In your stomach or in your nectar? Disentangling the effects of two pollination-related yeasts on bumblebee behavior and foraging

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Abstract:

The study of plant-insect interactions and pollination has just begun to scratch the surface of the microbes that inhabit many of the surfaces involved in these interactions. While most microbial studies have focused on the roles of obligate nectar yeasts in mediating pollinator behavior and fitness outcomes in plants, there are entire communities of nectar and pollinator microbes that have been overlooked. Their involvement in these areas is no less important than that of nectar yeasts, but their roles are harder to investigate and quantify. Here, we investigated one such microbe: the bumblebee specialist, *Starmerella bombi*, which primarily lives in the nests and stomachs of bumblebees but can be dispersed to other bee nests via nectar. By inoculating the species-specific nectar analogue of *Corydalis caseana* ssp. *brandegeei* near the Rocky Mountain Biological Laboratory with *S.bombi*, we tested whether *Bombus appositus* foragers preferred *S. bombi* inoculated flowers over sterile control flowers and *M. reukaufii* inoculated flowers. Furthermore, we assessed whether these insects spend more time at *S. bombi*-inoculated flowers over sterile flowers and *M. reukaufii* inoculated flowers. We found that bumblebee foragers showed no preference for either of the focal yeast species when compared to sterile nectar, suggesting that inoculated nectar had no effect on foraging behavior in terms of number of flowers visited, time spent on individual plants, or time spent on individual flowers. Ultimately, this result will contribute to our understanding of how pollinator behavior could be mediated by insect-inhabiting microbes and the role they play in shaping the evolution of floral rewards and potential outcomes to plant fitness.

Keywords: floral microbes, plant-pollinator-microbe interactions, *Bombus appositus, Corydalis caseana*

Introduction:

Plant-insect-microbe interactions have been increasingly studied, especially regarding herbivorous insects (Giron et al. 2013, Biere & Bennett, 2013, Pineda et al. 2013), but our understanding of plant-pollinator-microbe interactions is still in its nascency. Specific microbes can play outsized roles in the fitness of their plant hosts, having a range of effects from negative to positive (Schaeffer & Irwin 2014, Vannette 2020, Herrera et al. 2013). Because of the value of the ecosystem service pollinators provide, understanding the context-dependency and dynamics of these mutualisms is of vital importance to humankind (Porto et al. 2020, FAO 2018). Microbes present in floral nectar or on other parts of plant tissue can also impact pollinators and their behavior. Schaeffer et al. 2017 found that the presence of a ubiquitous nectar yeast, *Metschnikowia reukaufii,* encouraged visitation and increased the foraging time of bumblebees (*Bombus appositus* and *B. bifarius*) on inoculated *Delphinium nuttallianum* plants. *M. reukaufii* is becoming increasingly well characterized as an actor in these mutualisms, although there are many other plant/pollinator-associated microbes needing study, including other yeasts, such as *Stamerella bombi,* bacteria, and viruses (Cullen et al. 2021). The bumblebee-associated yeast, *S. bombi,* is of particular interest to research on the pollination mutualism; it is thought to overwinter in the gastrointestinal tracts (GI) of bumblebee queens before being passed on horizontally to her offspring. Its primary habitats are bumblebee colonies and GI tracts. Then, as bumblebee individuals visit different flowers, they introduce the yeast to their nectar, where it can then be passed on to bumblebees from other colonies as well as bumblebees of other species (Brysch-Herzberg 2004, Pozo et al. 2018). Thus, *Starmerella bombi* dispersal is mediated by pollinating bumblebees (*Bombus* spp.), and, likely to a much lesser extent, by air, wind, and rain (Martin et al. 2022). Numerous studies have investigated and characterized nectar yeast communities (Vannette et al. 2013, de Costa Neto & de Morais 2020, Herrera et al. 2008), and others have investigated the role of specific microbes on nectar, pollinator behavior, and outcomes for plant hosts (Schaeffer et al. 2019, Schaeffer & Irwin 2014, Vannette 2020, Herrera et al. 2013). However, to our knowledge, no work has been done to characterize the impact of *Starmerella bombi* on the pollination behavior of bumblebees.

Our aim in this study is to elucidate the effects of the yeasts *S. bombi* and *M. reukaufii* on pollinator foraging behavior. We inoculated nectar analogues of the alpine wildflower Brandegee's fumewort (*Corydalis caseana* ssp. brandegeei) with live cultures of *S. bombi* and *M. reukaufi*i and observed the foraging behavior of *B. appositus* in an artificial array. Since its thought that floral visitors are the main means of dispersal of these yeasts, and furthermore, bumblebees are the main host of *S. bombi*, we hypothesized that both yeasts would have an effect on visitor behavior, with *S. bombi*-inoculated flowers having a significant effect on foraging behavior, followed by *M. reukaufii*-inoculated flowers and finally, sterile control flowers. Furthermore, bumblebee foragers would spend a longer time foraging/pollinating *S. bombi* inoculated flowers over *M. reukaufii* inoculated flowers and control flowers containing sterile nectar, since these interactions could confer a dispersal advantage to these yeasts. Due to the

complexity of this three-way mutualism, the success of one actor impacts the success of the other: a well-dispersed yeast attracts many pollinators, who gain significant energetic rewards from nectar; in turn, thorough and lengthy floral visits should benefit plant hosts as well with pollen dispersal/deposition and thus larger seed sets. In the long term, the mediation of pollinator foraging behavior by nectar yeasts and other microbes associated with this mutualism has the potential to shape plant fitness as well as the evolution of floral traits (Schaeffer et al. 2017).

Methods:

Study site

This research was carried out in the vicinity of the Rocky Mountain Biological Laboratory in Gothic, Colorado, USA (38°50′N, 106°50′W). Experimental trials were carried out in lower Washington Gulch, Gunnison County, Colorado, USA (2940 m, 38.940002, -107.023040) in a subalpine meadow approximately 2 km from the RMBL (Maloof 2000). This site was chosen due to a natural *Corydalis caseana* ssp. brandegeei population that occurs in the area (Figure 1).

Yeast cultures

Using a culture of *Starmerella bombi* collected from a population of *Ipomopsis aggregata* at Deer Creek in 2022 and a culture of *Metschnikowia reukaufii* collected from a population of *Corydalis caseana* ssp. *brandegeei* at the lower Washington Gulch site, we inoculated *Corydalis caseana* ssp. *brandegeei* synthetic nectar at a concentration of 1,000 cells/µl. Synthetic nectar had identical amino acid and carbohydrate profiles and concentrations as naturally occurring *Corydalis* nectar. Inoculated nectar was incubated for 48 hours prior to trials at 25℃.

Choice arrays

We conducted our experimental trials between July 15, 2023 and July 25, 2023. Each morning on the day of observations, 12 individual *B. appositus* workers were captured in 50ml Falcon tubes, stored and starved in a portable cooler on ice for 1 - 2 hours. Six *Corydalis* inflorescences, previously bagged to avoid visitors, were cut and trimmed to have 10 flowers per stalk, and were placed in green translucent waterpics in a custom made Rubbermaid storage bin flight cage (Figure 2). Waterpics were labeled 1 - 6 and each inflorescence was randomly placed in the waterpics according to one of two treatments (three inflorescences per treatment). The treatments included sterile control synthetic nectar, and synthetic nectar inoculated with either *S. bombi* or *M. reukaufii* yeasts. Using 25 µl microcapillary tubes (Thermo Fisher Scientific, Waltham, MA, USA), nectar was removed from each flower prior to injecting 5 µl of treated nectar using a 50 µl Hamilton syringe (Hamilton Co., Reno, NV, USA). Syringes were cleaned and sterilized daily

using 10% bleach/Alconox solution and UV light; syringes were kept separate and used exclusively for either yeast or sterile treatments.

Bumblebee observations

Bumblebees were introduced individually to the array and allowed time to react. When the bumblebee began to fly, we allowed it 10 minutes to make a choice. Once an individual landed on a flower, we recorded flower choice, flower handling time, and number of flowers probed until the end of each bout using a hand held Olympus (model VN - 541PC) digital voice recorder. As backup, the entire bout was recorded using a SONY Handycam (model HDR-CX405) video recorder. Foraging bouts were considered over when the individual flew away from the plants although we allowed ~2 minutes in case it returned to the array. After a visit to a given plant, every flower was then replenished with 5ul of nectar according to its treatment. At the conclusion of trials, bumblebees that interacted with *Starmerella*-inoculated plants (n=5) were killed and surveyed for their tongue and body surface microbes.

Data analysis

Video recordings were used to record time spent on the whole plant, on each flower, and plant/flower treatment. Statistical analysis was conducted in R using R 4.2.0 (Vigorous Calisthenics). Due to the strongly skewed nature of the data, we used a one-sided non-parametric Wilcoxon Rank Sum Test (R: wilcox.test, from the package *stats*) between treatments to assess whether yeast treatments had significantly higher median times per flower, median times per plant, and median numbers of flowers visited by pollinators than control treatments. By nature of our array's design, we could only statistically compare the *Metschnikowia* treatment with its respective control treatment and the *Starmerella* treatment with its respective control treatment. We hypothesized that bumblebee foragers would spend more time at each yeast-inoculated flower, more time at each yeast-inoculated plant, and would visit more yeast-inoculated flowers, as compared to controls.

Results:

A total of 62 bumblebees were tested, of which 23 made a choice and visited at least one flower within 10 minutes of beginning to fly within the array (18 on controls, 8 on *Starmerella* and 8 on *Metschnikowia*). We found that workers of *B. appositus* showed little preference for either yeast when they encountered them in the nectar of *C. caseana*.

Our trials revealed that *B. appositus* workers did not visit yeast-inoculated flowers more often than control flowers (Fig. 3; *Starmerlla* treatments versus control: $W = 63$, $p = 0.7104$, $n_1 = 86$, $n_2 = 128$ | *Metschnikowia* treatment versus control: $W = 61.5$, $p = 0.4868$, $n_1 = 109$, $n_2 = 111$).

Bumblebees visited a total of 86 *Starmerella*-inoculated flowers with a median number of flowers visited of 5.00 +/- 2.09 flowers (median +/- SE) , 111 *Metschnikowia*-inoculated flowers with a median number of flowers visited of 9.00 +/- 3.01 flowers, 109 sterile control flowers in the *Metschnikowia* array with a median number of flowers visited of 8.00 +/- 1.76 flowers, and 128 sterile control flowers in the *Starmerella* array with a median number of flowers visited of 11.50 +/- 2.08 flowers.

Furthermore, *B. appositus* workers did not spend significantly more time at yeast-inoculated flowers than control flowers (*Starmerlla* treatments versus control: W = 5490, p = 0.513, $n_1 = 86$, $n_2 = 128$| *Metschnikowia* treatment versus control: W = 5078.5, p = 0.9802, $n_1 = 109$, $n_2 = 111$) (Figure 4). In the *Starmerella* array, workers spent a total of 534.03 seconds on *Starmerella*-inoculated flowers over 86 visits, with a median time per visit of 4.06 +/- 0.65 seconds; additionally, workers spent a total of 762.13 seconds over 128 visits on control flowers, with a median time per visit of 4.56 +/- 0.46 seconds. In the *Metschnikowia* array, workers spent a total of 688.98 seconds visiting *Metschnikowia*-inoculated flowers over 111 visits, with a median time per visit of 4.24 +/- 0.84 seconds; additionally, workers spent a total of 908.67 seconds on control flowers over 109 visits, with a median time per visit of 5.46 +/- 1.08 seconds.

Finally, *B. appositus* workers did not spend significantly more time at yeast-inoculated plants than control plants (*Starmerlla* treatments versus control: W = 89, p = 0.7487, $n_1 = 13$, $n_2 = 16$ | *Metschnikowia* treatment versus control: W = 55, p = 0.7707, $n_1 = 11$, $n_2 = 13$) (Figure 5). In the *Starmerella* array, workers had a median time per visit to *Starmerella*-inoculated plants of 19.12 +/- 12.35 seconds, while they had a median time per visit to control plants of 38.63 +/- 9.66 seconds. In the *Metschnikowia* array, workers had a median time per visit to *Metschnikowia*-inoculated plants of 45.74 +/- 18.71 seconds, while they had a median time per visit to control plants of 53.39 +/- 17.33 seconds.

Discussion:

Despite the recent evidence that bumblebee pollinators respond positively to the presence of yeasts in floral nectar (Herrera et al. 2013, Schaeffer et al. 2014, Schaeffer et al. 2017), we found that our focal yeast species have little effect on the behavior and foraging strategy of *Bombus appositus* workers. *Corydalis* flowers have a complex morphology, with hooded flowers and long nectar spurs (Maloof 2000); this has two potential ramifications for the effects of yeasts present in floral nectar and their impacts on forager behavior: first, complex morphologies have been characterized as a floral signal to deter inefficient pollinators and reward successful foragers with plentiful nectar rewards, while at the same time reducing cross-species pollen deposition by flower visitors (Krishna & Kaesar, 2018). Despite yeast inoculated nectars having distinct volatile profiles (Rodriguez & Martin, pers. comm.), it is possible that *B. appositus* foragers simply ignore these signals given the specialized handling technique and the known

profitable reward from such complex flowers. In other words, by the time floral visitors recognize different yeasts in the nectar associated with *Corydalis*, they have already committed to foraging on that flower (Krishna & Keasar 2018), and opt to do so regardless of the yeast content of the nectar; second, this morphology likewise could prevent the dispersal of yeast volatiles, which bumblebees use as signals of nectar quality (Schaeffer et al. 2019). Thus, they may not have demonstrated a preference or distaste for flowers or plants inoculated with yeasts because they may have been unable to detect these volatiles, and in turn, their associated yeasts. However, the role that flower morphology plays in microbial volatile diffusion and transmission is poorly understood (Adler et al. 2021), and future research is needed to understand how flower morphology may affect the transmission of microbial signals from floral nectar/surfaces to floral visitors.

Second, it has been demonstrated that throughout the season, *M. reukaufii*'s presence increases, primarily in bumblebee-pollinated plant communities (De Vega & Herrera, 2012). In some cases, such as in the nectar of *Helleborus foetidus*, *Metschnikowia reukauffi* was present in almost 50% of sampled individuals (Brysch-Herzberg 2002). In the case of *Starmerella bombi*, the horizontal transmission of this yeast from bumblebee individuals of the same hive likely leads to increasing yeast density as hives grow in size throughout the summer (if host density increases, yeast density will also increase) (Hale & Valdovinos 2021). We performed our experiments in late summer, where the densities of both yeasts are potentially high, and foragers were inundated with yeast volatile signals, potentially causing these bumblebees to ignore the treated plants within the array. In contrast to Schaeffer & Irwin (2014), where they found our focal bumblebee species to react positively to the presence of *M. reukaufii* in flowers of *Delphinium barbeyi*, we tested the preferences of workers late in the season rather than early summer emerging queens. Similarly, Pozo et al (2019) report increased visitation of yeast-inoculated artificial flowers by captive *Bombus terrestris* early in their experiment and in the season. This early season increased attraction to yeast-inoculated nectars could be due to yeast-associated volatiles assisting bumblebee foragers in locating floral provisions prior to forming foraging associations with specific areas (Pozo et al. 2019). Indeed, later in the season, such as when our experiment took place, individuals have already formed these associations, and may no longer rely on yeast-associated volatiles to find floral provisions.

Nevertheless, an important caveat to consider in our results is that we re-treated plants following a successful visit, rather than discarding visited individual inflorescences and replacing them with new inflorescences. Past research has shown that bumblebees can be attracted to plants that contain bumblebee-associated cuticular hydrocarbons, and by reinoculating plants and reintroducing them to our experimental array, we potentially biased our findings (Witjes et al. 2009). However, the fact that bumblebees appear to visit control and treated plants in similar proportions suggest that they too may have ignored these cues.

Despite not finding an obvious signal of these yeasts to foraging workers of *B. appositus*, multiple studies have shown some degree of response by floral visitors to nectar microbes, with positive, negative and neutral consequences to plant fitness (Eisikowitch et al. 1990, Herrera et al. 2013, Schaeffer & Irwin 2014, Vannette et al. 2013). How these microbes are dispersed and deposited into otherwise sterile nectar remains widely unknown and would likely elucidate some of the mechanisms that mediate plant-pollinator-microbe interactions. Given the current biodiversity crisis due to habitat loss, climate change and the increasing use of pesticides, improving our understanding of the dynamics mediating pollination is vital both for ecosystem functioning and human subsistence. We have only begun to scratch the surface of the potential influence floral microbes have on pollinator behavior and plant fitness. This area of research could benefit from further studies to disentangle the context-dependent interactions of microbes, plants, and pollinators.

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Figures:

Figure 1. ArcGis LandSat image of field site in Gunnison County, CO, near the RMBL, where experimental trials took place (Coordinates: 38.940002, -107.023040).

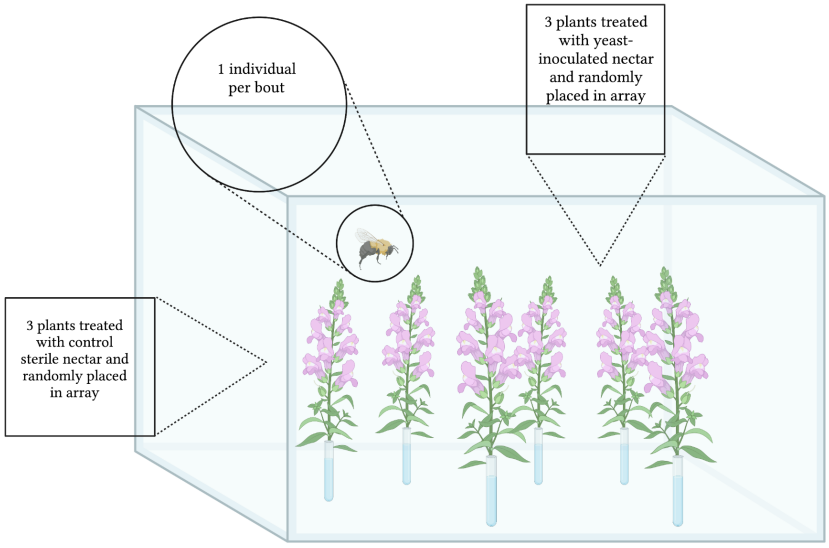


Figure 2. Experimental array in a flight cage for bumblebee behavioral trials.

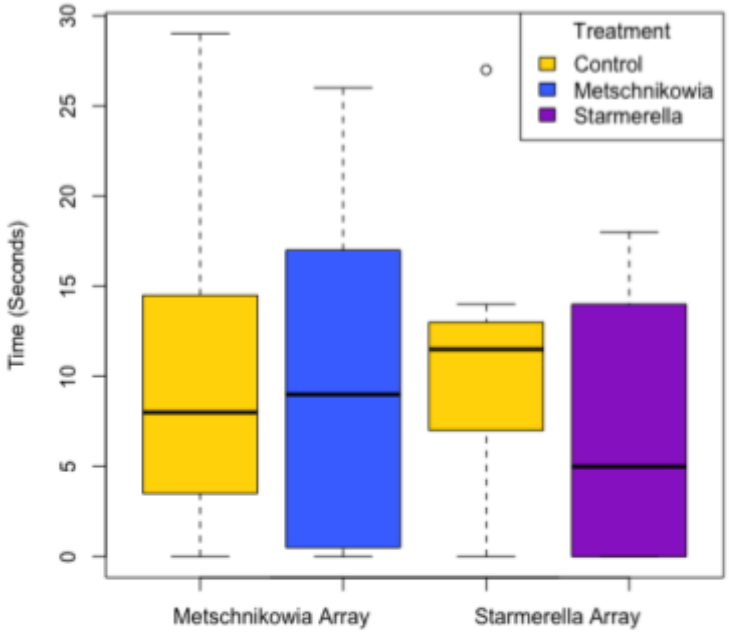


Figure 3: Boxplot of flowers visited by treatment by bumblebee foragers. Wilcoxon Rank-Sum Test: *Starmerella* treatments versus control: W = 63, p = 0.7104, $n_1 = 86$, $n_2 = 128$ | *Metschnikowia* treatment versus control: W = 61.5, p = 0.4868, $n_1 = 109$, $n_2 = 111$. Blue indicates *Metschnikowia* treatment, purple represents *Starmerella* treatment, and yellow indicates sterile control treatment.

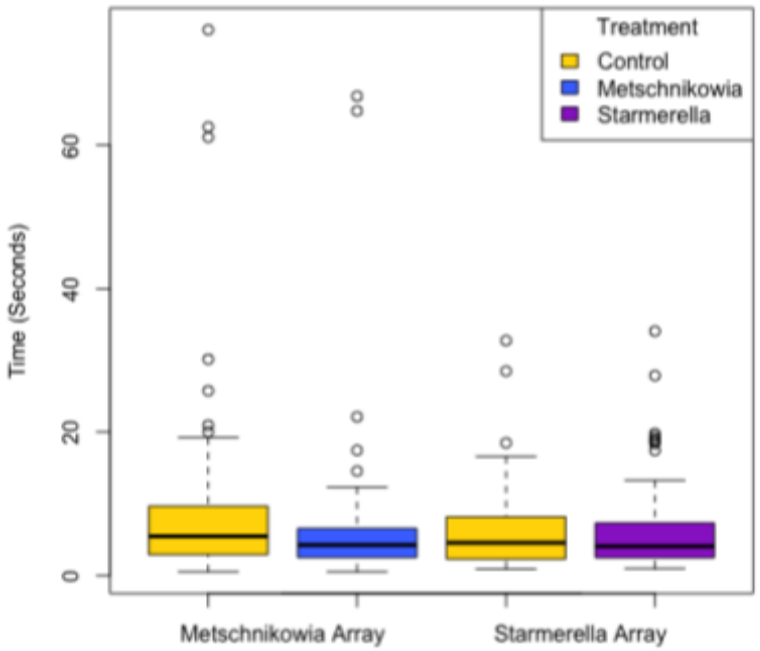
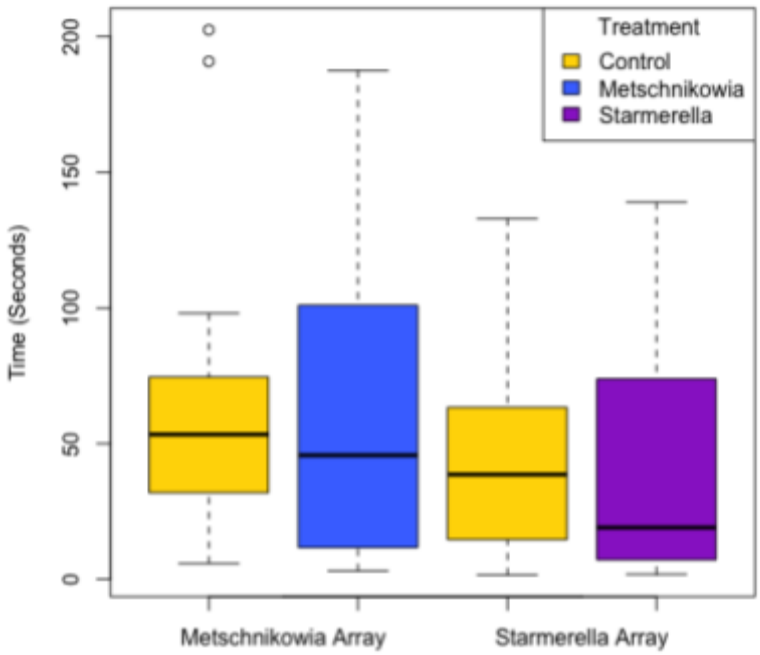


Figure 4: Boxplot of time per flower by treatment by bumblebee foragers. Blue indicates *Metschnikowia* treatment, purple represents *Starmerella* treatment, and yellow indicates sterile control treatment. *Starmerlla* treatments versus control: W = 5490, p = 0.513, n_1 = 86, n_2 = 128| *Metschnikowia* treatment versus control: W = 5078.5, p = 0.9802, n_1 = 109, n_2 = 111.



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