

Fit or Unlit: using quantum dots to investigate the effects of a floricolous yeast and nectar robbing on male fitness in *Ipomopsis aggregata*

Abstract

The interaction network between pollinators, plants, and the microbes that inhabit them is not yet widely understood. Most studies of floral microbes have focused on the role of nectar yeasts (e.g., *Metschnikowia reukaufii*) in the behavior of bumblebee pollinators and how they affect the fitness of plant subjects. However, little is known about the effects of these microbes on the hummingbird-pollinated wildflower *Ipomopsis aggregata*. We used a full factorial experimental design with two treatment levels: supplementation with sterile vs. *M. reukaufii*-inoculated nectar analogue and artificial nectar robbing vs. no nectar robbing. We used fluorescent quantum dots to quantify how these factors affected the dispersal of pollen grains from donor plants by comparing pollen donation across the four plant treatments. Our results suggest potential male fitness advantage on robbed plants, evidenced by significantly higher pollen donation from robbed donor plants to neighboring individuals, irrespective of the presence of yeast. This indicates that nectar robbing in *I. aggregata* may promote outcrossing, impacting hummingbird visitation and pollen dispersal.

Introduction

Despite increasing research on the effects of microbes on plant-pollinator interactions, this area of study remains an emerging field. Yeasts are a ubiquitous group of such microbial organisms that colonize floral nectar for its high concentrations of glucose and sucrose, using bees as vectors for dispersal (Rutkowski et al. 2023). It has previously been found that foragers of the bumblebee species *Bombus impatiens* prefer flowers with yeast-inoculated nectar over those with yeast-free analogues (Rutkowski et al. 2023). Additionally, Schaeffer et al. (2017) determined that, across all flower visits to *Delphinium nuttallianum*, foragers spent a higher proportion of time at inflorescences with yeast-inoculated nectar. The results of these studies and others indicate that there is a positive relationship between the yeast species *Metschnikowia reukaufii* and nectar intake rates of bumblebee pollinators (Schaeffer et al. 2017). However, no significant effect of nectar yeasts was detected on the nectar intake rates of hummingbird pollinators visiting artificial flowers in the sole study to date investigating yeast-plant-hummingbird pollinator interactions, leaving this subject rather unexplored (Vannette et al. 2013).

Yeast species of the *Metschnikowia* genus are single-celled organisms that reproduce by budding from vegetative cells and have been found in a wide range of hosts including flowers, fruits, flower-pollinating insects, and lacewings (Kang et al. 2017). *Metschnikowia reukaufii* and *M. gruessii* are the two species most commonly found in flower nectar and are dispersed by a variety of floral visitor species (Kang et al. 2017). When the nectar of *D. nuttallianum* is inoculated with *M. reukaufii*, there is a positive relationship with enhanced male fitness as

suggested by the observation of higher pollen donation rates in those with yeast-inoculated nectar (Schaeffer et al. 2014).

This male component of reproduction can be measured by pollen donation to conspecifics, acting as a proxy for the male function and fitness of plants (Schaeffer et al. 2013). Studying pollen donation is an effective way of quantifying male fitness in flowering plants because an increase in pollen donation is a good indicator that the reproductive success of the plant will be greater. This is due to the fact that there is a greater chance for reproduction if the plant has more pollen grains distributed to surrounding flowering plants (Ashman 1998). Therefore examining how the presence of *M. reukaufii* affects pollen dispersal may be an effective way of quantifying how a common floral yeast impacts male fitness.

The subalpine herb *Ipomopsis aggregata*, commonly known as scarlet gilia, is one such species in which the effects of *M. reukaufii* on male fitness remains unknown. With its red, tube-like flowers, *I. aggregata* is reliant on hummingbird visitors to disperse its pollen (Meléndez-Ackerman et al. 1997). Yet, this flower, like *D. nuttallianum*, is subject to nectar robbing by bumblebees which likely introduce microbes that could play an additional role on pollinator attraction and so, reproductive success (Richman et al. 2017). Nectar robbing is the act of acquiring nectar from holes cut by foragers in the corolla tissue instead of ‘legitimately’ through the entrance of the flower (fig. 1; Inouye 1980). This behavior occurs at high rates in flowering plants and has been shown to affect the behavior of legitimate pollinators that acquire nectar from the entrance of the flower (Bronstein et al. 2017). Specifically in *I. aggregata*, Irwin and Brody (1998) found that hummingbirds visited significantly fewer plants with nectar robbing holes (Irwin & Brody 1998). When further investigated, it was determined that nectar robbing did not significantly affect male or female fitness in *I. aggregata* (Irwin & Brody 2011). However, because there is evidence that nectar robbing is a significant factor in determining total number of *I. aggregata* pollinators to a plant on a given day, it is an important variable to take into account when studying the microbial impact on plant reproduction and male fitness in this species (Irwin & Brody 1998). These *M. reukaufii* microbes are commonly dispersed by nectar robbers making the addition of nectar yeasts a more realistic treatment than robbing alone.



Figure 1. Representation of *Ipomopsis aggregata* flower with robbing hole (Ryan Stuart).

The purpose of this study is to investigate whether the presence of the nectar yeast *M. reukaufii* and nectar robbing affect the male reproductive success of *I. aggregata* individuals. We employed a recently developed pollen staining technique using quantum dots to distinguish pollen deposition on stigmas from treated pollen donors. A quantum dot is a fluorescent crystal bound to a lipophilic ligand. Quantum dots are dissolved in a nonpolar solvent such as hexane that can be applied to the anthers of *I. aggregata*. When the solvent evaporates, the quantum dots are left attached to the pollen grains by ligands that bind to the lipids on the pollen grain surface, essentially staining individual pollen grains according to each specific treatment (Moir & Anderson 2023). When the excitons in the dots are excited with UV radiation, light energy is emitted causing the individual pollen grains to fluoresce. This technique can then be used to track how pollen donation varies between treatments. Thus, unlike with traditional male plant fitness studies which replace pollen with dye particles on focal plants, we will be able to track real pollen donation without affecting the pollen grain placement on pollinators or the receiving stigmas (Minnaar & Anderson 2019).

Because hummingbird pollinators visited fewer flowers with yeast-inoculated nectar in a 2023 behavior experiment (Souto-Vilarós, *et al.* unpublished results), we hypothesized that the rate of pollen donation in *I. aggregata* plants would be greater in those supplemented with sterile nectar analogue than in those inoculated with *M. reukaufii* (Vannette et al. 2013). Furthermore, we hypothesized that the rate of pollen donation in *I. aggregata* would be greater in plants that did not experience nectar robbing than those with robbing wounds. As a result, we expected that unrobbed plants would display higher levels of male reproductive fitness than plants that exhibit evidence of robbing.

Methods

Study Site and Species:

This study was conducted near the town of Mount Crested Butte, Colorado and the Rocky Mountain Biological Laboratory (RMBL) in the Elk Mountains of Colorado (38.881810, -106.956475). *Ipomopsis aggregata* (Fig. 2) is commonly found in and around the Rocky Mountains of Colorado and is primarily self-incompatible - i.e., its flowers are unable to be pollinated by pollen from the same plant (Grant 1956). To determine the effects of the yeast *Metschnikowia reukaufii* on male fitness in *I. aggregata*, a culture of *M. reukaufii* collected from an *I. aggregata* population at Deer Creek in 2022 was used.



Fig. 2. *Ipomopsis aggregata*, Sierra Nevada mts. (Original).
(Habit x 0.4; flower x 0.8; seed x 9).

Figure 2. Botanical illustration of the *Ipomopsis aggregata* plant subject of study (Grant 1956).

Yeast Cultivations and Nectar Preparation:

Metschnikowia reukaufii were cultured from stock on yeast malt agar plates and incubated for 48 hours at 25°C to allow budding and growth of new colonies following standard microbiology protocols. Following the 48 hour period of incubation the new yeast colonies were used to prepare the nectar treatments inoculated with *M. reukaufii*. To standardize the concentration of yeast cells to the volume of synthetic nectar, we used a hemocytometer under a compound microscope to count the number of cells per microliter. Then, we diluted the nectar accordingly to produce a 1 mL solution with a concentration of 1,000 cells per 1 μ L of nectar. This solution was then incubated for 48 hours at 25°C before treating focal plants.

Flower Treatment:

Our sample size consisted of 20 donor *I. aggregata* plants, with five plants randomly assigned to each of four treatments. For each plant, we collected and recorded the initial plant height, root width, and plant density of a 1.5 m surrounding radius, in addition to performing a twice weekly count of the number of flowers on each donor and recipient plant and nectar treatments. The treatments were 1) robbed with yeast-inoculated nectar, 2) unrobbed with yeast-inoculated nectar, 3) robbed with a sterile nectar analogue, and 4) unrobbed with a sterile nectar analogue. For robbed treatments, a small hole was cut in the corolla of the flower using sharp forceps. We then used a 10 μ L Hamilton syringe to inoculate each open flower on the plant with 1 μ L of the respective treatment, yeast-inoculated nectar or the sterile nectar analogue. Treatments were performed on Tuesdays and Thursdays for 4 weeks from July 2, 2024 to July 25, 2024. 2 μ L of a 1 mg/mL quantum dot (Strem Chemicals; Newburyport, MA, USA) and hexanes solution were applied to the anthers of each flower on the donor plant using a 2 μ L pipette (Gibson; Middleton, WI, USA). This stained the pollen grains with a color according to one of four treatments. The four colors used for this experiment were labeled as Red #1, Red #2, Orange, and Yellow with peak fluorescence at 360, 680, 590 and 550 $\pm$ 10 nm, respectively. The first week of the experiment, the anthers of donor individuals were stained with quantum dots on both July 2nd, and July 4th. Following this first week of treatments, the anthers of donor individuals were

stained with quantum dots once weekly. Bi-weekly, the color associated with the treatment was randomized in order to minimize any confounding variables related to the color of the pollen.

Stigma Collection, Mounting, and Pollen Quantification:

The first week of treatment, up to 3 open and receptive stigmas were collected from each donor and recipient plant on July 7, 2024. Following the first week of treatment and collection, on alternating treatment days over a 4 week period, up to 10 open and receptive stigmas from 5 *I. aggregata* individuals within a 1.5 m radius surrounding the donor plant were collected. Up to 10 stigmas from the donor plant were also collected. The collected stigmas were mounted on a glass microscope slide and sealed with Sally Hanson clear nail varnish for storage in a -20°C freezer until further quantification under a dissecting microscope and custom-made UV excitation box per Minaar and Anderson (2019). Photos of each stigma were captured using a Leica S60 camera connected to a Leica dissecting microscope and Leica 4.12 software. Individual dyed pollen grains were counted according to each quantum dot/floral treatment combination to calculate the distribution of pollen grains from each treatment.

Statistical Analysis

Because of the high number of stigmas collected with zero fluorescent pollen grains, we performed a zero-inflated negative binomial generalized linear mixed model (GLMM) analysis using R's "zeroinfl" function in the *pscl* package of R version 1.5.9 to test for a difference in the pollen deposition and occurrence of counts of zero between treatment groups (Jackman, 2024). This model provided a better fit than other models tested (highest AIC value) and prevented overdispersion. Visualization of pollen donation was performed using the *ggplot2* package in R Studio using R version 4.4.1 "Race for your Life" (Wickham, 2016).

Results

This experiment aimed to determine the effect of the nectar yeast *M. reukaufii* and nectar robbing on the male fitness of *I. aggregata* by tracing pollen dispersal using quantum dots. After capturing photos of all of the stigmas from the first two weeks of collection, the pollen grains appeared to fluoresce clearly under the UV excitation box (Figure 3).

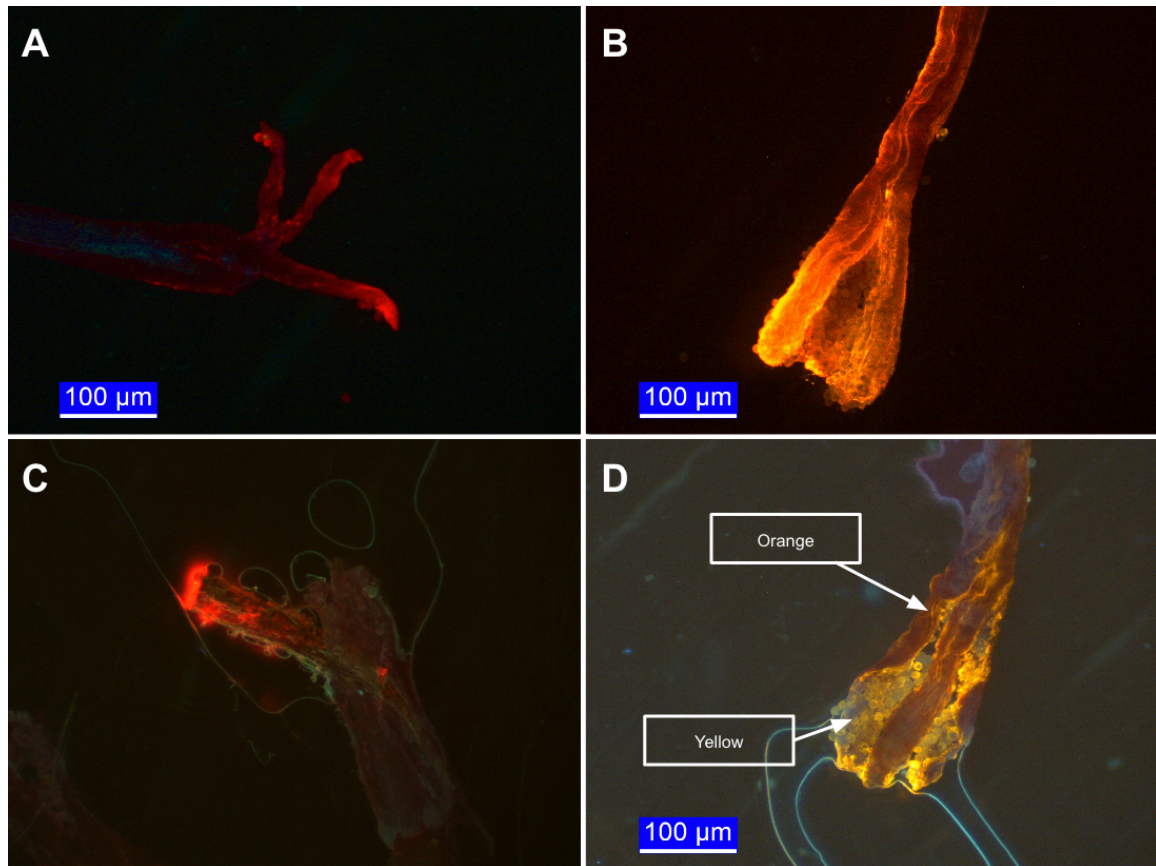


Figure 3. Fluorescing pollen on stigmas in UV-excitation box under dissecting microscope. A) Stigma collected from donor individual 013 with pollen stained with Red #1 quantum dots, B) Stigma collected from donor individual 001 with pollen stained with Orange quantum dots, C) Stigma collected from recipient 05 of donor individual 014 with pollen stained with Red #2 quantum dots, D) Stigma collected from donor 014 with pollen stained with orange and yellow quantum dots.

From the 259 stigmas collected from recipient individuals on July 7th, 82.1% of them had zero fluorescent pollen grains. From the 631 stigmas collected from recipient individuals on July 11th, 72.6% of them had zero fluorescent pollen grains. To account for this, we performed a zero-inflated negative binomial distribution with a log link to analyze the number of pollen grains donated to recipient plants. In the count model (Table 1), there was no significant effect of any treatment on the number of pollen grains donated from the July 7th collection. However, for the July 11th dataset there was a statistically significant positive effect of nectar robbing on the number of pollen grains donated from the robbed yeast-inoculated plants compared to the sterile unrobbed plants ($p = 0.0357$, Table 1).

July 7 Count Model	Estimate	Standard Error	P - value
--------------------	----------	----------------	-----------

Sterile : Unrobbed (intercept)	1.0662	0.5849	0.0705
Yeast-Inoculated	0.4006	0.6073	0.5095
Robbing	0.5219	0.6082	0.3909
Yeast-Inoculated : Robbing	-1.4531	0.8581	0.0904
July 11 Count Model	Estimate	Standard Error	P - value
Sterile : Unrobbed (intercept)	2.6115	0.2810	$2.0 * 10^{-16}$
Yeast-Inoculated	-0.6819	0.3827	0.0748
Robbing	-0.0189	0.3384	0.9554
Yeast-Inoculated : Robbing	1.0654	0.5072	0.0357

Table 1. Count model of the zero-inflated negative binomial GLMM with log link on recipient stigmas collected July 7 and July 11. The estimate represents the estimate of the parameter in the linear model. The intercept is the sterile and unrobbed treatment.

The zero-inflated portion of the zero-inflated negative binomial GLMM assesses the effects of treatments on the occurrence of the excess zeros in each treatment group (Table 2). There was no significant effect of any treatment on the occurrence of zero pollen donation from the July 7th collection. However, running the same analysis on the July 11 data revealed a statistically significant negative effect of robbing on the occurrence of zeros in both sterile ($p = 0.00358$) and yeast-inoculated ($p = 0.01541$) donor plants.

July 7 Zero-Inflated Model	Estimate	Standard Error	P - value
Sterile : Unrobbed (intercept)	0.5273	0.7939	0.507
Yeast-Inoculated	0.4694	0.5755	0.415
Robbing	0.5047	0.5750	0.380

Yeast-Inoculated : Robbing	-1.3558	0.9447	0.151
July 11 Zero-Inflated Model	Estimate	Standard Error	P - value
Sterile : Unrobbed (intercept)	1.1141	0.2762	5.49 * 10⁻⁵
Yeast-Inoculated	-0.2433	0.3798	0.52175
Robbing	-1.0450	0.3587	0.00358
Yeast-Inoculated : Robbing	1.2640	0.5218	0.01541

Table 2. Zero-inflated model of the zero-inflated negative binomial GLMM analysis on recipient stigmas collected July 7 and July 11. The estimate represents the estimate of the parameter in the linear model. The intercept is the sterile and unrobbed treatment.

When visualizing the data, no significant effect of inoculation with yeast or nectar robbing was detected on the number of fluorescent pollen grains distributed to stigmas of recipient individuals from the July 7th collection date (Figure 4A). In contrast, the July 11th stigma collection yielded a significant positive effect of nectar robbing in both yeast-inoculated and sterile donor individuals on the number of fluorescent pollen grains detected on stigmas from (Figure 4B).

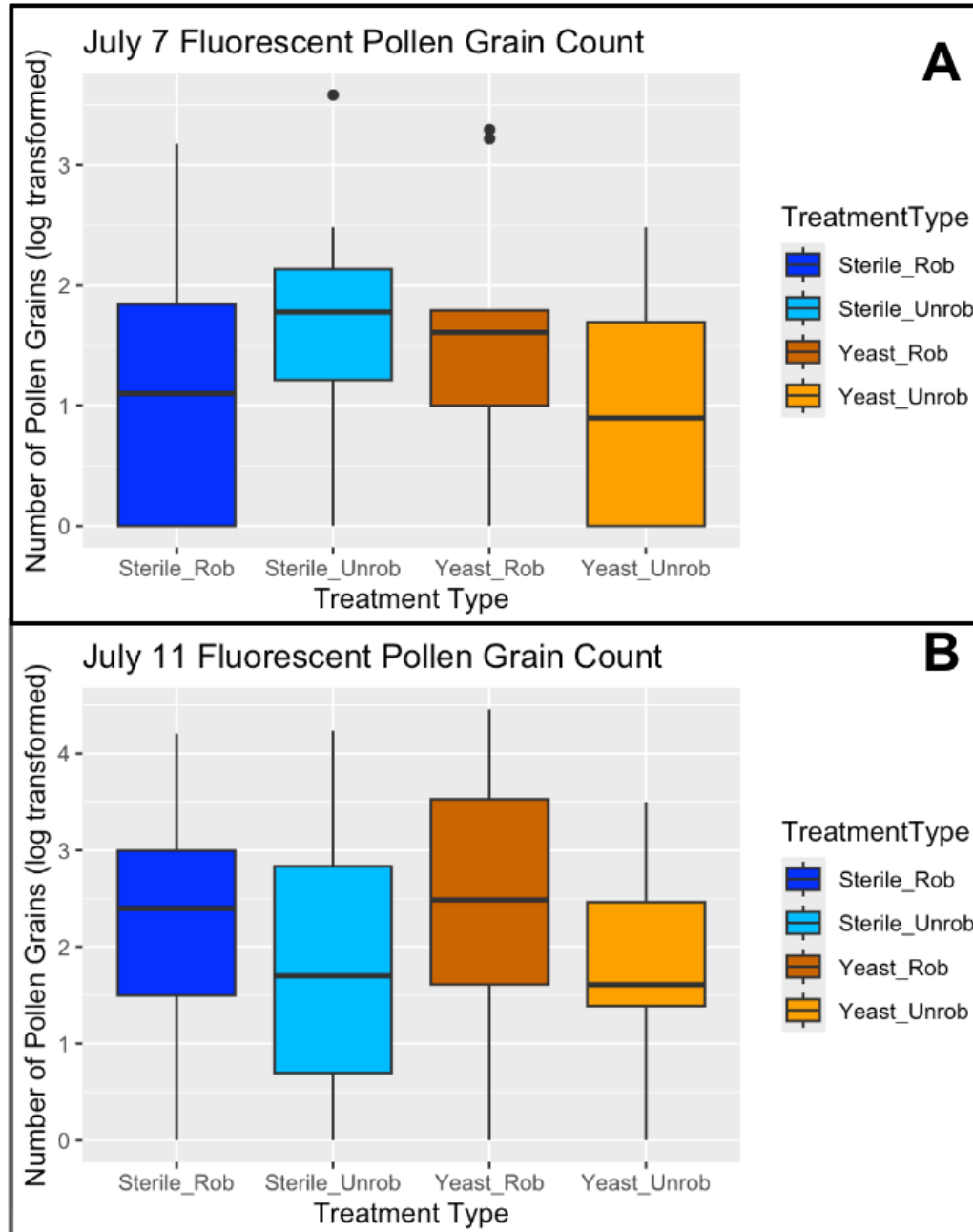


Figure 4. A) July 7 fluorescent pollen grain count from recipient *I. aggregata* plants; B) July 11 fluorescent pollen grain count from recipient *I. aggregata* plants. The dark blue, light blue, dark orange and light orange box plots represent fluorescent pollen grains counted on recipient stigmas for sterile robbed, sterile unrobbed, yeast-inoculated robbed and yeast-inoculated unrobbed donor treatments, respectively. The error bars represent the standard deviations from the means.

Discussion

This project aimed to determine the effects of inoculation with the nectar yeast *Metschnikowia reukaufii* and nectar robbing on the male fitness of *I. aggregata*. In this experiment, we treated 20 donor plants with yeast-inoculated nectar or a sterile nectar analogue and artificial nectar robbing or no robbing. Using quantum dots to stain pollen from the donor plants according to each individual's treatment, we collected open and receptive stigmas from five randomly selected *I. aggregata* recipient plants within a 1.5 m radius from each donor. This allowed us to quantify and trace the distribution of pollen from plants assigned to the four experimental treatments and, therefore, better understand how they impact the male fitness of this species. In quantifying the first half of the data collected during this project, we found that, when treated with artificial nectar robbing, there was a significant increase in pollen donation from donor plants to surrounding recipients. However, there was no trend observed with or without the presence of *M. reukaufii*.

These results are not consistent with those previously established by Souto-Vilarós, et al. concluding that hummingbirds show preference for sterile nectar over yeast-inoculated nectar in *I. aggregata* plants (Souto-Vilarós, *et al.* unpublished results). Instead, we find that hummingbirds show no preference for yeast-inoculated nectar nor the sterile nectar analogue. Our findings on nectar robbing are also contrary to those of Irwin and Brody (1998), stating nectar robbing does not have an impact on male or female fitness in *I. aggregata* (Irwin & Brody, 1998). From the data we have acquired thus far, there is a trend that implies nectar robbing wounds have a positive effect on the male fitness of *I. aggregata*. Because hummingbird pollinators distributed more pollen from flowers of donor plants with nectar robbing, our results indicate that the pollinators left the robbed donor plants after less visitation to other flowers from the donor. This can be interpreted as hummingbirds showing a preference for unrobbed over robbed flowers. Therefore, we can conclude that robbing likely promotes outcrossing in the species *I. aggregata*.

From this research, we can also conclude that quantum dots are an effective way to quantify and trace pollen dispersal and layering. Currently, there are only four colors of quantum dots commercially available. These have been employed to track pollen transfer and layering in Stellenbosch, Western Cape, South Africa by Minnaar and Anderson (2019) and near Caledon in the Western Cape Province of South Africa by Moir and Anderson (2023). However, prior to our research, little experimentation on using quantum dots to dye individual pollen grains in environments of high UV exposure from the sun and high temperatures. While some photobleaching of quantum dot-stained pollen grains may be one confounding variable in this study, it is evident from our data that this method is still an effective tactic in understanding the full scope of pollination and the methods behind it.

However, there are some caveats that must be taken into consideration with the interpretations of these results. To begin, the complete dataset of all stigma collections has not yet been fully

quantified. Additionally, the procedure for treatment and stigma collection was not standardized until the second week of the experiment which may also play a role in the final results of the experiment. Because the data collected on July 7th was considered to be a trial of the treatment and collection methods, it will likely not be included in the final dataset and may, therefore, play a role in the final conclusions from this experiment. With the number of fluorescent pollen grains dispersed from donor plants fully accounted for, we will be able to get a more accurate and complete understanding of the relationship between nectar yeasts, nectar robbing, and plant male fitness.

Conclusion

Acknowledgements

We thank t

References

- Ashman, T. (1998). Is relative pollen production or removal a good predictor of relative male fitness? An experimental exploration with a wild strawberry (*Fragaria virginiana*, Rosaceae). *American Journal of Botany*, 85(9): 1166-1171.
- Bronstein, J. L., Barker, J. L., Lichtenberg, E. M., Richardson, L. L., Irwin, R. E. (2017). The behavioral ecology of nectar robbing: why be tactic constant?. *Current Opinion in Insect Science*, 21: 14-18.
- Grant, V. (1956). A Synopsis of Ipomopsis. *Aliso: A Journal of Systematic and Floristic Botany*, 3(3): 9.
- Inouye, D. (1980). The Terminology of Floral Larceny. *Ecology*, 61(5): 1251-1253.
- Irwin, R.E. & Brody, A. K. (1998). Nectar robbing in *Ipomopsis aggregata*: effects on pollinator behavior and plant fitness. *Oecologia*, 116: 519-527.
- Irwin, R. E. & Brody, A. K. (2011). Additive effects of herbivory, nectar robbing and seed predation on male and female fitness estimates of the host plant *Ipomopsis aggregata*. *Oecologia*, 166: 681-692.
- Jackman, S. (2024). *pscl: Classes and Methods for R Developed in the Political Science Computational Laboratory*. University of Sydney, Sydney, Australia. R package version 1.5.9, <https://github.com/atahk/pscl/>.
- Kang, Y. M., Choi, J. E., Komakech, R., Park, J. H., Kim, D. W., Cho, K. M., Kang, S. M., Choi, S. H., Song, K. C., Ryu, C. M., Lee, K. C., Lee, J. (2017). Characterization of a novel yeast species *Metschnikowia persimmonesis* KCTC 12991BP (KIOM G15050 type strain) isolated from a medicinal plant, Korean persimmon calyx (*Diospyros kaki* Thumb). *AMB Express*, 7: 199.
- Meléndez-Ackerman, E., Campbell, D. R., Waser, N. M. (1997). Hummingbird behavior and mechanisms of selection on flower color in *Ipomopsis*. *Ecological Society of America*, 78(8): 2532-2541.

- Minnaar, C. & Anderson, B. (2019). Using quantum dots as pollen labels to track the fates of individual pollen grains. *Methods in Ecology and Evolution*, 10: 604-614.
- Moir, M. & Anderson, B. (2023). Pollen layering and male-male competition: Quantum dots demonstrate that pollen grains compete for space on pollinators. *American Journal of Botany*, 110(6): e16184.
- Richman, S. K., Irwin, R. E., Nelson, C. J., Bronstein, J. L. (2017). Facilitated exploitation of pollination mutualisms: fitness consequences for plants. *Journal of Ecology*, 105: 188-196.
- Rutkowski, D., Weston, M., Vannette, R. L. (2023). Bees just wanna have fungi: a review of bee associations with nonpathogenic fungi. *FEMS Microbiology Ecology*, 99: 1-16.
- R Core Team (2024). R a language and environment for statistical computing [4.2.2]. R Foundation for Statistical Computing, Vienna, Austria.
- Schaeffer, R. N. & Irwin, R. E. (2014). Yeasts in nectar enhance male fitness in a montane perennial herb. *Ecology*, 95(7): 1792-1798.
- Schaeffer, R. N., Manson, J. S., Irwin, R. E. (2013). Effects of abiotic factors and species interactions on estimates of male plant function: a meta-analysis. *Ecology Letters*, 16: 399-408.
- Schaeffer, R. N., Mei, Y. Z., Andicoechea, J., Manson, J. S., Irwin, R. E. (2017). Consequences of a nectar yeast for pollinator preference and performance. *Functional Ecology*, 31: 613-621.
- Vannette, R. L., Gauthier, M-P. L., Fukami, T. (2013). Nectar bacteria, but not yeast, weaken a plant-pollinator mutualism. *Proceedings of the Royal Society*, 208: 20122601.
- Wickham H (2016). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York. ISBN 978-3-319-24277-4, <https://ggplot2.tidyverse.org>.