Characterizing the ant-*B. stricta* relationship: How does plant investment in extrafloral nectar production alter ant recruitment?

RMBL Summer Education Program

Abstract

Extrafloral nectaries are commonly associated with protective mutualisms whereby the nectar-producing plant rewards predatory arthropod symbionts (commonly ants) in exchange for protection from herbivory. *Boechera stricta*, a self-pollinating perennial closely related to the model species *Arabidopsis thaliana*, is known to produce extrafloral nectar, but the benefit this conveys to the plant is not yet understood. In this study, we investigated the effect of plant investment in nectar production on ant recruitment using a synthetic nectar experiment. Synthetic nectar with varying levels of sucrose concentration was applied to post-flowering *B. stricta* plants, followed by observation of ant visitation. We then compared results of this experiment to pre-existing data on seed predator load and naturally occurring nectar volume in *B. stricta*. The goal of this research was to characterize aspects of the ant-*B. stricta* relationship; specifically by analyzing 1) how investment in extrafloral nectar alters ant visitation rates, and 2) how ant visitation, herbivory (seed predator load), and nectar volume influence one another at the population level. While we found evidence for a significant increase in ant visitation as a result of the addition of synthetic nectar, there was no difference in ant visitation to plants that received a high sucrose concentration (high investment) treatment compared to a low sucrose concentration (low investment) treatment. Furthermore, this study found no evidence for differentiating amounts of seed predation or naturally occurring nectar volume between *B. stricta* populations, meaning we are unable to define the relationship between these two response variables and ant visitation as of the completion of this study. This research nevertheless paves the way for future investigation into this study system; and provides evidence that variation in sucrose content in *B. stricta* extrafloral nectar does not significantly affect ant recruitment to the plant. Understanding the external factors that influence the fitness of *B. stricta* provides important knowledge for future research incorporating this model system.

Introduction

A primary way in which plants facilitate specific interactions with animals is through the production of nectar (Pacini et al., 2003). Nectar is a liquid composed of sugars and water, as well as small amounts of important amino acids and other molecules (Calixto et al., 2018; Del-Claro et al., 2016; Liao et al., 2025; Pacini et al., 2003). Nectaries, the organs that produce nectar, can be divided into two types: nectar produced in *floral* nectaries is commonly a reward for pollination, while nectar produced in *extrafloral* nectaries (EFNs) is typically associated with protective mutualisms. (Del-Claro et al., 2016; Liao et al., 2025; Pacini et al., 2003). This protective mutualism consists of the recruitment of predatory arthropod symbionts via the excretion of an extrafloral nectar reward, thereby decreasing herbivory on the plant (Calixto et al., 2018; Del-Claro et al., 2016; Liao et al., 2025; Pacini et al., 2003).

Ants are known to be the most common arthropod to engage in EFN-facilitated mutualisms (Calixto et al., 2018; Del-Claro et al., 2016; Pacini et al., 2003). These mutualisms tend to be quite generalized (Apple & Feener, 2001; Fagundes et al., 2017), and facultative as opposed to obligate (Fagundes et al., 2017), with considerable variation in the benefit the plant receives (Calixto et al., 2018; Del-Claro et al., 2016; Fagundes et al., 2017; Apple & Feener, 2001). One source of this variation comes from differences in mutualistic behavior, with the species of the mutualist ant being a key predicator of the protection they may afford a plant partner (Calixto et al., 2018, Fagundes et al., 2017).

Another important predictor of the efficacy of the ant-plant protective mutualism is the quantity and quality of the nectar reward provided by EFN-bearing plants (Alves-Silva & Del-Claro, 2013; Apple & Feener, 2001; Del-Claro et al., 2016; Fagundes et al., 2017; Lange et al., 2017). For example, Fagundes et al. (2017), in a study comparing multiple EFN-bearing plant species in Brazil, found that sugar

concentration of extrafloral nectar was a significant factor in the "protection effectiveness" a plant species was afforded by ants. Furthermore, Apple and Feener, in a 2001 study, found that a plant species with fewer EFNs received comparatively decreased visitation and protection from ants. While more nectar with higher concentrations of sugar is linked with more effective ant-plant mutualisms, nectar production is nevertheless a cost to the plant (Pacini et al., 2003) and represents an investment that must be appropriately balanced against the benefits of the protective mutualism.

Environmental factors are known to influence nectar production; among these are temperature and water availability as they relate to the production of sugars within the plant (Pacini et al., 2003). Similar to floral nectar, there is observable variation in the production of extrafloral nectar that may arise as a result of external factors. While EFN nectar production is less studied than its floral counterpart, a 2017 study shows that EFN nectar concentration may be impacted by temperature, and has been found to vary in multiple plant species over the course of the day (Lange et al., 2017). Furthermore, EFN production may be an *inducible* defense, with increases in nectar production associated with plant damage from herbivory (Calixto et al., 2018; Del-Claro et al., 2016).

Study Species

The focal plant for this study, *Boechera stricta* (Brassicaceae), is a small flowering perennial (Anderson et al., 2015; Anderson & Gezon, 2015; Song et al., 2006) with an extensive geographic and elevational range throughout the Rocky Mountains (Anderson et al., 2015). Prior research has proved that *B. stricta* is, by and large, diploid and sexually reproducing (Schranz et al., 2005), and typically self-pollinating (Anderson et al., 2015; Song et al., 2006). *B. stricta*, during its flowering stage, secretes extrafloral nectar (Rushworth et al., 2022) at the base of its flowers. Although it has not been extensively investigated, it is likely that this species's production of extrafloral nectar (as an inherent cost to the plant) is linked to a protective mutualism. In this study, we focused primarily on ants (*Formicidae*) as a predatory arthropod mutualist because they are the most common and well-researched protective plant mutualist, and because preliminary observations have shown ants drinking the nectar secreted by *B. stricta*.

Experimental Approach

This study consists of two approaches to investigate the role of plant investment into nectar production on ant recruitment to *B. stricta*. First, a synthetic nectar application experiment was carried out in naturally occurring *B. stricta* individuals. In this experiment, we aimed to discern the effect of nectar concentration (either no nectar application, 75% sucrose nectar, or 95% sucrose nectar) on ant recruitment to *B. stricta*. Our second approach was a population-level analysis comparing ant recruitment, seed predator load, and natural nectar production across the four *B. stricta* populations included in this study. Together, these approaches enabled a more thorough understanding of the role of plant nectar investment on *B. stricta* ant recruitment and the downstream effect of that relationship on seed predation at a population level.

This study sought to answer two questions: 1) Does the degree of plant investment in extrafloral nectar production affect ant recruitment to *B. stricta*? In response to this question, we predict that, in the *B. stricta* model system, nectar with higher sucrose concentration will result in higher ant recruitment. 2) Is there population-level evidence for or against the existence of an EFN-facilitated protective mutualism between ants and *B. stricta*? In response to this second question, we hypothesize that populations observed here to have higher ant visitation counts will also have lower seed predator loads and higher volumes of natural nectar production.

One goal of this study is to investigate how plant-ant interactions change in response to site-level environmental factors. This is an especially important question given the context of anthropogenic climate change and its effect on local environment, including temperature. Another aspect of the significance of this work is to better understand *B. stricta* as a model organism. Because *B. stricta* is an important model organism for scientific research, it is necessary to understand not only its biology as an individual organism, but also the symbioses it engages with that may shape its fitness in ways we cannot currently

predict. The presence of extrafloral nectaries in *B. stricta* is an understudied phenomenon that, judging from the role these nectaries play in other plants, may provide key insights into the relationships that *B. stricta* holds with other species.

Methods:

Synthetic Nectar Experiment

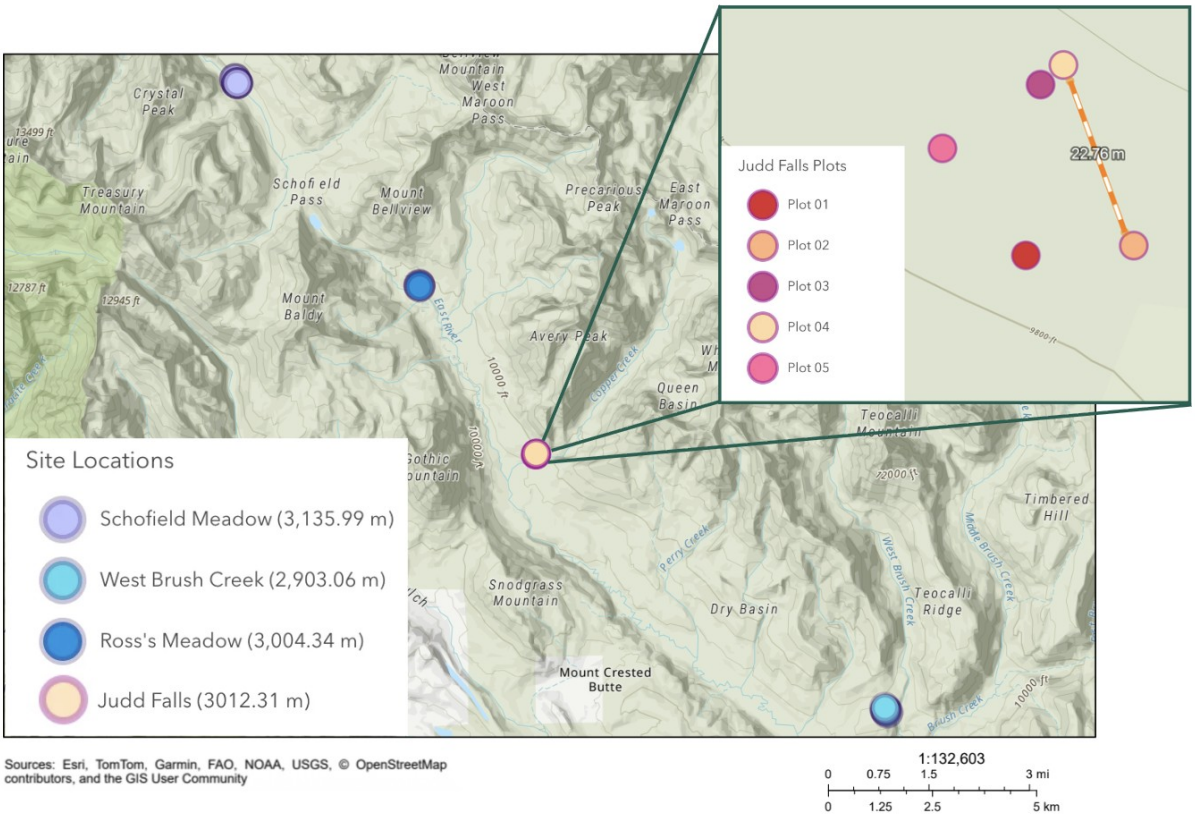

Figure 1: Map of sites included in this study. The elevation of each site is recorded in the legend on the bottom left. Plot arrangement is shown for Judd Falls site; this is similar across sites as plots were opportunistically established to encompass >2 B. stricta individuals until a total of 18 individuals were included at each site.

This study was conducted at the Rocky Mountain Biological Laboratory and in the surrounding Elk Mountains, and was carried out between the 8th and 17th of July, 2026. Data collection took place at four research sites established by the Carley Lab: West Brush Creek (-38.903, -106.884), Judd Falls (-38.96, -106.986), Ross's Meadow (-38.996, -107.0143), and Schofield Meadow (-39.0404, -107.0669) These sites, varying in elevation from 2,903.06 m (West Brush Creek) to 3,135.99 m (Schofield Meadow) were chosen to investigate the potential differences in the ant-*B. stricta* relationship that may arise as a result of large-scale habitat differences (including altitude) and population-level differences arising from geographic separation. In this study, *B. stricta* occurring within the same study site (Figure 1) are defined as members of the same population, and sites are used as a proxy for observing population-level differences in *B. stricta*.

Within each site, plots (each one square meter) were established opportunistically to include at least two *B. stricta* plants each (Figure 1). Eighteen *B. stricta* were included in this study at each site, with the number of plots per site ranging from five to seven. Plots were marked with a small orange stake in

each corner. To differentiate between plots, stakes were labeled numerically and GPS coordinates for each plot were entered into a GPS device (Garmin InReach; (Garmin Ltd.; Olanth, Kansas) or Trimble Catalyst Handle; (Trimble Inc.; Westminster, Colorado)).

Synthetic Nectar Treatments:

Synthetic nectar was utilized in place of natural nectar for this study to control for sugar concentration, with this solution being applied to the siliques (seed pods) of *B. stricta*. This experimental design reflects unpublished observations (by myself and other members of the Carley Lab) of ants climbing and inspecting *B. stricta* even after flowering (and therefore natural nectar production) had ceased.

Plants included in this study were assigned at random to one of three treatments (Figure 2): a control treatment with no synthetic nectar applied to the plant; a low 75% sucrose concentration treatment (0.75g sucrose per 1.0mL distilled water), or a high 95% sucrose concentration treatment (0.95g sucrose per 1.0mL distilled water). These values were based on prior observation during methods testing of a natural sucrose concentration of >80% in *B. stricta.* Individual plants were marked with small screws pressed into the soil for identification of the treatment the given plant was to receive. An attempt was made to have at least one plant of each treatment in each plot, but more dispersed *B. stricta* populations made this impossible in some cases. Twelve out of twenty-four plots have at least one plant of each treatment.

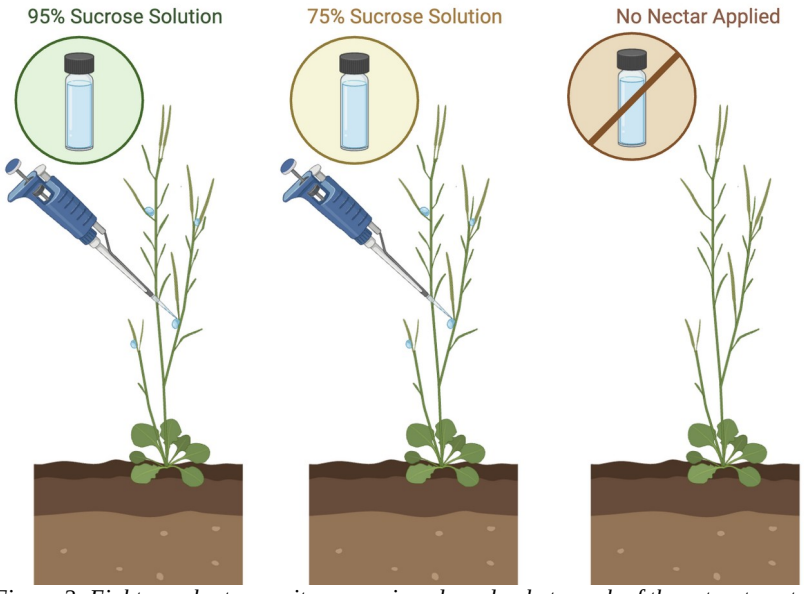

Figure 2: Eighteen plants per site are assigned randomly to each of three treatments: no nectar application (control), application of 95% sucrose solution (high concentration nectar treatment), or application of 75% sucrose solution (low concentration nectar treatment).Figure created in https://BioRender.com

To produce the synthetic nectar used in this experiment, table sugar and distilled water were combined, heated (via microwave for ~15 seconds) to dissolve, and stored in small glass vials for transportation to field sites. Prior studies have established the use of synthetic nectar in nectar research, from investigating the role of nectar concentration on pollination behavior (Maldonado et al., 2023) to analyzing the nectar microbiome (Mueller et al., 2023).

Extrafloral nectar is secreted in multiple small droplets at the external base of the flower in *B. stricta* nectar production. To maintain a degree of similarity to what is observed in natural nectar production of *B. stricta*, four 0.5µL droplets were placed as close as possible to the base of each of four mature siliques utilizing a p10 pipette (Eppendorf Research Plus, 0.5-10 µL; Eppendorf, Hamburg,

Germany). Nectar application was carried out twice per day (once in the morning, and once in the afternoon) and followed by ant observation surveys. The methods described were repeated over two days at each site, with the exception of Ross's Meadow, where multiple days of afternoon rains prevented afternoon surveys from taking place. Synthetic nectar was not manually removed from plants between surveys, given the unlikeliness of relocating all droplets, and that the removal process may alter the plants or add excess moisture to the surrounding area (as water would be the most effective method of removing concentrated sucrose solution).

Ant Surveys:

After nectar was placed, observational surveys of ant visitation were performed. The variable of interest was a count of ant visitation events occurring on each plant (which, in some cases, reflects multiple visits by the same individual ant) over the survey period. Plants occurring in the same plot were observed simultaneously. Each plot within a site was observed for five minutes. Ants were *not* identified to species level due to difficulty and uncertainty of in-field identification.

Observations took place at least ~20 minutes after nectar placement occurred to allow adequate time for ants to discover the nectar reward (minimum 18, maximum 41, and mean 26.92 minutes between application and survey). This ~20 minute mark was based on methods testing, during which ant count was instantaneously surveyed 5, 15, 25, and 35 minutes post nectar application at the West Brush Creek site to investigate how long it took for nectar to be discovered by ants when applied to *B. stricta*.

The following additional information was recorded on each individual plant: height (summed over all stalks if multiple stalks occurred on one plant), number of siliques occurring on the whole plant, and the number of stalks the plant possessed. Stalks are defined as diverging at the base of the plant, at the rosette. Maximum and minimum daily temperature were obtained for each survey time from either Crested Butte (West Brush Creek) or Gothic (Judd Falls, Ross's Meadow, and Schofield Meadow) weather data. Crested Butte weather data was obtained from the National Weather Service, and Gothic weather data was collected by billy barr.

Population-Level Analysis:

Pre-existing data specific to the *B. stricta* population of each of the Carley Lab's demographic sites (including the four utilized in this study) includes a survey of arthropod larvae occurring *within* the siliques of *B. stricta* (as a measure of seed predator load) and a survey of extrafloral nectar volume across a subset of individual plants. Ant visitation by population was compared with these two metrics to test for possible population-level correlation.

Seed predation methodology:

Data on seed predation was collected during the summer of 2025 at research sites utilized by the Carley Lab in the vicinity of the Rocky Mountain Biological Laboratory, including the four sites used for the above ant visitation methodology. At each site, between 42 and 60 mature siliques were collected from a random subset of *B. stricta* at each site (while avoiding plants occurring in permanent transects used by the Carley Lab). Siliques were analyzed at the University of Chicago by the Carley Lab for the following metrics: silique length, count of seed predator larvae found in the silique, and a count of seeds found in the silique. Seed predator larvae were preserved in ethanol.

Natural extrafloral nectar production methodology:

Data on extrafloral nectar production in *B. stricta* was collected by the Carley Lab (led by graduate student Greta Thoresen) during May and June of 2026 at the same research sites utilized by the Carley Lab for seed predation data collection. For the subset of those sites included in this project, fifteen plants per site were selected semi-randomly for nectar volume analysis while again avoiding plants found in permanent transects at those sites (except for Schofield Meadow, where ten individuals were measured). Only plants with a measurable quantity of liquid nectar were included in data collection;

representing a subset of the total population. For each of these plants, nectar volume was summed over the entirety of the plant using graduated microcapillary tubes.

Data Analysis:

Nectar concentration as a predictor of ant visitation:

To answer Question 1 posed in this study, a series of generalized linear mixed effect models with a negative binomial distribution were fit with ant visitation count over a five minute survey period as the response variable, and the nested random effects of plot and individual plant. Synthetic nectar treatment (low concentration, high concentration, or control), time of day (morning vs afternoon survey), plant height (as a metric of usable plant area), and temperature for the given survey date and location were also included as possible predicator variables. Site was taken into account as a predictor variable, as significant variation in habitat characteristics (including elevation) are found between sites. It is important to note that it is impossible to disentangle the genetic effect of population (given that each site is geographically separated by distance and home to a different population of *B. stricta*) from the effect of environmental factors that differ between sites. Models fit with package glmmTMB (Brooks et al., 2017).

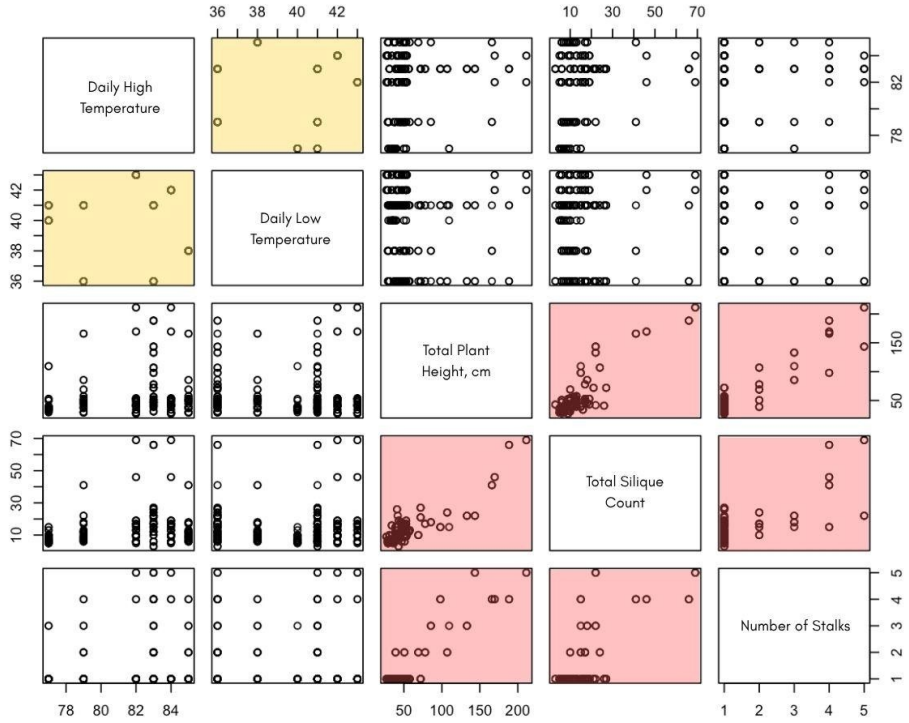

Figure 2: Pairwise comparison of numeric predictor variables, testing for multicollinearity prior to fitting ant visitation models.

When testing for multicollinearity (see Figure 2), it was visually determined that plant height, number of stalks, and silique count were highly correlated and therefore could not be included in the same model. Plant height was chosen as the metric of plant morphology to include in modelling, as it best reflects the usable plant area available to visiting ants. While daily high temperature and daily low temperature were not clearly correlated, this may have been due to a low sample size. The decision was made to not include both temperature metrics in the same model.

A null model was fit, as well as a model for each individual predictor variable and each of four potential interactions: the effect of treatment interacting with the effect of site, the effect of treatment interacting with the effect of survey time, and the effect of temperature (daily high or daily low) interacting with the effect of treatment. No other interactions were identified as being plausible given the

nature of the study system. Two additive models were fit that included all predicator variables; one with daily low temperature and one with daily high temperature. All models can be seen in Table 1.

Table 1: List of fitted models for ant visitation with AIC values listed. Variables found to have significant effect (p-value < 0.05) are highlighted blue. The top model's AIC value is highlighted in green, and the null models (as well as models that failed to outperform the null, being <2 ΔAIC different from the null) are highlighted in red.

Model Ranking	Model Formula	Model AIC
1	glmmTMB(ant_visits~Treatment + daily_low_temp + Height_summed_cm + general_time + Site + (1|PlotID/Unique.ID), data = visitplant, family = nbinom2)	318.1
2	glmmTMB(ant_visits~ Treatment * daily_low_temp + (1|PlotID/Unique.ID), data = visitplant, family = nbinom2)	322.7
3	glmmTMB(ant_visits~ Treatment*daily_high_temp + (1|PlotID/Unique.ID), data = visitplant, family = nbinom2)	331.6
4	glmmTMB(ant_visits~Treatment + (1|PlotID/Unique.ID), data = visitplant, family = nbinom2)	334.9
5	glmmTMB(ant_visits~ Treatment*general_time + (1|PlotID/Unique.ID), data = visitplant, family = nbinom2)	336.3
6	glmmTMB(ant_visits~ daily_low_temp + (1|PlotID/Unique.ID), data = visitplant, family = nbinom2)	337.8
7	glmmTMB(ant_visits~ Site + (1|PlotID/Unique.ID), data = visitplant, family = nbinom2)	340.1
8	glmmTMB(ant_visits~ general_time + (1|PlotID/Unique.ID), data = visitplant, family = nbinom2)	345.5
9	glmmTMB(ant_visits~1 + (1|PlotID/Unique.ID), data = visitplant, family = nbinom2)	346.7
10	glmmTMB(ant_visits~ daily_high_temp + (1|PlotID/Unique.ID), data = visitplant, family = nbinom2)	347.2
11	glmmTMB(ant_visits~ Height_summed_cm + (1|PlotID/Unique.ID), data = visitplant, family = nbinom2)	347.6
12	glmmTMB(ant_visits~Treatment + daily_high_temp + Height_summed_cm + general_time + Site +(1|PlotID/Unique.ID), data = visitplant, family = nbinom2)	No Output (Failed to Converge)
13	glmmTMB(ant_visits~ Treatment*Site + (1|PlotID/Unique.ID), data = visitplant, family = nbinom2)	No Output (Failed to Converge)

Models were ranked in accordance with Aikake's Information Criterion (AIC), and a top model (the model with the lowest AIC value) was chosen. The model with the lowest AIC value was the full additive model (including all predictor variables) with daily low temperature as opposed to daily high temperature (AIC = 318.1). No other models were considered as top models, because no other models occurred within 2 ΔAIC of 318.1. Stepwise comparison of models was used to determine if any additive combination of predictor variables would outperform the full additive model. This analysis returned the full additive model as the model of best fit, confirming its selection as the best performing model for this data.

Goodness-of-fit for the top model was determined using the DHARMa package in R (Hartig et al., 2024). Model fit showed no evidence of zero-inflation, underdispersion, or overdispersion and was determined to be adequate (Figure 3).

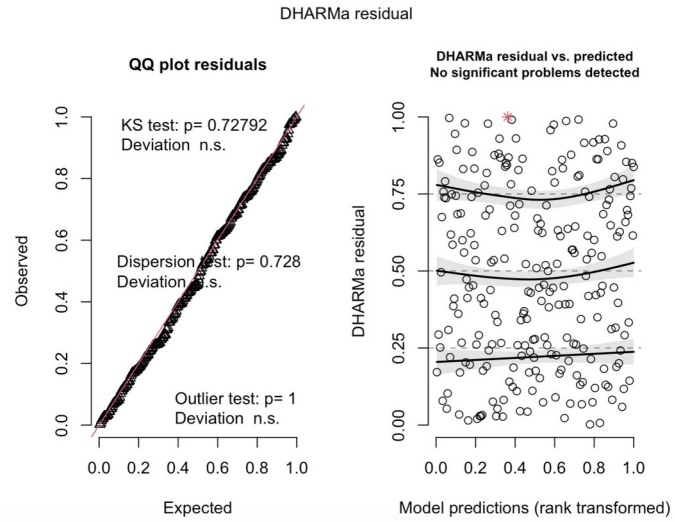

Figure 3: DHARMa goodness-of-fit analysis of the full additive model (with daily low temperature instead of daily high temperature) for ant visitation.

Analysis of pre-existing data

To answer the second question of this study, two series of models were fit: one with seed predator larvae count as a response variable, and the other with naturally occurring nectar volume as a response variable.

Predictor variables fit to nectar volume include site, general time of day that the sample was collected (morning or afternoon), and average flower length of the nectar-producing flowers of the sampled plant. Linear models were fit to this data, as the response variable (nectar volume) was log-transformed to achieve a Gaussian distribution (Shapiro-Wilkes test, W = 0.98974, p-value = 0.9193).

To analyze seed predation, a series of generalized linear models were fit with the count of seed predator larvae per silique as a response variable. Silique length was included as a potential predictor variable alongside site. General linear models were fit with a negative binomial distribution using package glmmTMB (Brooks et al., 2017). Although there was some cases of non-independence in this data, with 19 out of 193 sampled plants having more than one silique included in this dataset, this potential random effect was deemed unlikely to confound model outcomes based on visual analysis and given that the vast majority (79.8%) of siliques included in this dataset came from plants that were only sampled once.

As above, a null model and a full additive model were fit in addition to a single-variable model for each of the predictor variables (see Table 2 for nectar volume models and Table 3 for seed predation models) for both analyses. An interaction between silique length and source site was tested in addition to the aforementioned models. Aside from this, no other interactions were deemed plausible in either study system.

Table 2: List of models fitted to nectar volume data with AIC values listed. Variables found to have significant effect (p-value < 0.05) are highlighted blue. The top model's AIC value is highlighted green, and the null models (as well as models that failed to outperform the null) are highlighted red.

Model Ranking	Model Formula	Model AIC
1	glm(lognectar ~ avg_flr_length, family = gaussian, data=nectarunique)	110.01

2	glm(lognectar ~ 1, family = gaussian, data=nectarunique)	113.1
3	glm(lognectar ~ generaltime, family = gaussian, data=nectarunique)	114.4
4	glm(lognectar ~ Site + generaltime + avg_flr_length, family = gaussian, data=nectarunique)	117.16
5	glm(lognectar ~ Site, family = gaussian, data=nectarunique)	117.65

Table 3: List of models fitted to seed predator data with AIC values listed. Variables found to have significant effect (p-value < 0.05) are highlighted blue. The top model's AIC value is highlighted green, and the null models (as well as models that failed to outperform the null) are highlighted red.

Model Ranking	Model Formula	Model AIC
1	glmmTMB(larvaecount ~ 1, data = seednona, family = nbinom2)	531.8
2	glmmTMB(larvaecount ~ fruit_length, data = seednona, family = nbinom2)	533.2
3	glmmTMB(larvaecount ~ site, data = seednona, family = nbinom2)	536.8
4	glmmTMB(larvaecount ~ fruit_length + site, data = seednona, family = nbinom2)	538.2
5	glmmTMB(larvaecount ~ fruit_length*site, data = seednona, family = nbinom2)	543.0

Models were then ranked, with the top model being that with the lowest AIC value. As the only model to outperform the null (>2 ΔAIC less than the null model), the top model that explained variation in natural nectar volume was the single-variable model for average flower length as a predictor variable. Stepwise comparison confirmed this model as having the best fit when compared to all other combinations of the three predictor variables. Goodness-of-fit was determined visually using a Q-Q plot (Figure 4), which showed good model fit for the top model, and adequate fit for the full additive model. No fitted model for seed predator larvae count outperformed the null model. Stepwise comparison was not employed in this case, as all possible variable combinations are accounted for in the above fitted models (Table 3). Goodness of fit for the full additive model fitted to seed predator larvae count (utilizing DHARMa package in R) demonstrated adequate fit, with no evidence for overdispersion or zero inflation.

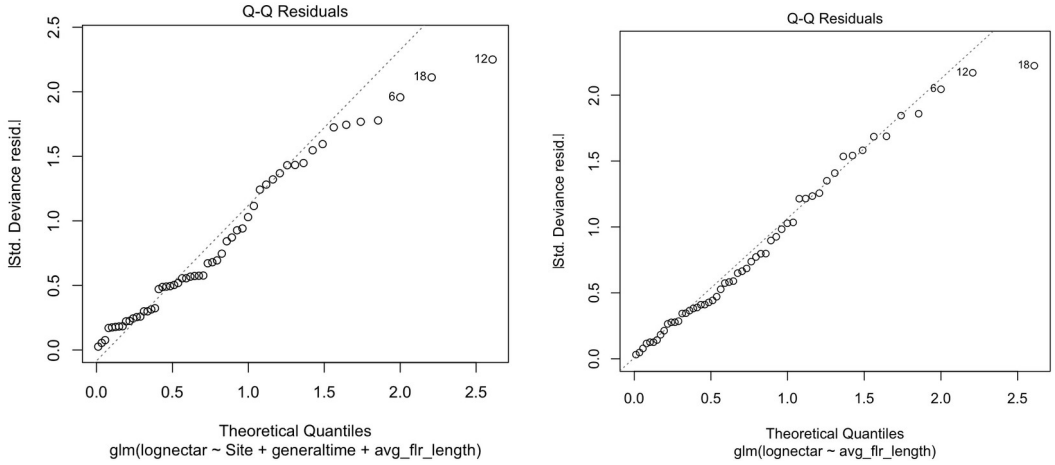

Figure 4: Q-Q Plot demonstrating model fit of the top performing model (left) and full additive model (right) for nectar volume data.

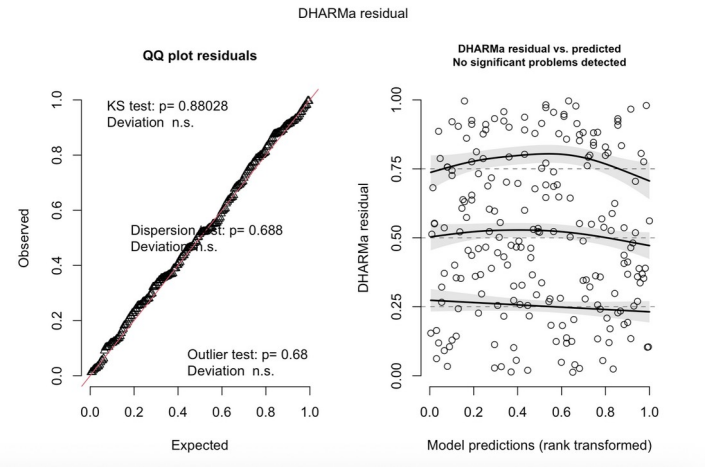

Figure 5: DHARMa model fit for full additive model with seed predator load as response variable

All statistical analysis took place in R (version 4.4.1, R Core Team). When constructing the code for data analysis, the following resources were used as reference: code written for RMBL education program workshops, class notes from BIOS 338 ("Analysis and Visualization of Biological Data") at Rice University, non-AI web resources (Stack Exchange, R Documentation, etc.), and code written by Caroline Pollan and Dr. Andrea Rummel for another research project as part of BIOS 310 ("Independent Research") at Rice University. Some sections of code written for BIOS 310 were corrected for errors using AI resources (primarily Google Gemini) or otherwise written with assistance from those same tools.

Results:

Nectar concentration as a predictor of ant visitation:

Treatment, daily low temperature, and site had significant effects on ant visitation, while plant height and the time of day the survey took place did not (Table 1). While there was no significant difference in ant visitation to plants that received the 75% sucrose concentration nectar treatment and 95% sucrose concentration nectar treatment (p-value = 0.5346), both the 75% sucrose concentration nectar treatment (p-value = 0.0006) and 95% sucrose concentration nectar treatment (p-value = 0.0075) had a significant positive effect on ant visitation when compared to the control (no nectar applied) (Figure 6) (Emmeans pairwise comparison, R package emmeans, Lenth et al., 2025). Increasing daily low temperature had a significantly positive effect on ant visitation (p-value = 0.000207). The only significant difference in ant visitation between sites occurred between Schofield Meadow and Judd Falls, with Judd Falls experiencing significantly more ant visitation (p-value = 0.0242, Figure 5) (Emmeans pairwise comparison, R package emmeans, Lenth et al., 2025).

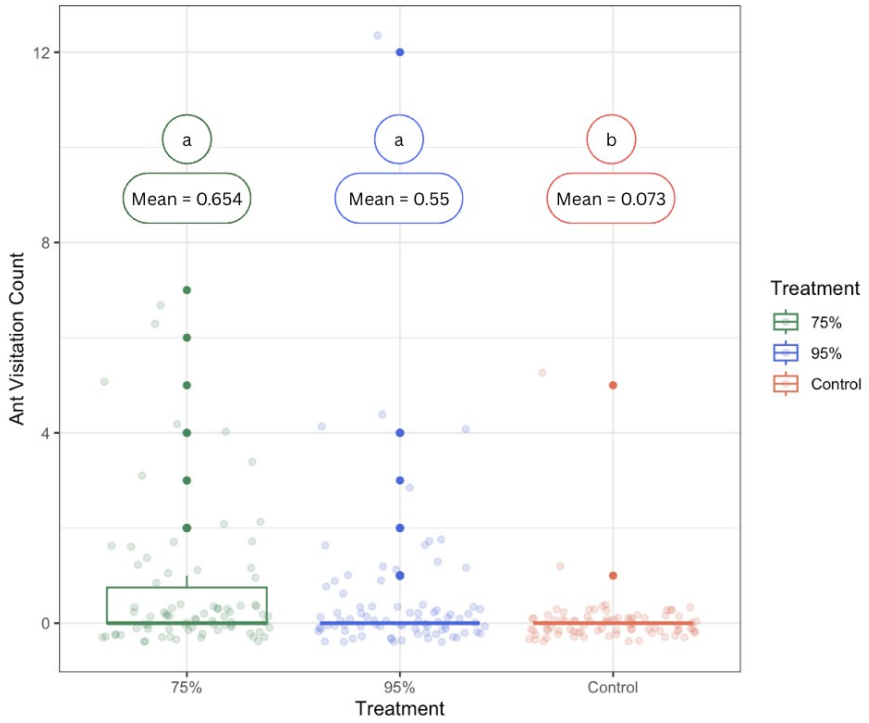

Figure 5: Boxplot of ant visitation count by site. JUD = Judd Falls, ROS = Ross's Meadow, SCH = Schofield Meadow, WBC = West Brush Creek. The mean value of ant visitation at each site (across treatments) is noted at the top of the graph. Letters "a" and "b" delineate significant statistical difference between ant visitation at different sites in accordance with the full additive generalized linear mixed model. Plots made with package ggplot2 (Wickham et al., 2016).

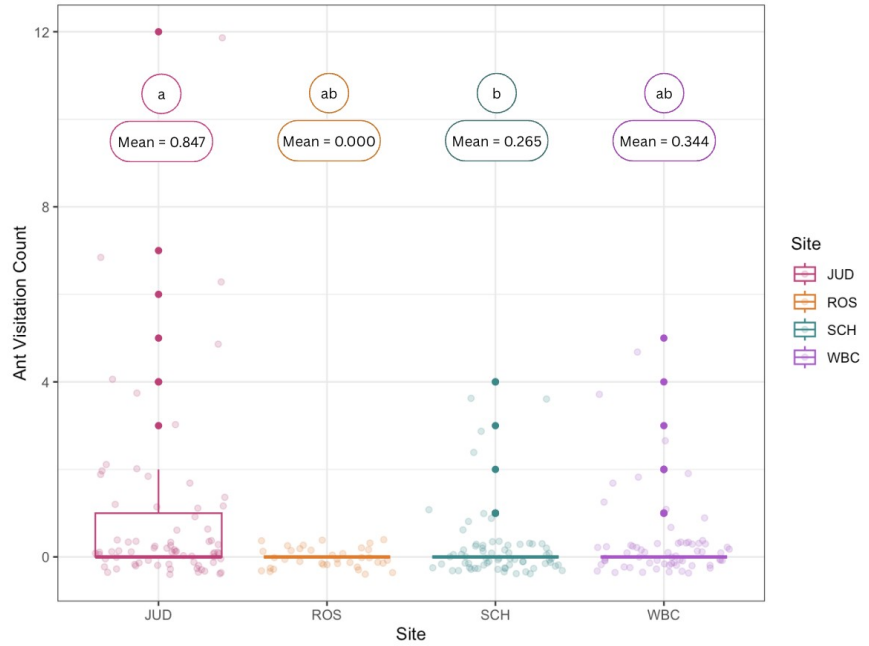

Figure 6: Boxplot of ant visitation count by treatment. 75% = 75% sucrose solution application, 95% = 95% sucrose solution concentration, Control = no synthetic nectar applied. The mean value of ant visitation for each treatment is noted at the top of the graph. Letters "a" and "b" delineate significant statistical difference between ant visitation in accordance with the full additive generalized linear mixed model.Plots made with package ggplot2 (Wickham et al., 2016).

Population-level comparison:

Because the inclusion of site-specific population as a predictor variable did not improve upon the null model, it can be concluded that there is no evidence for differences in natural nectar production or seed predator load between site-specific populations of *B. stricta*. However, increasing flower length did have a significant positive effect on nectar volume (p-value = 0.0274). None of the tested predictor variables had any significant effect on seed predator load, with all fitted models failing to improve upon the null model. While population was found to have a significant effect on ant visitation, this was only true in on pairwise comparison (Judd Falls population compared to Schofield Meadow population), with no evidence for differentiation between Judd Falls, Ross's Meadow, and West Brush Creek or Schofield Meadow, Ross's Meadow, and West Brush Creek.

Discussion:

Two key studies demonstrate the role that sugar concentration of extrafloral nectar plays in the ant-plant mutualism: a study by Fagundes et al. came to the conclusion that sugar concentration of extrafloral nectar significantly altered "protection effectiveness" demonstrated by ant mutualists, but it is important to note that this study occurred across *multiple* EFN-bearing plant species (Fagundes et al., 2017). Alves-Silva and Del-Claro's study, on the other hand, investigated this relationship within the same species (*Banisteriopsis campestris*), finding that the abundance of ant mutualists increased with higher sugar concentration in extrafloral nectar in both control individuals and resprouted individuals in fire-altered landscape (Alves-Silva & Del-Claro, 2013). While it has been found in prior research that the ant-plant mutualism is influenced by the concentration of the nectar reward, our results do not align with this finding. In this study, we found that ant visitation significantly increased with synthetic nectar application. However, the degree of plant investment (modelled here as the concentration of sucrose in the synthetic nectar solution) did not appear to affect ant visitation, with no significant difference detected among plants that received a 75% sucrose synthetic nectar and those that received 95% sucrose synthetic nectar.

One potential reason for this discrepancy is the fact that both the low (75% sucrose) and high (95% sucrose) concentration treatments, while based on the actual sucrose concentration of *B. stricta* extrafloral nectar, were still quite high compared to other published extrafloral nectar sugar concentrations. For example, Alves-Silva and Del-Claro (2013) reported a range of 0.04 to 0.48 mg/microliter in reprouting *B. campestris* plants, and a range of 0.05 to 0.35 mg/microliters in the control group (4-48% and 5-35% sugar concentration, respectively). In addition, a study characterizing the extrafloral nectar concentration of the cowpea (*Vigna unguiculata*) found a range of 127 to 244 mg/mL of sucrose in one type of EFN, and a range of 100 to 271 mg/mL in another (for context, this equates to 12.7% to 24.4% sucrose in the first EFN and 10% to 27.1% sucrose in the other) (Pate et al., 1985). It could certainly be that the sucrose concentrations modelled in this study are too high to have an observable effect on ant preference, with both being potentially quite appealing to ants.

While there was no significant difference in ant visitation between plants that received low or high concentration synthetic nectar, the application of the nectar itself *did* result in significantly higher ant recruitment. From this result, we can see that this extrafloral nectar resource is desirable to ants, with plants possessing this resource being sought out in comparison to those that lacked it. This was true even considering the fact that this synthetic nectar was a fairly simplified version of the real thing: natural nectar is a complex solution consisting of *multiple* sugar types in addition to certain amino acids (Calixto et al., 2018; Del-Claro et al., 2016; Liao et al., 2025; Pacini et al., 2003). It would be interesting to analyze whether altering other key components of nectar, such as amino acid concentration or the concentration of other sugars, would significantly alter ant visitation patterns or show ant preference for higher or lower levels of certain key nectar components.

Another factor found to significantly affect ant visitation in this study was daily low temperature, with higher daily lows correlating with increased ant visitation. Interestingly, daily high temperature (ranging from 77 to 85 degrees Fahrenheit) did not have any significant effect on ant visitation, suggesting that the range of daily low temperatures (36 to 43 degrees Fahrenheit) is a better predictor of ant foraging activity in the subalpine environments where this study was conducted. It must be

emphasized, however, that ants are *incredibly* diverse, and this is reflected in variation in their thermal constraints: for example, a review by Roeder et al. notes differences in thermal constraints between diurnal and nocturnal ant species, as well as diurnal and nocturnal castes of the *same* species (Roeder et al., 2021). Interestingly, this review also reports that multiple studies have found a correlation between increased cold tolerance in ants with increasing elevation, with a lack of agreement in the literature on the relationship between *heat* tolerance in ants and elevation (Roeder et al., 2021). This aligns with the findings of my study in suggesting that cold minimum temperatures common at high altitudes may place more stringent thermoregulatory stress on ants than high maximum temperature.

The only significant difference found between populations occurred in ant visitation between the Judd Falls population and the Schofield Meadow population, with the Judd Falls population receiving significantly more ant visitation across treatments. Interestingly, Schofield meadow did experience more ant visitation than Ross's Meadow (with Ross's Meadow having zero recorded ant visits). However, because data was only able to be collected from Ross's Meadow in the two morning surveys due to afternoon rains, the sample size for that population is smaller than the other three. This could lead to the lack of statistical evidence for significant differentiation between this population and the others. In terms of naturally occurring nectar volume, there was no statistical evidence for differentiation between populations, which could in part be due to a smaller sample size (10-15 individuals per site). There was also found to be no significant difference in seed predator load of *B. stricta* between site-specific populations. Because nectar volume and seed predator load did not significantly vary between the four sites included in this study, it is impossible to draw any conclusions on how ant visitation, nectar volume, and seed predation relate to one another at the population level. This certainly does not mean that there *isn't* some link between ant visitation, nectar production, and herbivory on *B. stricta*: this study was limited in scope on this matter, and further investigation into this system would provide a more definitive answer as to how these three variables are related.

There are three primary future research directions recommended in this study system: the first would be investigating whether there is an identifiable fitness benefit to ants and *B. stricta* engaging in this interaction. The second would be analyzing the effect of other nectar components on ant recruitment to *B. stricta*. Lastly, we would recommend a study linking multiple forms of herbivory, nectar production (both volume and composition), and ant visitation within the same year and on the same individual plants.

Very little is known about the role of extrafloral nectar in *B. stricta*. Based on what is known about extrafloral nectar production in other plants, it is likely that EFNs in *B. stricta* facilitate protective mutualisms. This study proves that ants were reliably recruited to *B. stricta* by the presence of synthetic nectar in three out of four sites, even in the absence of floral cues. Furthermore, this study provides evidence that differences in sucrose concentration of extrafloral nectar in *B. stricta* did not affect the degree of ant visitation afforded to a plant. These findings begin to illuminate the nature of the ant-*B. stricta* relationship, paving the way for further research on this potential symbiosis in a model organism.

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