

Elucidating the influence of a nectar yeast and nectar robbing on bumblebee foraging tactic constancy

Abstract:

Animal pollination services are vital for plant reproduction, with approximately 90% of plant species benefitting from this mutualistic relationship. Nectar is one of the most common floral rewards offered by plants in exchange for pollination services. Similarly, nectar is vital for bumbles as they consume it for flight fuel and nourishment for larvae and their colonies. However, not all floral visitors are pollinators. A forager may choose to forage legitimately (pollinate) or may choose to forage illegitimately (nectar rob). Nectar robbers bypass anthers at the floral opening and collect nectar by chewing a hole on the nectar spur or finding a pre-existing hole. Nevertheless, it has been shown that foraging patterns can be influenced by microbial presence and community structure implying that the presence of nectar inhabiting microbes (NIMs) can influence floral visitors' behavior. We investigate how *Metschnikowia reukaufii* plays a role within this relationship, looking to further understand how NIMs may affect quality and signal of floral rewards. By manipulating the presence of *M. reukaufii* in the nectar of flowers of *Corydalis caseana*, we can observe the foraging patterns of bumblebees which can facultatively act as both legitimate pollinators and secondary robbers and are therefore capable of tactic switching. This research will elucidate the influence, if any, of NIMs on bumblebee's foraging strategies which may disrupt pollination services by promoting floral larceny.

Introduction:

Pollinating insects use flower nectar as flight fuel and nourishment for larvae (Fenner *et al.* 2022). Nectar is one of the most common floral rewards offered by plants in exchange for pollination services (Fenner *et al.* 2022). Nectar foragers that pollinate engage in mutualistic relationships with plants with approximately 90% of plant species pollinated by animals (Ollerton *et al.* 2011). However, not all floral visitors are considered pollinators. Floral larcenists display nontypical behaviors such as primary and secondary robbing, where foragers access the nectar reward avoiding pollination altogether (Inouye, D., 1980; Richman *et al.* 2021). Floral visitors may choose to bypass the floral opening and collect the flower's nectar by chewing a hole on the nectar spur, as primary robbers, or taking advantage of pre-existing robbing holes as secondary robbers (Richman *et al.* 2021). Antagonistic behaviors such as nectar robbing can be a threat to the mutualism's stability (Lichtenberg *et al.* 2018). The terms legitimate and illegitimate have been used to distinguish between mutualistic and antagonistic floral visitors, respectively (Inouye, 1980).

Foragers can maintain a single foraging strategy through multiple floral visits, or switch between legitimate and illegitimate visits (Bronstein *et al.* 2017). Tactic switching has been found to be context-dependent; past research indicates that tactic switching may arise in situations where the efficiency of foraging may be improved, for instance, when a forager determines that the nectar volume is less in robbed flowers (Lichtenberg *et al.* 2018). It has been shown that individual foragers of some floral visitor species can freely switch between foraging tactics but often do not, rather remaining constant for prolonged periods of time (Bronstein *et al.* 2017; Francis *et al.* 2021). Research by Lichtenberg *et al.* (2018) found that bumblebee foragers display a high degree of tactic constancy with most observed free flying bumblebees on *M. ciliata* either consistently foraged legitimately, or a mix of both primary and secondary robbing. However, captured bees readily switched between tactics (robbing and legitimately

foraging) when offered the opportunity to use either tactic on inflorescences in a flight cage. Their results suggest that for bumblebees capable of legitimate and illegitimate foraging behaviors the minimal increase in handling time while legitimately foraging does not significantly affect foraging efficiency (Lichtenberg et al. 2018). What causes switches in foraging tactics remains an understudied phenomenon with potential implications for both foraging efficiency, and plant reproduction (Lichtenberg et al., 2020).

There is much research regarding pollination mutualisms, but to fully understand these plant and animal interactions we need to study their interactions with microbial organisms (Francis et al. 2021; Martin et al. 2022; Luizzi et al. 2024). The roles of flower-inhabiting microorganisms have not been fully explored (Klaps et al. 2020). Although microorganisms within nectar can alter nectar's chemical composition (Francis et al. 2021; Luizzi et al. 2024), their influence on bumblebee foraging behavior remains relatively understudied. While the presence of nectar-inhabiting microbes has been shown to have negative, neutral or positive effects on plant reproduction (Schaeffer & Irwin 2014; Schaeffer et al. 2016; de Vega et al. 2022), their influence on the behavior of floral visitors is not well understood. Some researchers suggest they could potentially promote tactic switching (Francis et al. 2021), since microbial activity has been shown to alter floral phenotypes, in particular, volatile composition (Rering et al. 2018). Additionally, nectar-inhabiting yeasts have been found to modify nectar's pH, amino acid concentration, temperature and sugar content potentially affecting the quality, quantity, and signal of floral rewards (Schaeffer et al. 2017; Rering et al. 2018; Klaps et al. 2020). Research at RMBL in 2023 found that bumblebee foragers could more quickly find robbing holes on *Corydalis caseana* flowers when the flowers were treated with yeast-inoculated nectar analogues than when the flowers were treated with sterile nectar analogue (Souto-Vilarós et al. in prep). In foraging environments where efficiency is the name of the game, minimizing the time to reach nectar rewards may mean that the presence of microbes could facilitate tactic switching in bumblebee foragers. However, to date, little work has been done to elucidate the influence of nectar-inhabiting microbes on foraging tactics of bumblebees.

The purpose of this study was to investigate the influence of the nectar specialist *Metschnikowia reukaufii* on the tactic constancy of bumblebees foraging nectar from *Corydalis caseana*. *Metschnikowia reukaufii* is a widespread nectar specialist yeast which depends exclusively on floral visitors for dispersal (Pozo et al. 2018; Rukowski et al. 2023). Additionally, *M. reukaufii* overwinters in the digestive tract of bumblebee queens suggesting these two have a tight relationship with both floral hosts and potential dispersers (Pozo et al. 2018; Rukowski et al. 2023). By manipulating the presence of *M. reukaufii* in the nectar of otherwise sterile flowers of *Corydalis caseana*, we observed the foraging patterns of bumblebees which can act as both legitimate pollinators and secondary robbers and are therefore capable of tactic switching. We expected that offered flowers containing *Metschnikowia*-inoculated nectar would prime legitimate foraging bumblebees to switch tactics since the presence of yeast may act as a signal promoting robbing. We also suspected that secondary robbers would remain constant to nectar robbing tactics of the yeast-inoculated flowers and that these secondary robbers would be able to locate them more quickly (regardless of their foraging tactics prior to the trial). Lastly, we predicted both foraging types would visit more inflorescences and flowers per inflorescence in the arrays with the yeast-inoculated flowers.

Methods:

Behavioral assays were conducted in 3 locations in lower, middle, and upper Washington Gulch, Crested Butte, Colorado (Fig. 1) within a *Corydalis caseana* ssp. *brandegeei* wildflower populations. These perennial forbs often grow in large patches of hundreds to thousands of plants in subalpine meadows of New Mexico and Colorado, typically near a water source (Maloof, 2008). *Corydalis* is frequently robbed by short-tongued bumblebee species such as *Bombus flavifrons* and *B. bifarius*, both of which act as facultative nectar robbers (Maloof, 1999).

The two yeast isolates we used for inoculations were originally sourced from the lower and upper Washington Gulch *Corydalis* populations in 2022 (Martin *et al.* in prep). The isolates were grown on yeast malt agar plates supplemented with the antibacterial, chloramphenicol, and incubated at 25°C in a bench top incubator for 48 hours. Sterile nectar analogs with the same amino acid and sugar concentrations as *Corydalis* nectar were inoculated with *M. reukaufii* cells at a concentration of 1,000 cells/ μ L. To achieve this, we used an inoculation loop to collect yeast cells from the agar plate and suspend them in the *Corydalis* nectar analog. This stock yeast nectar solution was then placed under a hemocytometer to quantify the number of yeast cells per volume of nectar analog. Using this stock solution, we will dilute it accordingly to achieve 1,000 cells/ μ L before incubating the inoculated nectar for 48 hours at 25°C.

Two flight boxes were constructed from Rubbermaid plastic containers with the side and top panels replaced with mesh to allow breathability. Each box contained 6 translucent waterpics to hold experimental inflorescences. Before bloom, inflorescences were covered using fine mesh bags to avoid visitation, and thus microbial dispersal, and later chosen at random for the experiment. All inflorescences were trimmed to have 10 flowers and treated according to each flight box assay. One flight box array contained flowers treated with 5 μ L each of sterile nectar analogue and the other treated with 5 μ L of yeast-inoculated nectar analogue. All flowers were artificially robbed by cutting a hole on the spur of each flower using sharp forceps, mimicking the robbing wounds created by primary robbers. Individuals of *B. flavifrons* and *B. bifarius* that were both foraging legitimately, or secondary robbing were collected using a standard hand net and placed in plastic vials. Each bumblebee was placed randomly in either the sterile treatment or yeast treatment and allowed up to ten minutes to make a choice within the array. Once the bee reacted to the inflorescences within, using a hand-held voice-recorder, we recorded the bumblebee's behavior. The following foraging behaviors were monitored: which inflorescence they visited in the array, landing location on the flower, foraging type (legitimate or illegitimate), the time spent per flower (head in and out of corolla or tongue in and out of robbing hole), movement to a new flower on the same inflorescence or movement to a new inflorescence. A foraging bout was considered over when the bumblebee had visited every available flower, or it was clear that the individual had lost interest in the array by flying to the side panels of the flight box. Every inflorescence visited by the individual was discarded and replaced with a new one to avoid potential leftover signals (e.g., cuticular hydrocarbons) which can influence subsequent focal individuals. After testing, each individual was marked with a dot nontoxic POSCA paint on its thorax to avoid testing individuals more than once.

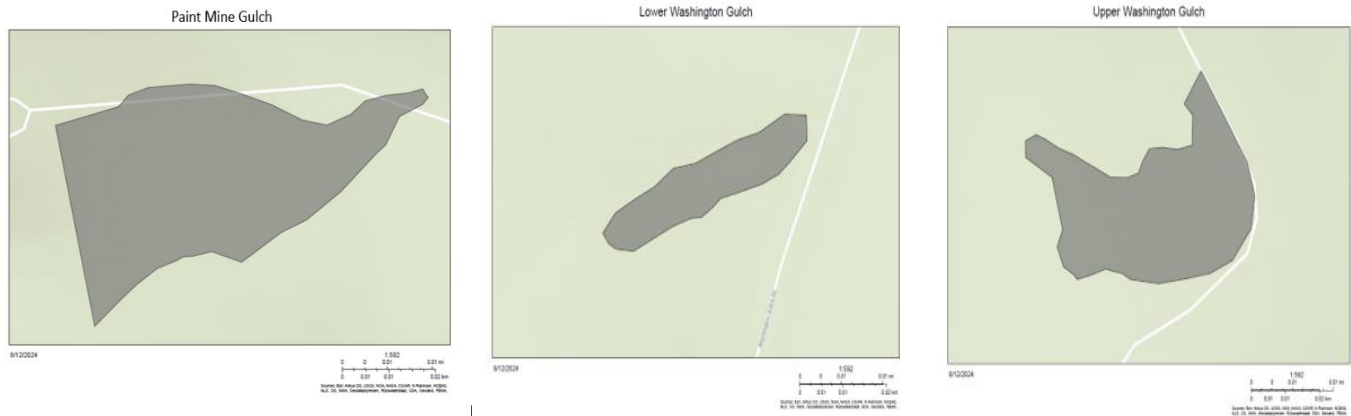


Figure 1. *Corydalis caseana* ssp. *brandegeeii* populations (shaded grey) at the middle (38.949455, -107.028584), lower (38.940679, -107.023121) and upper (38.969977, -107.045447) Washington Gulch, Crested Butte, Colorado (left to right, respectively).

Results:

We trapped and tested a total of 99 bumblebees, of which 37 of them reacted within the behavioral arrays. For each trial, we recorded the time to rob or visit each flower, the time spent feeding, and the foraging strategy employed on each flower visit. We tested 11 *B. bifarius* of which 1 were legitimate visitors and 10 secondary nectar robbers, as well as 26 *B. flavifrons* of which 5 were legitimate foragers and 21 secondary robbers respectively.

Both bumblebees were faster at finding robbing holes in control plants when compared to yeast inoculated plants (Fig. 2A; ANOVA $F_{1,403} = 24.41$; $p < 0.001$); however, the time to rob was not significantly different between species (Fig. 2B), with *B. flavifrons* taking 7.41 ± 0.58 seconds to find the robbing hole on yeast-inoculated plants, compared to 4.82 ± 0.40 seconds on control plants and *B. bifarius* taking 7.20 ± 0.94 seconds to and 3.44 ± 0.42 seconds to find the robbing hole in yeast inoculated plants and control plants respectively. Similarly, prior behavior did not have a significant effect on the time it took a bumblebee to rob the flowers within the array (Fig. 2C)

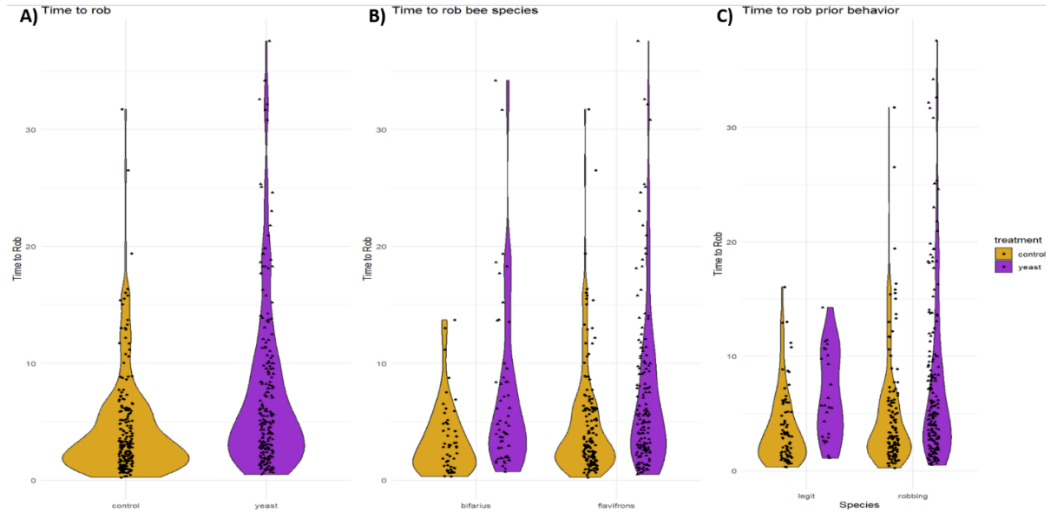


Figure 2. Violin plots displaying time to rob according to treatment (A), separated by species (B) and according to prior behavior (C). Plots in orange display time to rob on control flowers; in purple flower treated with yeast-inoculated nectar.

Feeding time was not significantly different between treatments, however, we found a significant difference when we separated the data by bumblebee species (Fig. 3) with *B. bifarius* feeding on control and yeast inoculated flowers for 3.37 ± 0.47 and 3.96 ± 0.58 seconds respectively and *B. flavifrons* feeding for 6.50 ± 0.56 and 7.36 ± 0.64 seconds on control and yeast inoculated flowers, respectively.

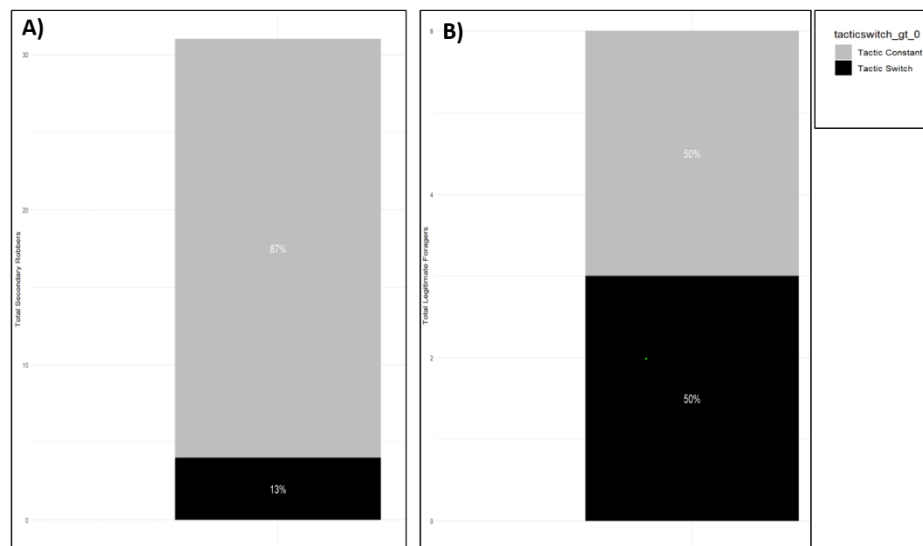


Figure 3. Percentage of foraging bumblebees either switching tactic or remaining tactic constant according to prior foraging strategy. A) Secondary robbers; B) Legitimate foragers.

Finally, regarding foraging strategy, an interesting pattern arises. When looking at the foraging strategy of each bumblebee individual, it seems that robbing visitors remain constant to their strategy, with 81% of tested individuals continuing to rob flowers within the array (fig. 3). When looking at legitimate

foragers, the pattern is less clear with 50% of bumblebees switching tactic to robbing and 50% foraging legitimately. In both cases, it appeared that treatment of flowers did not affect this pattern

Discussion:

We did not find evidence that the nectar specialist *Metschnikowia reukaufii* had an influence on visitors' tactic constancy. Nevertheless, it had been found that the robbing time varied among treatment but not species. This indicates that bumblebees were able to find the robbing hole within the control treatment faster. On the other hand, these robbing bees remained tactic constant and did not tend to switch foraging behavior while on the other hand we found legitimate foragers to switch tactics half the time.

In this study we analyzed bumblebee behavioral arrays of two treatments over 3 weeks. We used two species of bumblebees *B. flavifrons* and *B. bifarius* which have been shown to be facultative secondary robbers in *Corydalis casseana* (Newman, 2005), within a nectar yeast treatment and sterile nectar. Despite some evidence indicating that yeast presence in floral nectar could affect floral signals, which in turn could be used to influence foraging behavior (Zemenick et al, 2018; Morris et al, 2020) we did not find evidence of the presence of yeast on enticing tactic switching on legitimately foraging bumblebees.

From this experiment we had predicted that bumblebees would be more likely to switch foraging tactics within the array due the yeast microbes' ability to alter the nectar chemistry (Francis, 2021). Studies have shown that these microbes can modify the quality and quantity as well as signals of floral reward (Schaeffer *et al.* 2017; 2019). A forager is able to feed in multiple way per plants, but studies still do not fully understand what influences their foraging decision (Bronstein et al. 2017). This had led us to believe that yeast nectar would influence floral cues and bumblebee tactic switching but based on our findings in Fig. 3, bees remained tactic constant. Bronstein et al. 2017, proposes the idea that visitors would prefer to stay tactic constant because floral constancy appears to reduce foraging time, allowing bumblebees to forage more efficiently (Bronstein et al. 2017). Although bumblebees have the ability to switch foraging tactics they often choose not to (Francis *et al.* 2021). We are also inclined to think that the bumblebees maintain tactic constancy once they switch to robbing, since they most likely learn how to rob at the beginning of the season, particularly in *Corydalis caseana*, where approximately 100% of flowers appear to be robbed by the end of their flowering season in mid-July (Lichtenberg, 2020). Despite finding that approximately half of legitimate visitors appear to switch tactic within our array, our sample size was severely limited (n = 6) and ideally, testing many more legitimate foragers would allow us to conclude whether or not yeast presence in *Corydalis* flowers prime legitimate foragers to switch to robbing. Repeating our behavioral assays early on in the flowering season, while the *Corydalis* patches are not totally robbed could allow us to test more legitimate foragers. Since *M. reukaufii* is primarily dispersed by bumblebees, and it is known that nectar robbers are responsible for the dispersal of distinct microbial communities when compared to legitimate visitors (Zemenick *et al.* 2018), it is possible that by the time we preformed our experiments, most foragers had already encountered robbing related signals, and as such we were unable to detect tactic switching in our assays.

Nevertheless, we did find that bumblebees appeared to find the robbing holes faster in the control flowers than on yeast-inoculated flowers, contrasting the results found on a similar experiment the previous year (Souto-Vilarós *et al.* in prep). These results suggest that perhaps foraging behavior may not directly depend on nectar-inhabiting microbes or that bumblebees may respond to yeast cues differently between years. Regardless, microbial activity has been shown to alter the floral phenotype

and as such, remains an interesting question to pursue regarding how these changes affect flower visitor behavior which will ultimately affect plant reproduction.

Timeline:

Activity:	Duration	Date
Preliminary Fieldwork (Site determination and inflorescence bagging)	~ 1 week	6/24-7/1
Experimentation (Bumblebee trials)	~ 3 weeks	7/3-7/26
Analysis (Collection of data from video data, statistical analysis)	10 days	7/25-8/1
Writing (Completing final paper/ PowerPoint)	~ 1 week	8/1-8/13

Major Professional Objectives:

This summer has deepened my understanding of the mutualistic relationships between pollinators, plants, and their relationship with yeast microbes. Specifically, I hoped this summer helped to bridge a gap studying how these interactions influence bee behavior and floral signaling. By exploring this intricate relationship this summer this research will add to the continuing plant-pollinator research at RMBL highlighting the importance of microbial interactions and ecology. My time at RMBL has helped provide me with valuable experiences in scientific writing through an independent study. This research in an area of study interest, pollinators has helped guide me and prepare me for further academic pursuits and necessary skills for this field.

Projected Outcomes:

From this REU I developed a new skill set by studying the plant-pollinator interactions and microbial ecology. My goal was to complete my field data collection and produce a comprehensive final paper to add to RMBL's publication database. I collaborated and worked with my mentors Rebecca Irwin, Robert Schaffer, Valerie Martin, Daniel Souto, Nicholas Dabagia and peers, Greta Thoresen and Oriana Gutierrez to complete these tasks. I will be a part of a coauthorship on bumblebee behavior and *Ipomopsis aggregata* papers.

Data Archiving:

As part of the RMBL Data Archiving, Curation, and Vouchers, I will participate in workshops and training for spatial and non-spatial metadata and archiving. My Data Management Plan includes a description of the datasets being archived, the dissemination methods, and the timeline for data-sharing. I will use the RMBL template metadata form. I will use the default data repository Environmental Data Initiative (EDI), <https://environmentaldatainitiative.org/> unless my mentors request a different repository.

Environmental Impacts:

We expect that the project will have minimal impact on nature. For this project we used a non-lethal sampling method. Bees were marked after the behavioral assays to ensure we continued to see the bumblebees foraging even after experimental array suggesting minimal stress. We kept our flower

sampling to less than 5% of inflorescences produced by the population over the bloom period and avoided sampling from the same individual plants to avoid flower clearing. Short period impacts may cause stress to the bumblebees. We tested 99 bumblebees with no fatalities. We do not think that we will have any long term impacts due to renewal of worker bees and regrowth of the flowers.

References:

- 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.
- Fenner, E. D., Scapini, T., da Costa Diniz, M., Giehl, A., Treichel, H., Álvarez-Pérez, S., & Alves, S. L. (2022). Nature's Most Fruitful Threesome: The Relationship between Yeasts, Insects, and Angiosperms. *Journal of Fungi*, 8(10), Article 10. <https://doi.org/10.3390/jof8100984>
- Francis, J. S., Tatarko, A. R., Richman, S. K., Vaudo, A. D., & Leonard, A. S. (2021). Microbes and pollinator behavior in the floral marketplace. *Current Opinion in Insect Science*, 44, 16–22.
- Inouye, D. W. (1980). The Terminology of Floral Larceny. *Ecology*, 61(5), 1251–1253.
<https://doi.org/10.2307/1936841>
- Klaps, J., Lievens, B., & Álvarez-Pérez, S. (2020). Towards a better understanding of the role of nectar-inhabiting yeasts in plant–animal interactions. *Fungal Biology and Biotechnology*, 7(1), Article 1.
<https://doi.org/10.1186/s40694-019-0091-8>
- Lichtenberg, E. M., Irwin, R. E., & Bronstein, J. L. (2018). Costs and benefits of alternative food handling tactics help explain facultative exploitation of pollination mutualisms. *Ecology*, 99(8), 1815–1824.
- Lichtenberg, E. M., Richman, S. K., Irwin, R. E., & Bronstein, J. L. (2020). Competition for nectar resources does not affect bee foraging tactic constancy. *Ecological Entomology*, 45(4), 904–909.

Luizzi, V. J., Harrington, A. H., Bronstein, J. L., & Arnold, A. E. (n.d.). *Nectar robbers and simulated robbing differ in their effects on nectar microbial communities*. Retrieved August 13, 2024, from <https://esj-journals.onlinelibrary.wiley.com/doi/10.1111/1442-1984.12446>

Maloof, J. E. (2000). Reproductive biology of a North American subalpine plant: *Corydalis caseana* A. Gray ssp. brandegei (S. Watson) G. B. Ownbey. *Plant Species Biology*, 15(3), 281–288.
<https://doi.org/10.1111/j.1442-1984.2000.00047.x>

Maloof, J. E. L. (1999). *The ecological effects of nectar robbers, with an emphasis on the reproductive biology of Corydalis caseana*. University of Maryland, College Park.

Pozo, M. I., Bartlewicz, J., van Oystaeyen, A., Benavente, A., van Kemenade, G., Wäckers, F., & Jacquemyn, H. (2018). Surviving in the absence of flowers: Do nectar yeasts rely on overwintering bumblebee queens to complete their annual life cycle? *FEMS Microbiology Ecology*, 94(12), fiy196.
<https://doi.org/10.1093/femsec/fiy196>

Richman, S. K., Barker, J. L., Baek, M., Papaj, D. R., Irwin, R. E., & Bronstein, J. L. (2021). The sensory and cognitive ecology of nectar robbing. *Frontiers in Ecology and Evolution*, 9, 698137.

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(8), fiad077.

Schaeffer, R. N., & Irwin, R. E. (n.d.). *Yeasts in nectar enhance male fitness in a montane perennial herb*.
<https://doi.org/10.1890/13-1740.1>

Schaeffer, R. N., Mei, Y. Z., Andicoechea, J., Manson, J. S., & Irwin, R. E. (n.d.). *Consequences of a nectar yeast for pollinator preference and performance*. Retrieved August 13, 2024, from
<https://besjournals.onlinelibrary.wiley.com/doi/10.1111/1365-2435.12762>

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(3), 613–621.

<https://doi.org/10.1111/1365-2435.12762>

Vega, C. de, Albaladejo, R. G., Álvarez-Pérez, S., & Herrera, C. M. (n.d.). *Contrasting effects of nectar yeasts on the reproduction of Mediterranean plant species*. <https://doi.org/10.1002/ajb2.1834>

Zemenick, A. T., Kula, R. R., Russo, L., & Tooker, J. (2019). A network approach reveals parasitoid wasps to be generalized nectar foragers. *Arthropod-Plant Interactions*, 13(2), 239–251.

<https://doi.org/10.1007/s11829-018-9642-9>

Effects of nectar robbing on nectar dynamics and bumblebee foraging strategies in Linaria vulgaris (Scrophulariaceae)—Newman—2005—Oikos—Wiley Online Library. (n.d.). Retrieved August 13, 2024, from <https://nsojournals.onlinelibrary.wiley.com/doi/abs/10.1111/j.0030-1299.2005.13884.x>

Legitimate visitors and nectar robbers of Aquilegia formosa have different effects on nectar bacterial communities—Zemenick—2018—Ecosphere—Wiley Online Library. (n.d.). Retrieved August 13, 2024, from <https://esajournals.onlinelibrary.wiley.com/doi/full/10.1002/ecs2.2459>

Microbial abundance, composition, and function in nectar are shaped by flower visitor identity | FEMS

Microbiology Ecology | Oxford Academic. (n.d.). Retrieved August 13, 2024, from

<https://academic.oup.com/femsec/article/96/3/fiaa003/5700281>