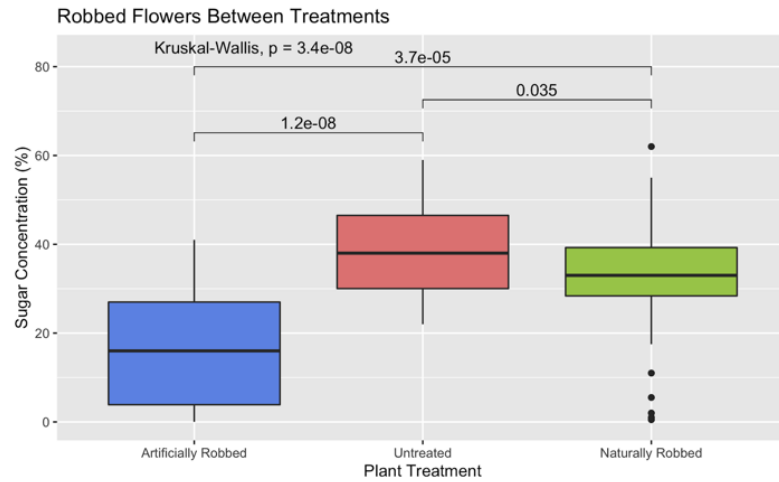


Figure 3: Robbed flowers between treatments. This figure compares sugar concentrations between robbed flowers (or, in the control plants, unrobbed flowers) in each of the plant treatments. The robbed flowers have significantly lower sugar concentrations than the unrobbed ones.

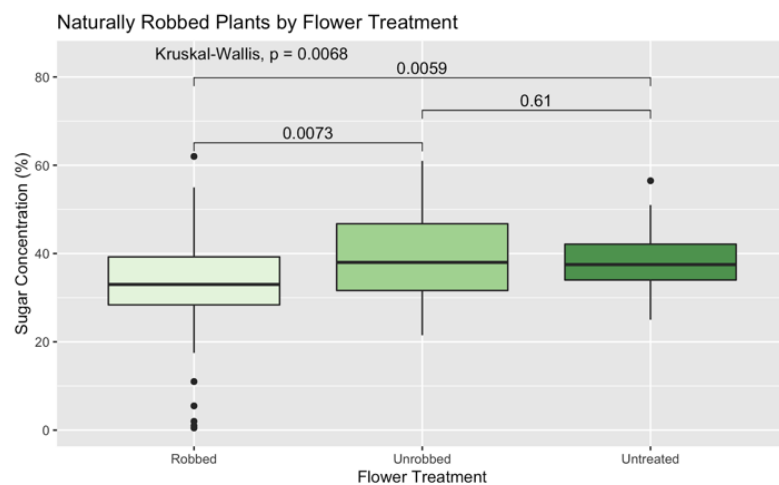


Figure 4: Naturally robbed plants by flower treatment. Robbed flowers have significantly lower sugar concentrations than unrobbed or untreated flowers.

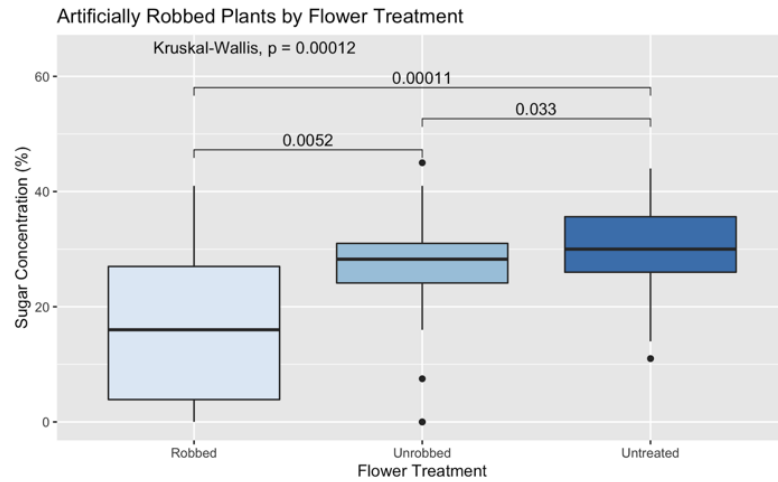


Figure 5: Artificially robbed plants by flower treatment. Robbed flowers have significantly lower sugar concentrations than unrobbed or untreated flowers, and unrobbed flowers also have significantly lower concentrations than untreated flowers.

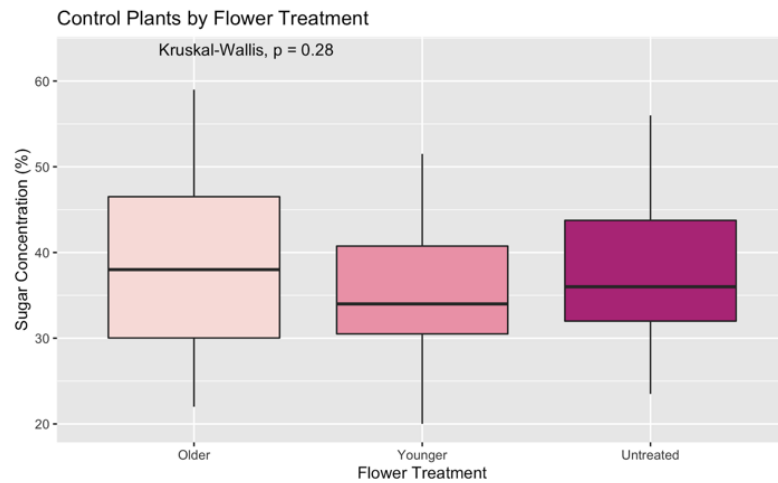
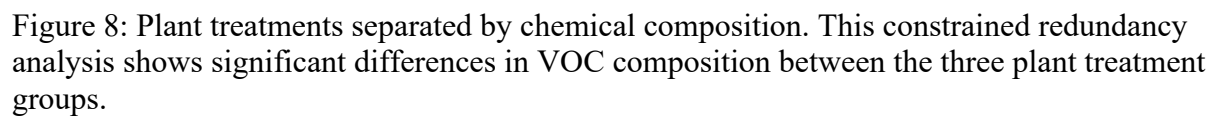


Figure 6: Control plants by flower treatment. There are no significant differences in sugar concentration between control flowers.



Compound	p-value
Acetic acid, 2-phenylethyl ester	0.1484
Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-	0.01416
D-Limonene	0.4866
Ethyl Acetate	0.04102
.beta.-Myrcene	0.06848
Butanoic acid, 2-methyl-, methyl ester	0.02775
Cyclopentanone	0.6229
Cyclopentanone, 2-ethyl-	0.3868
trans-.beta.-Ocimene	0.09755

Figure 9: Significant compounds. These compounds are important in the NMDS analysis, but not all are present in significantly different concentrations between treatment groups.

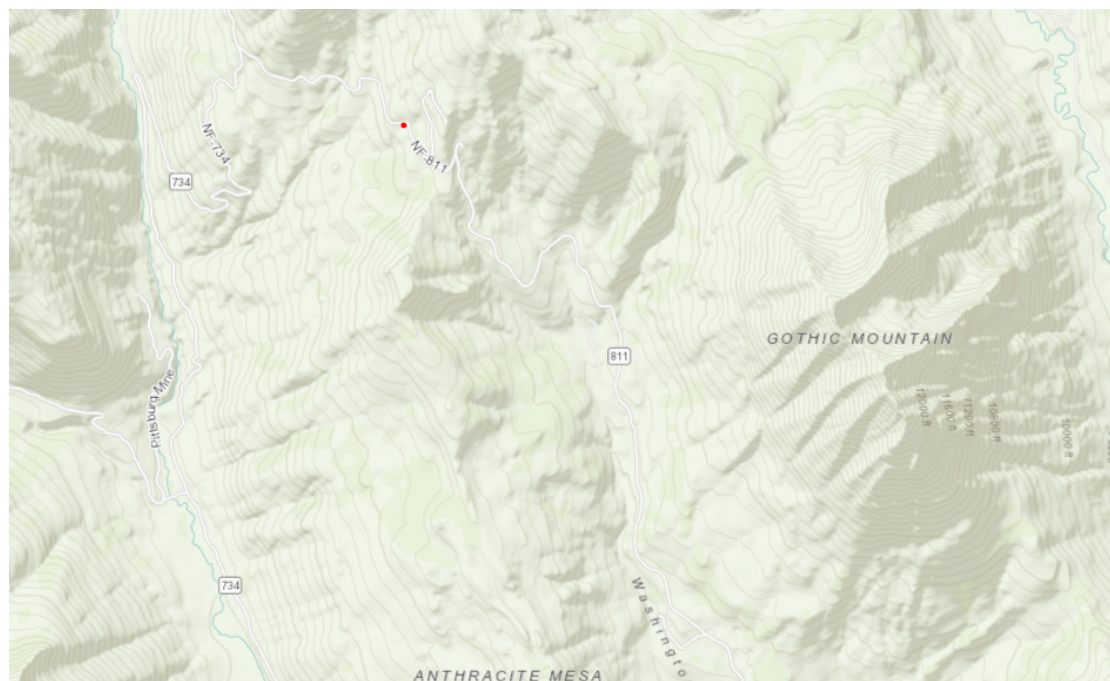


Figure 10: Study site. This site is located in Washington Gulch near the 403 trailhead.

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