

Observing the effects of a nectar-inhabiting bacterium, *Pantoea sp.*, and nectar robbing on male fitness in *Ipomopsis aggregata*

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Abstract

There is still much unknown about how microbes within flower nectars, specifically bacteria, impact pollinators. Hummingbirds use gustatory and visual cues to determine which flowers are most rewarding. The diversity in colonization of nectar-inhabiting microbes, such as bacteria, can alter the scent, taste, and chemical composition of a plant's nectar. Additionally, antagonistic nectar-robbing by insects can further alter the composition of nectar and affect a pollinator's perception of a floral reward. Despite this, there is very little research looking at the combined effect of nectar-inhabiting bacteria and nectar robbing on pollen movement and male flower fitness, within a natural system. We aim to observe this effect through inoculation of *Pantoea sp.* bacteria and application of a robbing treatment on *Ipomopsis aggregata*, a hummingbird-pollinated plant. We will track pollen donation using quantum dot fluorescent crystals. We expect to see lower male fitness and less pollen donation in plants that were robbed and inoculated with bacteria because *Pantoea* has been seen to ferment sugars within nectar, and robbing has previously been seen to deter pollinators.

Introduction

Plant-pollinator mutualisms are a key feature in the ecological world that provide beneficial goods and services to each individual taking part, while also promoting biodiversity (Bronstein 1994). Disruptions to these relationships can occur when organisms engage in exploitative, cheating behaviors such as nectar-robbing. This involves organisms extracting nectar from flowers either by creating a hole in the base of the flower (primary robbing) or by accessing the nectar through a hole that is already present (secondary robbing) (Irwin et al. 2010). In doing this, they bypass the reproductive structures of the flowers, causing both direct and indirect effects on flower fitness. Indirect effects of nectar-robbing include decreased pollinator visitation due to reduced nectar volume or reduced attractiveness of the floral phenotype via alterations to scent, visual cues, nectar taste, or other components of floral display (Wang et al. 2023).

When pollinators and robbers visit flowers, they deposit various microbes onto the floral structures, pollen, and nectar. Microbes such as yeast and bacteria can occupy up to 70% of individual flowers (Vannette 2020). These microbes can impact various aspects of the nectar chemistry and floral scent, ultimately altering how floral visitors perceive flowers. Pollinators like hummingbirds use gustatory cues to find nectar with high concentrations of sugar, showing a

specific preference for high concentrations of sucrose while avoiding nectar with high fructose concentrations. A past study by Vannette and colleagues observed that when the nectar specialist bacterium, *Neokomagatea sp.*, inhabited nectar, it reduced the pH and total sugar concentrations, specifically decreasing glucose concentrations and increasing fructose concentrations. The nectar's change in taste profile resulted in reduced pollination success, seed set, and nectar consumption by hummingbirds. While the lower seed set highlights a decrease in female fitness in bacteria-inoculated flowers, this experiment was unable to measure bacterial effects on pollen transport or male reproductive success in the flowers.

Most studies look at the individual effects of floral microbes or nectar-robbing on plant reproductive fitness, with none to our knowledge exploring their combined effects. Our study seeks to fill this gap by investigating the effects of both nectar robbers and a nectar-inhabiting microbe on male reproductive success of plants (Irwin et al. 2010, Vannette 2020). Male reproductive fitness can be measured as pollen donation (i.e., transport) to conspecifics by pollinators. Pollen donation can provide a quantitative estimate for the distribution of pollen grains from one flowering plant to others in surrounding areas, with the greater the number of pollen grains reflecting a greater chance for reproduction (Opedal et al. 2023). To track pollen movement, we will utilize quantum dots: fluorescent crystals made with a lipophilic ligand. These can be dissolved in a nonpolar solvent such as hexane and applied to the anthers of a flower. Once the hexane evaporates, the ligands within the quantum dots will bind to the lipids on the pollen grain surface, essentially staining each individual pollen grain (Minnaar & Anderson 2019). When the quantum dot is exposed to UV radiation, the electrons & electron-hole pair in a bound state (called an exciton), can become excited and upon settling to the ground state emit a fluorescent light. Using an epifluorescent microscope, we can use this technique to precisely quantify the amount of pollen transported from donors to recipients. This quantification was not possible when replacing the pollen with dye particles. This older practice allowed for the tracking of movement but did not represent the pollen itself (Schaeffer & Irwin 2014).

We will investigate how the presence of the bacterium *Pantoea* in nectar affects the male reproductive fitness of *Ipomopsis aggregata*, a hummingbird-pollinated flower. *Pantoea* is a gram-negative, non-spore-forming bacterium that is a common plant epiphyte. *Pantoea* can be found throughout the phyllosphere of a plant, but can be transferred to the nectar by rain, wind, and flower visitors via a robbing wound. While the effect of *Pantoea* on *I. aggregata* has yet to be investigated, *Pantoea* strains have been seen to ferment glucose, lactose, and sucrose (Cusumano et al. 2023). We then expect *Pantoea* to alter nectar flavor profiles to those undesirable by hummingbird pollinators and, as a result, see lower male fitness, or fewer pollen grains present in surrounding flowers. Additionally, we would expect to see lower male fitness in flowers with evidence of robbing, as Irwin and Brody (1998) found that hummingbirds visited significantly fewer *I. aggregata* with nectar robbing holes.

Methods

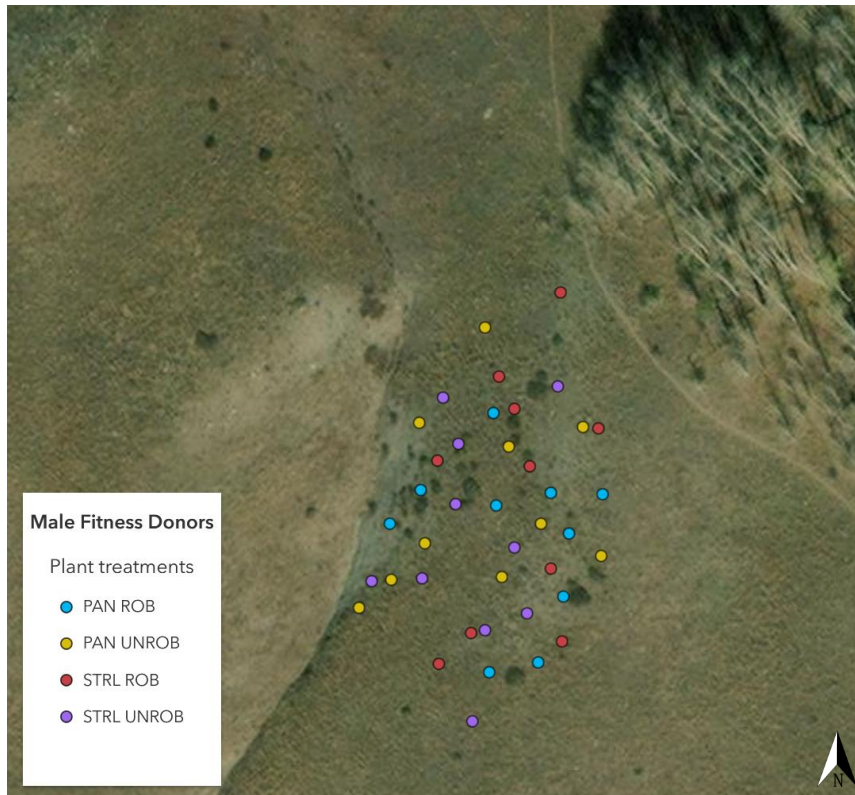


Figure 1. Map of donor plant along Deer Creek Trail near Crested Butte

Study System:

For this experiment, we used *I. aggregata* as our subject of study because it experiences both nectar robbing and bacterial colonization within its nectar. *I. aggregata*, more commonly known as scarlet gilia, is a perennial herb native to the western United States. It can be identified by its red, tubular flowers, usually between 2 and 4 centimeters long. The openings of the corolla are narrow, preventing bumble bees from accessing the nectar through the legitimate entrance. As a result, bumble bees such as

Bombus occidentalis and *Bombus bifarius* will rob these flowers of their nectar. The primary pollinator (i.e., pollen vector) of *I. aggregata* is the broad-tailed hummingbird, *Selasphorus platycercus*.

We aimed to study the effect of a naturally occurring bacterium on male fitness in *I. aggregata*. For this project, we were able to isolate a strain of *Pantoea* sp., which was isolated from an *I. aggregata* flower that was robbed by a single *Bombus bifarius* queen at the Deer Creek Trail site (38°56'46.3"N 106°58'37.4"W). Using Sanger sequencing of the 16S rRNA gene, we were able to identify our bacterium as *Pantoea* sp. The resulting sequences were analyzed using NCBI BLAST, with the search restricted to 16S ribosomal RNA genes from Bacteria and Archaea type strains. Both genetic testing forward and reverse sequences resulted in a 100% match to multiple plant-associated *Pantoea* species including *Pantoea agglomerans*, *Pantoea eucalypti*, and *Pantoea brenneri*.

Nectar Preparation:

Synthetic nectar was prepared for all flower treatments, half of which was inoculated with *Pantoea*. For the sterile analog, mimicked the natural sugar and amino acid concentrations of *I. aggregata* nectar (Valerie Martin, unpublished data). This was formulated in the lab and filter-sterilized using a 0.2 μm filter. Once prepared, the nectar analog was stored at -80°C . To inoculate the analog with *Pantoea*, we will add a two-day old culture of *Pantoea* sp. which has been prepared on Luria-Bertani agar. It was added at an optical density at 600 nm of 0.02-0.04 (corresponding to field-realistic nectar bacterial densities; Valerie Martin, unpublished data). This bacterial solution was then incubated at 20°C for 2 days until use.

Field Site & Treatments:

This study was conducted in a meadow just off Deer Creek Trail (38.94970°N , $106.97870^{\circ}\text{W}$) near Rocky Mountain Biological Laboratory in Gothic, Colorado. 40 naturally occurring *I. aggregata* plants were identified as donors in this experiment. Each donor was spaced roughly 5 meters away from the next closest donor. Within a 2m radius of each pollen donor plant, five recipient *I. aggregate* individuals were randomly selected and flagged for a total of 200 recipient plants. The 40 donors were divided among four treatments, with 10 donors per treatment. The treatments were 1) robbed with *Pantoea*-inoculated nectar, 2) unrobbed with *Pantoea*-inoculated nectar, 3) robbed with sterile nectar, and 4) unrobbed with sterile nectar. Nectar and robbing treatments were conducted weekly. For each plant, we will record root diameter, plant height, number of flowers, as well as corolla length and diameter. We will also measure the distance of each recipient plant to the nearest donor. If a donor was browsed or had zero flowers, a nearby plant was selected and restaked as the new donor. The recipient's distance to the donor and the donor plant's height and root width were measured for the new plant.

When inoculating the flowers with nectar, we used a 10 μL Hamilton syringe to inject each open flower on the plant with 1 μL of the respective treatment, *Pantoea*-inoculated nectar or the sterile nectar analogue (Hamilton Company; Reno, NV, USA). For the robbing treatment, we created a visual cue by pinching a small hole in the corolla with fine forceps. No nectar was removed as part of the robbing treatment. Treatments were performed once per week beginning June 25, 2025, and continuing until July 23, 2025. A 20 μL pipette (Gibson; Middleton, WI, USA) was used to apply 4 μL of a 3mg/mL Cadmium Selenide (CdSe) quantum dot (Strem Chemicals; Newburyport, MA, USA) and hexane solution to the anthers of each flower on a donor plant. When the hexane evaporates, the quantum dots bind to each individual pollen grain. The four colors of quantum dot that was used for this experiment was Red #1 (fluorescence at $360 \pm 10\text{nm}$), Red #2 ($680 \pm 10\text{nm}$), Orange ($590 \pm 10\text{nm}$), and Yellow ($550 \pm 10\text{nm}$). The color treatments were randomly assigned to a treatment group at the start of each week to minimize any confounding effect associated with a specific color causing more or less attraction for hummingbirds.

Sample Collection:

Two days after treatments were applied, up to four open and receptive stigmas were collected from each recipient and donor plant using a pair of forceps. Stigmas from each plant were placed in a 1.5 mL microcentrifuge tube, transported from the field to the lab on ice, and then stored at -20°C until they were mounted on microscope slides.

Microscopy:

Collected stigmas were mounted and sealed on glass microscope slides using clear nail varnish. They were stored in the -20°C freezer until they could be quantified under an epifluorescent microscope. The slides were exposed to a Nikon HB 10103AF mercury lamp UV source located at Western Colorado University. We will use a Nikon Eclipse E600 scope to observe the fluorescence and a Zeiss AxioCam 305 Color camera to record the fluorescence. Individually dyed pollen grains were counted from each donor and recipient plant stigma. For each week, we were able to sum up the number of pollen grains donated from each treatment/quantum dot color.

Statistics:

We predicted a high number of stigmas collected with zero fluorescent pollen grains. Therefore, we used a zero-inflated negative binomial general linear mixed model (GLMM) analysis. We used the “zeroinfl” function within the pscl and MASS packages in R version 4.5.1 to determine whether there are differences in the distribution of pollen treatments among recipient stigma (Jackman 2024, Venables & Ripley 2002). We used a Kruskal-Wallis rank sum test to observe significant differences in the number of pollen grains donated to recipient plants (Kruskal & Wallis 1952). We then use a pairwise Wilcoxon rank-sum tests with Benjamini-Hochberg correction to identify differences between treatment pairs (Benjamini & Hochberg 1995). We visualize our results using the ggplot2 package in R Studio (Wickham 2016)

Results

This experiment aimed to determine the effect of *Pantoea* sp. bacterium and nectar robbing on male fitness in *Ipomopsis aggregata*, as observed through pollen donation. Four weeks of data collection was performed. At present two weeks worth of data have been analyzed. There were 426 total stigma collected from the week of July 3. 259 of these stigma were confidently analyzed for the presence of quantum dots. 95.7% of these 259 stigma had zero fluorescent pollen. There were 604 total stigmas collected from the week of July 10. 393 of these stigma were confidently analyzed for the presence of quantum dots. 93.6% of the 393 analyzed stigma had zero evidence of quantum dots.

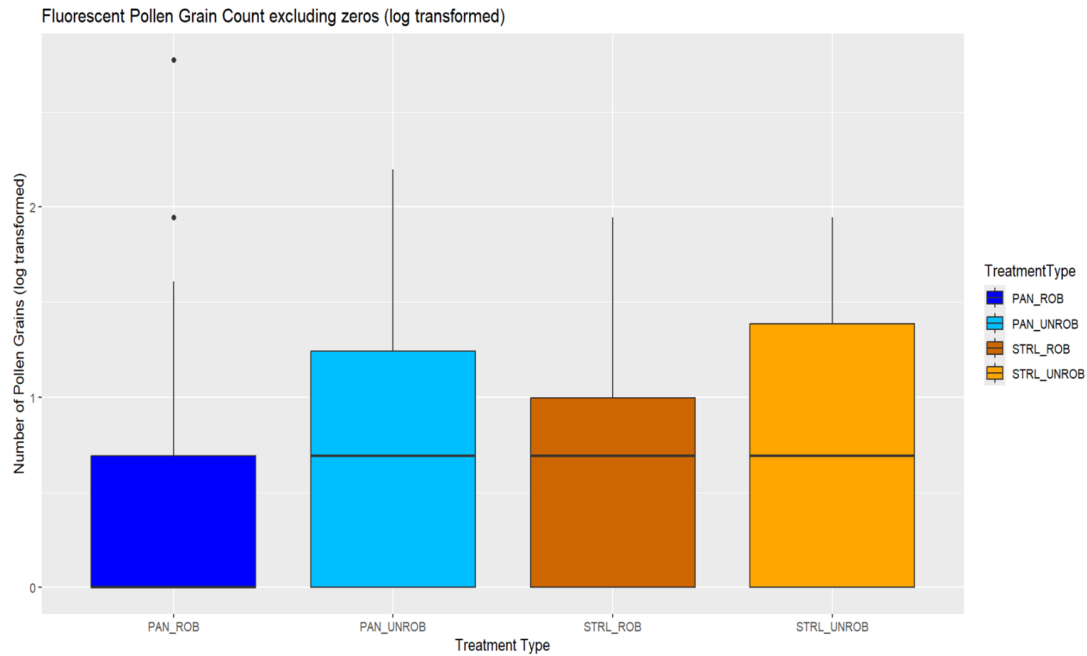


Figure 2. Pollen grain counts from donor and recipient *Ipomopsis aggregata* stigmas collected on July 3rd and July 10th. 652 stigma analyzed for these counts. The error bars represent the standard deviations from the mean. The dark blue represents *Pantoea* inoculated flowers with a robbing treatment. The light blue represents *Pantoea* inoculated flowers without a robbing treatment. The dark orange represents flowers inoculated with sterile nectar and a robbing treatment. The light orange represents flowers inoculated with sterile nectar and no robbing treatment.

Figure 2 shows the pollen counts for the stigmas collected on July 3rd and July 10th. There was no significant difference between the number of pollen grains observed (log transformed) across the different treatments.

To account for the high percentage of zeros, we used a zero-inflated negative binomial distribution with a log link to analyze the number of pollen grains donated to recipient plants. This was a two part model which investigated the effects of our treatments and covariates on both the number of pollen grains present (Table 1) and the occurrence of the excess zeros in each treatment group (Table 2). The count model shows that neither robbing, nor inoculation significantly impact the number of fluorescent pollen grains present on stigma. Similarly, the zero inflated model shows that neither robbing or inoculation had a significant effect on the occurrences of zeros present within the data. When testing whether our covariates such as number of flowers, recipient density, or distance to donor impacted the occurrence of zeros, we found that distance to donor and recipient density were significant in contributing to the likelihood of finding zero pollen grains on stigma ($p > 0.05$). Within this test, inoculation was also found to significantly impact the occurrence of zeros ($p > 0.05$).

Table 1. Count model of the zero-inflated negative binomial GLMM with log link on recipient stigmas collected July 3 and July 10. The intercept is the sterile and unrobbed treatment.

July 3 & 10 Count Model	Estimate	Standard Error	p-value
Intercept	0.636	0.973	0.514
Robbing	-0.070	0.341	0.838
Sterile Inoculation	-0.513	0.885	0.563
Robbing : Sterile inoculation	NA	NA	NA

Table 2. Zero-inflated model of the zero-inflated negative binomial GLMM analysis on recipient stigmas collected July 3 and July 10. The intercept is the sterile and unrobbed treatment.

July 3 & 10 Zero Inflated Model	Estimate	Standard Error	p-value
Intercept	-2.43	0.607	6.45e-05 *
Robbing	-0.347	0.224	0.122
Sterile Inoculation	-0.423	0.610	0.488
Robbing : Sterile inoculation	14.495	843.461	0.986

Table 2. Zero-inflated model of the zero-inflated negative binomial GLMM analysis on recipient stigmas collected July 3 and July 10. The intercept is the sterile and unrobbed treatment.

July 3 & 10 Zero Inflated Model	Estimate	Standard Error	p-value
Intercept	-1.337	0.914	0.144
Robbing	-14.756	825.128	0.986
Sterile Inoculation	-1.545	0.700	0.0274 *
N_flowers	0.004	0.015	0.804
RecipientDensity13	1.713	0.683	0.012 *

RecipientDensityN/A	2.470	0.817	0.003 *
Distance_to_donor	-0.003	0.001	0.016 *
Robbing : Inoculation	14.338	825.128	0.986

Discussion

The goal of this experiment was to determine how naturally occurring bacteria within flower nectar and the presence of nectar robbing could be affecting pollen movement within *I. aggregata*. We predicted that because our bacteria species, *Pantoea* sp. has been seen to ferment sugars within plant nectar, those plants inoculated with bacteria would ultimately be less desirable for hummingbird pollinators (Cusumano et al. 2023). This would then result in less pollen donation from bacteria inoculated plants to their neighbors and therefore, lower fitness. With the current data, we do not see an effect of nectar robbing or nectar inoculation on pollen donation. Our results show a high number of zeros, reflecting that the likelihood of pollen being picked up from donor flowers and transported to recipient flowers while the dyed pollen remained on the hummingbird beak was extremely unlikely. Most of the quantum dot observations were found on donor stigma, likely resulting from hummingbirds visiting all the flowers on one plant before moving onto another one. As we continue with this analysis, we will perform a specific geitonogamy test to determine how many pollen grains were moved within plants.

Although we have not yet observed an effect from *Pantoea* or nectar robbing, we did see some significance from the covariant zero inflated model analysis. Both distance to donor and recipient density were found to affect the number of zeros present. It would be expected that the more neighbors, and the closer they are to a donor, those plants would be less likely to have zeros occur. The distance to the donor has a slightly negative effect (-0.003) while opposite was found for the recipient density (1.713 & 2.47). Because both of these factors have the most significant effect on the presence of pollen grains, it is likely that the limiting factor for male pollen donors is whether or not neighboring plants were present and in close enough proximity to be pollinated. For hummingbirds, they want to maximize their feeding efficiency without spending too much energy. They will visit all the flowers in a given area before exerting more energy to visit farther flowers. This means donors with more and closer recipients would be more likely to see pollen

grains dispersed. *I. aggregata* often grows in meadows, where it will have hundreds of plant stalks within a smaller area. This helps to maximize plant fitness as it encourages the pollination of these areas at once with hummingbirds visiting many different plants at a time and spreading lots of pollen before having to make a much larger trip.

It is possible that because *Pantoea* is naturally occurring within the *I. aggregata* at Deer Creek, the hummingbirds in this area could be used to the potentially lower sugar concentrations. *Pantoea* could be affecting the sugar concentrations of the nectar to such a small degree that it is not enough to deter hummingbirds. In the future, analyzing how the presence of *Pantoea* is affecting the sugar concentrations within nectar and how it is altering the scent emitted from floral nectar will further showcase how these microbes are impacting the nectar community. Ultimately, continuing data analysis is needed to determine whether there is an effect of *Pantoea* and nectar robbing. By studying the effects of microbes on pollinators and plant fitness, we can better understand what smaller mechanisms are working to support ecosystem health and biodiversity.

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