

Nectar Dwelling Yeasts and their Effects on Pollinator Preference
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Abstract:

Pollinators are extremely important in the global ecosystem. Flowers have evolved attractive traits in order to draw these pollinators. Traits like colorful petals draw in pollinators to metabolic rewards such as nectar and pollen, and in return, pollinators transfer pollen between plants. Plants with better attractants or rewards are more likely to draw in more pollinators and thus are most likely to reproduce. Microorganisms have been found inside of the floral reward nectar that may skew the plant-pollinator reactions. This study looked at the distribution of nectar inhabiting yeasts in the floral nectar of the perennial herb *Delphinium barbeyi*, and pollinator reactions to those yeasts. This study found that pollinators are most likely the vectors for the transportation of nectar dwelling yeasts in the tall larkspur, and that *D. barbeyi* is unable to regulate the yeast concentration in its floral nectar. Finally, the results of nectar removal experiments, where either a sucrose solution or a yeast and sucrose solution was injected directly into the nectar spurs of the *D. barbeyi* flowers, showed that pollinators had no preference to yeasty nectar versus a sucrose control. Understanding how microbes in floral nectar effect plant-pollinator interactions is important in better understanding the evolutionary traits of flowering plants as well as the patterns behind pollinator preferences.

Introduction:

Bees like flowers; it's something that is known as an intrinsic truth. Bees like flowers because angiosperms, commonly known as flowering plants, bribe them with a reward, the most common being floral nectar (Simpson and Neff 1983), which they use for energy. This basic idea begs the question: are all nectars created equal? Recent studies have shown that many pollinators, specifically bees, frequent flowers with high yeast concentration in their nectar more often than those with low or no yeast (Herrera et al, 2013). Furthermore, studies suggest that yeast colonize the nectar of a substantial number of plant species (Herrera et al 2012), reducing pH levels, sucrose, and total sugar content of the nectar (Peay et al 2011). By changing the composition of nectar, yeasts may change the appeal of flowers to pollinators, which may in turn, affect pollinator behavior including the number and duration of visits per plant. If plants are pollen-limited for plant reproduction, these yeast-induced changes in pollinator behavior can affect overall plant fecundity (Herrera et al. 2013). Nonetheless, inconsistency has been shown in just how nectar-inhabiting yeasts affect plant fecundity; some studies state it increases male fitness (Schaeffer 2013), while others state it decreases fruit set, seed set, and seed mass, which are important in female flower and overall plant fecundity (Herrera et al 2013). By studying the effects of nectar-dwelling yeasts on pollinator behavior and their impact on plant reproduction, we can better understand the driving force behind the extremely important nature of plant-pollinator mutualisms.

This study investigated the extent of variation in nectar-dwelling yeast concentrations both within and among *Delphinium barbeyi* and also assessed how yeast concentration affected plant-pollinator interactions. Because *D. barbeyi* is closely related to another *Delphinium* species (*D. nuttallianum*) that has been shown to harbor nectar yeast (Schaeffer 2013) and both *Delphinium* share pollinators, I predicted to find yeast in the flowers of *D. barbeyi*. I also predicted that because pollinators can vary in their preference for individual plants, yeast concentrations would vary more widely among

than within plants. Finally, using pollinator preference and nectar removal experiments, I tested the prediction that pollinators would prefer flowers that have yeasts, given that pollinators of *D. nuttallianum* also prefer yeast-rich flowers (Shaeffer 2013).

Methods:

Delphinium barbeyi (Ranunculaceae), common name tall larkspur, is a long lived-perennial herb that blooms in from mid-July to late August. It is found mainly in wet meadows and bogs in the subalpine and montane regions of Wyoming, Utah and Colorado (Nelson 1992). *Delphinium barbeyi* grow multiple stalks of about 1.5m tall with inflorescence of purple flowers that are visited mainly by bumblebees and hummingbirds (Inouye 1978, Wright 1988, Kotliar 1996). Though *D. barbeyi* is self-compatible, it relies heavily on visitations by pollinators to increase seed set (Williams et al 2001) The flowers of *D. barbeyi* are protandrous or sequential hermaphrodites, where the flower shifts from male to female over time (Williams et al 2001).

In the area surrounding the Rocky Mountain Biological study, nectar-dwelling yeast has been collected from *Delphinium nuttallianum*. Over 60% of all flowers open to pollinators collected contained yeast (Shaeffer 2013). When identified, all samples collected were revealed to be the cosmopolitan nectar-dwelling yeast, *Metschnikowia reukaufii* (Scaheffer 2013).

This study was conducted at the Rocky Mountain Biological Laboratory in Gothic, Colorado, USA elevation: 2891.03m. I chose three sites for this study: the 401-Trailhead Meadow (Lat, Long: 38.965, -106.988), Marriage Meadow (Lat, Long: 38.967, -106.99), and The Beaver Dam Meadow (Lat, Long: 38.974, -106.993). All three sites were chosen based on their high abundance of *D. barbeyi*.

Variation of yeast within and among individuals

In order to determine whether *D. barbeyi* is capable of regulating yeast content in its floral nectar, nectar was collected by haphazardly choosing 10 plants per site and covering one stalk per plant with bridal veil netting for 48 hours. A mark was made underneath the lowest unopened flower bud. For each bagged stalk, all the flowers on one stalk underneath the mark made were collected in their own plastic bag. I removed the nectar by opening the end of the nectar spurs, drawing out the nectar into a micro-capillary tube, and estimating the remaining nectar using a caliper. The caliper gave the amount of nectar in millimeters, which was then converted to μl . The nectar collected was then stained with 9 μl of 95% ethanol diluted lactophenol blue. All of the dyed nectar collected from each flower was individually loaded into a hemocytometer and the yeast cells counted. This number was then standardized to yeast concentration for each flower by dividing the number of yeast cells counted by the amount of nectar in μl . The plant number, flower number, sex of the flower (male, female, or intermediate), and number of yeast cells were recorded. This was repeated twice for each site.

I analyzed the data in three ways. The data collected were not normal, and so the median rather than the mean was used in the statistical analyses. The coefficients of variation were calculated for three categories, individual, site, and date measured. An ANOVA was run comparing the coefficients of variation for these three categories to determine if there was variation in yeast concentration within individual plants, among sites, and temporally. Finally, to determine if there was significant variation among sites

and sampling dates, an ANOVA was run comparing sites and sampling dates to the median of the plant's yeast concentration.

Yeast concentration in virgin versus open flowers

Prior studies in other plant species have shown that flowers that have never been visited by pollinators remain free of yeast infection, while flowers that have been visited by pollinators have yeast, suggesting that pollinators are the vectors of yeast among flowers (e.g., Schaeffer 2013). Using *D. barbeyi*, I tested whether a similar pattern held. The same 10 plants from above were chosen and marked in the same way as indicated above. All the flowers above the mark that had bloomed during the 48 hour time period when covered by the bridal veil bags, were collected and placed in their own plastic bag and labeled as "virgin" (i.e., unvisited by pollinators). It was noted which stalk the flowers came from. The yeast concentration was obtained using the same methods as above. This was repeated twice for each site. I used a paired t-test comparing the median yeast concentrations of virgin vs. non-virgin yeast concentrations was conducted to determine if there was a significant difference between flowers to test they hypothesis that pollinators are the primary vectors of yeast.

Yeast impacts on pollinator preference

To determine whether pollinators preferred flowers with high concentrations of nectar-dwelling yeasts, which has been shown in previous studies (Herrera et al 2013, Schaeffer 2013), I conducted a nectar removal experiment. I made 50% w/w sucrose solution and added the yeast culture "inoculate one" to a subsample. I let that subsample incubate for four days and used it as my experimental solution. The 50% w/w sucrose solution without added yeast was used as a control. To collect yeast inoculates, 10 stalks at each site were bagged for 24 hours. Two flowers from each stalk were destructively picked and placed in their own plastic bags. The flowers were given unique identifiers and sexed and the nectar was removed in sterile conditions in the same manner as stated above, and blown onto YM agar plates. These plates were left to incubate. At the end of two days one colony of yeast was chosen at random and labeled as "inoculate one" and was used during the nectar removal experiments.

I haphazardly chose 22 blooming *D. barbeyi* at each of the three research sites on. Four flowers on each stalk were chosen haphazardly on two stalks on each plant, and were injected with either 2 μ l of the control or experimental solution, directly into the plant's nectar spurs using a 10 μ l glass syringe. Within each plant, each stalk received a separate treatment. These flowers were left for five hours to allow for pollinators to remove the nectar, and then collected at the end of the day. Each flower was given a unique identifier, and sexed. The remaining nectar was recorded in the same manner as above. This was repeated twice for each site. The data was analyzed in the program JMP using a T-test comparing the amount of nectar remaining in control flowers to artificially increased yeast flowers.

Results:

Variation of yeast within and among individuals

By comparing the coefficient of variation, which standardizes the variation of yeast concentration, of each individual plant measured to the total coefficient of variation at all three sites, there was no significant difference found ($F_{1,59}=1.71$, $P=.20$; Fig. 1). There was, however, a significant difference in the total coefficient of variation between the three sites ($F_{2,55}=7.33$, $P=0.0015$; Fig. 2). Moreover, the mean yeast concentration per flower per plant also varied among sites (Chi square=13.26, $DF=2$, $P=0.002$) and among dates sampled at each site (Chi square=13.26, $DF=1$, $P=.003$; Fig. 3). There was also a marked increase in yeast concentration temporally across all three sites (Fig. 4).

Yeast concentration in virgin versus open flowers

At all three sites, there was significantly lower yeast concentration in virgin flowers as compared to open flowers that had access to pollinators before being bagged ($DF=14$, $P=.03$; Fig. 5). Though some virgin flowers did have yeast counts, these numbers were extremely low as compared to flowers that had access to pollinators, and only 8% of virgin flowers had any yeast.

Yeast impacts on pollinator preferences

When flower nectar was artificially increased with either 2 μ l of 50% (w/w) sucrose solution as a control, or the same sucrose solution with incubated yeast, there was no significant difference in the nectar removed from flowers ($F_{1,129}= .17$, $P=0.68$). The mean nectar remaining in the control and the increased yeast respectively was: 0.74 μ l and 0.71 μ l. The standard error was respectively .048 and .053.

Discussion:

The goal of this study was to assess spatio-temporal variation in nectar yeast concentrations of *D. barbeyi* as well as determine whether nectar-dwelling yeasts had an impact on pollinator preference. The first aspect of this experiment looked to see if there was variation in the concentration of yeast within and among *D. barbeyi*. This was done to better understand if *D. barbeyi* is capable of regulating the fungal-microbes in its nectar as well as understand if yeast concentrations are dependant on local ecology. Should *D. barbeyi* be able to regulate, we would expect to see significantly higher variation among sites than within sites and among plants than within plants. Though there was higher variation among sites, no significant differences between the two were found. This indicates that *D. barbeyi* is most likely unable to regulate its floral yeast. This is important in understanding the symbiotic nature of yeasts and angiosperms. Yeasts concentration in floral nectar increases as the season progresses, and should yeast have a detrimental effect on plant fecundity, as was found in a study conducted by Herrera et al. (2013) as well as a study by Eikikowitch et al (1990, 1990), being able to regulate would be an evolutionary advantage. But, as yeast plays no significant role in the plant-pollinator interactions of *D. barbeyi*, there may not be a need to regulate the fungal-microbes in my study plants. An interesting future study would be to look at yeast regulation of plants where yeast is a significant variable in pollination.

Interestingly, yeast concentrations varied based on site and varied among individual sites based on dates sampled. Overall, there was more yeast found at each site as the season progressed. The significant difference in yeast concentration among sites indicates that concentrations of nectar-dwelling yeasts are heavily dependant on local

ecology. The difference in yeast concentrations temporally and spatially may be caused by a few different factors. The difference in yeast concentrations spatially may be attributed to possible variance in blooming times for *D. barbeyi*, or differences in yeast concentrations among pollinators. Yeasts may be overwintering with pollinators. Certain microclimates may be more suitable for the fungal microbes to overwinter or some pollinators in a region may be more effective at transferring yeasts from flower to flower. Temporally, yeast may be multiplying as time goes on, or there are more pollinators as the season goes on (Irwin, *unpublished*) and they are transferring more yeast to each flower. Pollinators may be collecting an increasing amount of yeast as the season progresses and are thus able to transfer more yeast to each flower they visit. It is important to note that the higher concentration of yeast in floral nectar, the more likely it will be picked up by a pollinator. And so, the largest chance of pollinators carrying yeast is at the end of the season, where the yeast can then be transferred to new blooming flowers, or overwinter with its pollinator host.

The second aspect of this study looked at possible modes of transportation for nectar dwelling yeasts. We assumed, based on other studies (Schaeffer 2013), that pollinators were the only vectors for the transfer of yeasts between plants, and without access to pollinators, virgin flowers should contain no yeast. The results were unexpected. Most virgin flowers fit this hypothesis and zero yeast was found, but as the season progressed, more virgin flowers were found to contain low levels of yeast. Some possible explanations for this unexpected result may be that yeasts are capable of becoming airborne, which would bypass the need for pollinators for transportation. Also, it could be that some yeasts are overwintering within the plant, and are present within the plant throughout the entire year. A third explanation that is more favored are small beetles found inside of the flowers. These small beetles were too small to completely remove before bagging, and were found inside of the *D. barbeyi* flowers after the stalks were brought to the lab. Because we were unable to remove these beetles, it is possible that when the virgin flowers bloomed, the beetles transferred yeast into the previously virgin flower nectar. To better understand how yeast is transferred between plants, this lab recommends culturing yeasts from floral nectar as well as yeasts that may be growing on a main pollinator's, such as bees, proboscis, and comparing the two cultures.

Finally, by setting up nectar removal experiments to determine whether pollinators favored and disfavored yeasty nectar, I found that pollinators did not favor either type of flower. This is an unexpected and interesting result. Previous studies that had used the cosmopolitan nectar dwelling yeast, *Metschnikowia. reukaefii*, noted that wild bumblebees removed significantly more yeasty nectar than the control from the *Helleborus foetidus* flower (Herrera et al. 2013), and bumblebees visited proportionally more yeast-injected flowers than the control in *D. nuttallianum* (Schaeffer 2013). Even more, studies have shown that yeast is capable of changing the chemistry of the nectar it inhabits (Peay et al 2011, Herrera et al 2013), and a decrease in sucrose or an increase in ethanol in floral nectar is something that we expected to significantly impact pollinator behavior. The difference in results could be a consequence of differing environments, flowers, and pollinators. Though around the Rocky Mountain Biological Laboratory where this study took place, *Bombus appositus* are the most common pollinators (Inouye, 1976) for my study plant *D. barbeyi*, other visitors were found on the flowers, such as hummingbirds, flies, and beetles (C. Phillips, *personal observation*), which may have

affected the outcome as well. The differing results among separate studies indicate variance in the role of yeast in plant-pollinator interactions. Different species of pollinators and the different flowers they favor may react in unique ways to the microbes in floral nectar. It is important to note that the mean remaining nectar for both the control and experimental set up were close to zero. Pollinators were draining both types of flowers by the end of the five-hour experimental design. It could be that pollinators are draining either the control or the experimental solutions faster. To determine this, I recommend pollinator observations as well as a shorter time period during a similar nectar removal experiment. Further study on the topic will give more insight as to whether or not floral yeasts exert net positive or negative effects on pollinator preference.

Floral yeasts have only begun to be studied, yet are important parts of plant-pollinator mutualisms. These fungal microbes are widespread throughout angiosperms and directly effect plant fecundity and pollinator preference (Herrera et al 2013, Schaeffer 2013). Even more, they may become a crucial role in the evolution of plants that are affected by nectar-dwelling yeasts. This study demonstrated that pollinator reactions to nectar-dwelling yeasts are not universal, and can vary by site, pollinator, and plant host. Because of the importance of these microorganisms, I recommend more studies on nectar-dwelling yeasts in order to cultivate a more complete understanding of plant-pollinator mutualisms.

Figures:

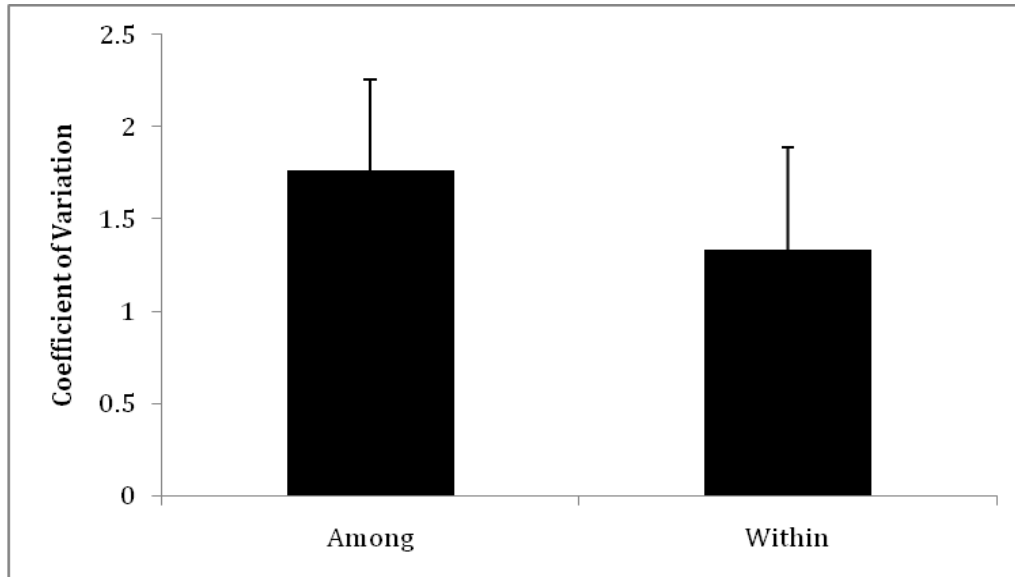


Figure 1. Variation within individual stalks on a plant as compared to variation of the sites studied.

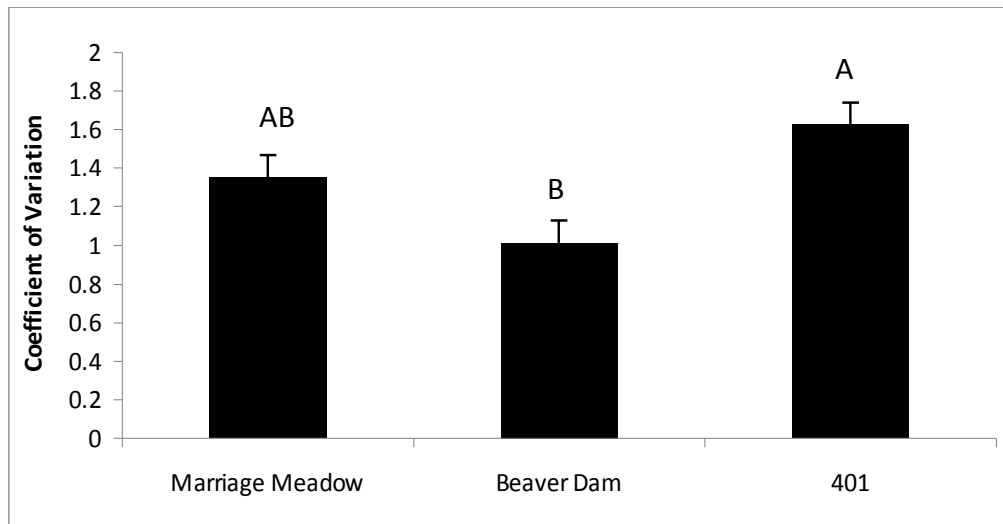


Figure 2. There was significant difference in the variation of yeast concentration at each site. The higher the coefficient of variation, the larger the amount of variation of all the plants at one site.

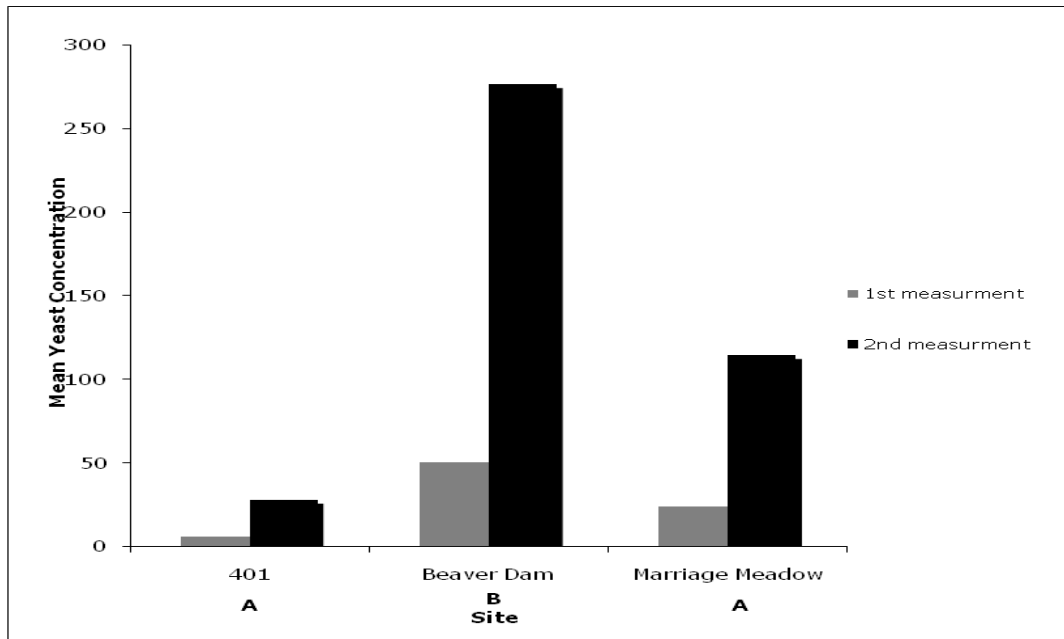


Figure 3. The mean yeast concentration varied among sites and varied at individual sites temporally.

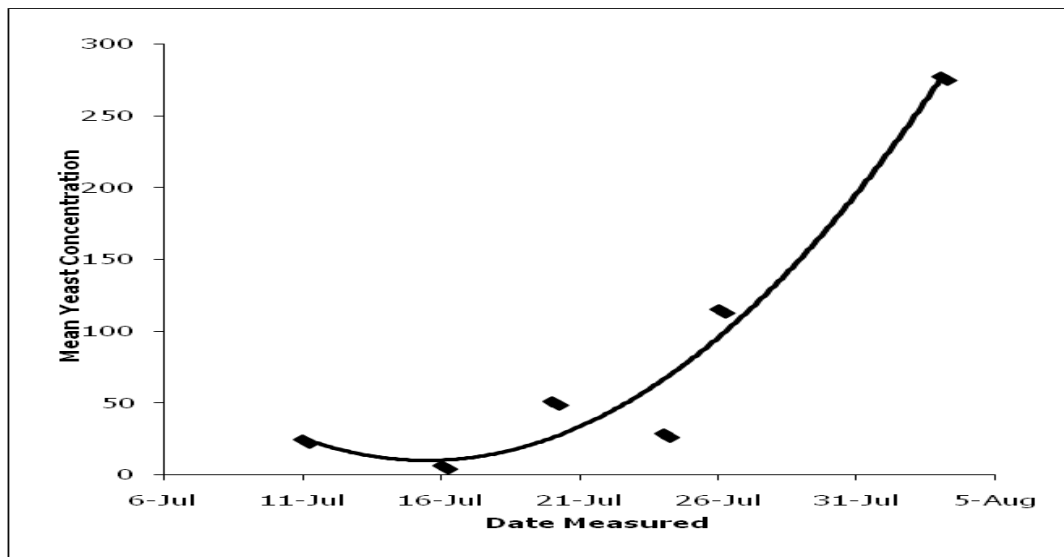


Figure 4. The mean yeast concentration at all three sites increased as the season progressed

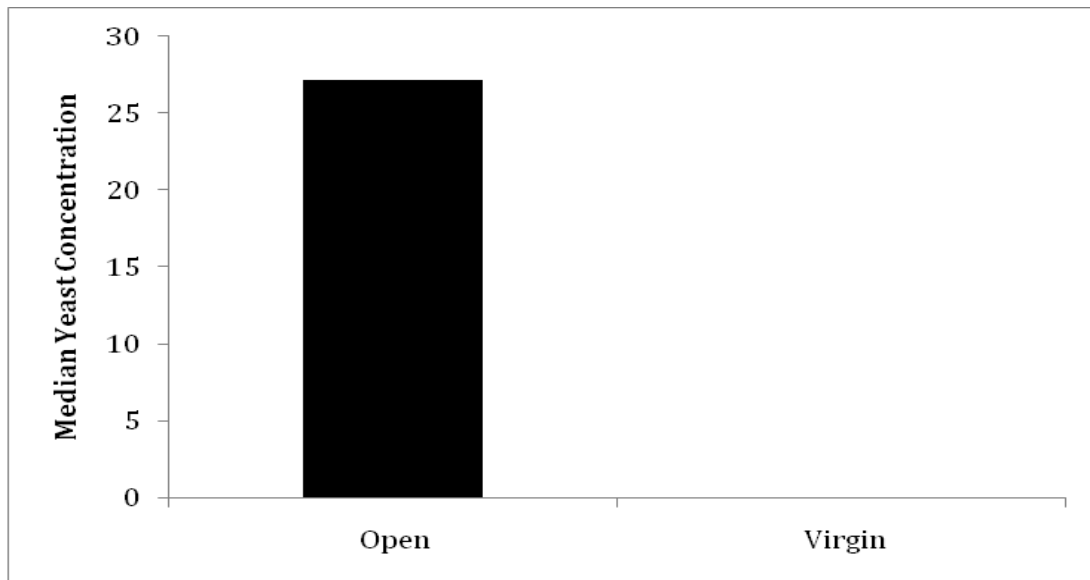


Figure 5. Open flowers, which had access to pollinators before being bagged had significantly higher yeast concentrations than closed, virgin flowers.

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