

Butterfly foraging and the dispersal of microbes among & within flowers

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## BUTTERFLY FORAGING & MICROBE DISPERSAL

Butterfly foraging and the dispersal of microbes among & within flowers

### Abstract

Dispersal is integral to the ecology and evolution of communities. Dispersal affects bacterial communities by impacting bacterial composition and diversity in many environments. One such environment is floral organs. Flowers are nutrient-rich environments for diverse and abundant microbes. Floral microbe community composition is important to pollinator visitation and plant reproduction because microbes alter nectar quality thus changing pollinator visitation. Pollinator visitation to flowers can change the composition and diversity of the floral microbial community and can also impact the spread of disease among plants and pollinators. There is a paucity of studies of rate at which pollinators acquire and disperse microbes among and within flowers. We asked how butterfly foraging affects dispersal of microbes and what factors contribute to microbe abundance on butterflies' bodies. We used *Pyrocoma crocea* (Asteraceae), *Glaucopsyche lygdamus* (Lepidoptera: Lycaenidae), and *Speyeria mormonia* (Lepidoptera: Nymphalidae) to test dispersal of a yellow bacteria via nectar foraging. Butterflies dispersed microbes differentially to different floral organs. The nectaries received significantly more microbes than the anthers, which received significantly more microbes than the stigma. There were not significant differences in microbe abundance on different butterfly parts including the proboscis, feet, and thorax. *Glaucopsyche lygdamus* had lower bodily microbe abundances than *Speyeria mormonia*. Dispersal between flowers depends on microbe abundance with more microbes resulting in higher rates of dispersal between flowers. Microbial transmission among and within flowers by generalist butterflies can thus depend on species, age, and microbe abundance.

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### **Introduction**

Flowers serve as nutrient-rich environments for diverse and abundant microbes and are central to plant reproduction (Alekkett, Hart, & Shade, 2014). Microbes in flowers can change the physical and chemical environment within flowers. For instance, nectar yeasts and bacteria can affect the composition of nectars. Yeasts can alter the composition and concentration of amino acids and sugars (Canto & Herrera, 2012), augment floral volatile emission (Raguso, 2004), and warm nectar (Herrera & Pozo, 2010). Bacteria can modify the physical and chemical traits of nectar such as sugar and amino acid content and concentration (Canto & Herrera, 2012), pH (Lenaerts et al., 2016), scent (Golonka, Johnson, Freeman, & Hinson, 2014), and presence of secondary metabolites (Vannette & Fukami, 2016). These changes can alter nectar quality which can in turn cause pollinators to avoid certain flowers (Herrera, García, & Pérez, 2008; Good, Gauthier, Vannette, & Fukami, 2014). Bacteria found in nectar can also weaken plant-pollinator mutualisms (Vannette, Gauthier, & Fukami, 2013). Floral microbe community composition is thus important to pollinator visitation and plant reproduction.

Pollinator visitation to flowers can change the composition and diversity of the floral microbial community (Ushio et al., 2015; Vannette & Fukami, 2017). Plant-pollinator interactions can also impact the spread of disease among plants and pollinators. Flowers aid dispersal and transmission of pathogens within and between pollinator species (Graystock, Goulson, & Hughes, 2015). There is a paucity of studies of rate at which pollinators acquire and disperse microbes among and within flowers. Additionally, the dispersal of floral microbes has been studied almost exclusively in the context of pollinators foraging for nectar (Herrera, García, & Pérez, 2008; Hausmann, Tietjen, & Rillig, 2017). However, flowers are complex structures comprised of many organs.

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Flower organs differ in morphology, function, and habitat suitability for microbes (Alekkett, Hart, & Shade, 2014; Steven, Huntley, & Zeng, 2018). Floral organs differ in their microbial communities (Junker & Keller, 2015). Pollinators interact with certain floral organs and flower species to differing degrees. Bumble bees disperse microbes at different rates to different floral organs while foraging for nectar and pollen (Russell, Rebolleda-Gómez, Shaible, & Ashman, 2019). Although most adult butterflies' main food source is floral nectar (Scott, 1946), not pollen, butterflies spread pollen much like bees do. Pollen has been found to be transferred by the proboscis, body, and legs of butterflies (Levin & Berube, 1972). These body parts could also be instrumental in microbe transfer. If microbes are transferred like pollen, butterflies could act as vectors for microbes like bees. It is unknown what butterfly body parts are involved in microbe transfer.

Butterflies have the potential to act as a microbe vector to an array of plant species. Generally, neither individual nor species feed on only one floral species (Scott, 1946). Butterfly body mass, wing loading, and proboscis length characterizes families or subfamilies of butterflies by the flowers they visit and feed on (Corbet, 2000). Since butterflies often feed on many species of flowers, they could act as a vector of plant microbes and pathogens to many flower species. The microbe dispersal rates of Lepidoptera are unknown.

We hypothesized that certain butterfly body parts such as the proboscis can be attributed to microbe dispersal more than others. This is because the proboscis enters the floret to extract nectar while the feet and thorax remain on the flower head (Scott, 1942). We predicted that butterfly size would affect dispersal; we hypothesized that smaller butterflies would disperse microbes less than larger butterflies. Since overall body size could limit microbe acquisition, we predicted that this would impact microbe dispersal. We also predicted that butterfly age would

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affect the number of microbes present on an individual butterfly. Since microbes are acquired over time from the environment, older butterflies could have more microbes present than younger butterflies. We also tested the hypotheses that butterflies disperse microbes to flowers readily and differentially among floral organs. We anticipated that the flower nectaries will have the highest microbe colony content because butterflies will contact the nectaries while foraging for nectar. Lastly, we predicted that microbe concentration within florets will affect the rate of dispersal because higher microbe concentrations could result in higher dispersal rates.

### Methods

#### Experiment 1: Butterfly Body Parts and Microbe Dispersal

We created YM broth by mixing 0.75 grams of yeast extract, 0.75 grams of malt extract, 1.25 grams of bacto peptone, and 2.5 grams of glucose to 250 milliliters of deionized water. We mixed the broth solution using a magnetic mixer in a 500 milliliter Erlenmeyer flask and autoclaved the mixture at 121°C for 15 minutes. We added 5 grams of agar to the YM broth after the solution cooled to the point where we could touch the flask. We mixed the solution with a magnetic mixer and autoclaved the mixture at 121°C for 15 minutes. We poured the YM agar into 60x15mm plastic petri dishes using sterile technique. We sealed the plates with parafilm (Suh, Zhang, Nguyen, Gross, & Blackwell, 2008).

We collected 10 *Glaucopsyche lygdamus* and 31 *Speyeria mormonia* from the Kettle Ponds area. We placed each individual in an autoclaved glassine envelope to prevent contamination. We recorded sex and wing wear as a proxy for age. Wing wear was quantified using a 1-5 scale with 0.5 gradations with 1 being fresh with incompletely dried wings and 5 being worn with wing loss. Using the YM agar plates, we plated samples using sterile technique from allowing butterfly to walk across plate, washing the thorax, and extending the proboscis. To

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ensure that the butterflies' legs do not touch the plate while extending the proboscis, we held the butterflies so that their body was perpendicular to the agar surface. We counted the microbe colonies that grew after 1-4 days of incubation at room temperature. To make sure colonies did not get recounted, we marked each colony with a permanent marker on the bottom of the plate while counting. We recorded a description of each colony and took photos of colonies of a given description for reference. After the colonies were counted, we stored the plates in the fridge to prevent plate overgrowth.

### **Experiment 2: Butterfly Microbe Dispersal to Different Floral Organs**

We created plates using the same technique as described in Experiment 1. We caught 26 *Speyeria mormonia* and kept them in an autoclaved envelope to starve overnight. We recorded sex and wing wear of each individual. We collected *Pyrrcoma crocea*, a composite flower that *Speyeria mormonia* commonly feeds on. To collect a flower head, we cut the flower stem an inch below the flower head. We collected a yellow bacterium from colonies found during Experiment 1. To isolate this bacterium, we transferred a colony edge onto a new plate. We repeated this process to isolate the yellow bacterium.

The trial arena consisted of a pop-up cage next to a window inside the lab with a wild-cut flower in tube with water to prevent wilting. We allowed the butterfly to feed on the training flower to see if any of the yellow bacterium was on the butterfly or within the flower (Fig. 1). Immediately after feeding, we removed floret that the butterfly fed on using needle-tip forceps. We plated samples from the stigma, anthers, and nectaries of that disk floret. To do this, we streaked the given flower organ in a “#” shape. To plate samples from the nectaries, we used needle-tip forceps to remove the corolla and expose the nectaries.

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We inoculated the donor flower with the isolated yellow bacteria. To inoculate the flower, we dispensed a solution of 8.5% saline and bacterial cells using a micropipette to fill each floret. Usually, this took about 1 microliter per floret. We allowed the flower to dry. We then placed the donor flower in the trial arena and allowed the butterfly to feed. Immediately after feeding, we plated samples from the stigmas, anthers, and nectaries of the donor floret that the butterfly fed on to get an initial microbe concentration. We then replaced the donor flower with recipient flower, formerly the training flower, and allowed the butterfly to feed on the recipient flower. This was done to allow the butterfly to transfer microbes to the recipient flower. Again, we removed and plated samples from the selected floral organs from the floret that the butterfly fed on. After incubating at room temperature for 1-4 days, we counted the number of microbe colonies of the yellow bacteria.

### Data Analysis

We analyzed the data using R v.3.6.1 (2019-07-05) and Systat 13.1. We excluded 1 female out of the 41 trials because this individual was caught on the ground in the mud and one of the only females. Mud puddling is very rare in female *Speyeria mormonia*, so we chose to exclude this individual.

*Experiment 1.-* To determine if there was an unequal dispersal of microbes to butterfly parts, we used a one-way ANOVA using the `aov()` function in R. To determine if there was a linear relationship between total microbe abundance on butterflies and butterfly age using wing wear as a proxy for age, we used a generalized linear mixed model in Systat with date being a random variable. We log-transformed the dependent variable to normalize the residuals. The response variable was the total number of colonies found on the proboscis, thorax, and feet, and the explanatory variable was wing wear. To determine if there were differences between species of

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different sizes, we used a Mann-Whitney U test for nonparametric data using the `wilcox.test()` function in R with *Glaucopsyche lygdamus*, the smaller butterfly species, having fewer total colonies than *Speyeria mormonia*. The data were not normally distributed before or after log-transformation. The Shapiro-Wilk test showed that the data for *Glaucopsyche lygdamus* not normally distributed ( $P = 0.073$ ). After log-transforming the data, the Shapiro-Wilk test showed that neither data set was normally distributed (data from *Glaucopsyche lygdamus*  $P = 0.80$ ; data from *Speyeria mormonia*  $P = 0.97$ ).

*Experiment 2.*-To determine if there was a linear relationship between the abundance of colonies in the donor flower florets and the recipient flower florets, we used a generalized linear mixed model using the `glmer()` function in R. We used a Poisson distribution for count data. The response variable was the number of colonies in the donor floret, the explanatory variable was the number of colonies in the recipient floret, and the random variable was date. To determine if there was an unequal dispersal of microbes to different floral organs, we used a one-way ANOVA assuming a Poisson distribution using the `aov()` function in R.

## Results

### Experiment 1

Butterfly body part did not significantly affect the quantity of microbes present (Fig. 2; ANOVA:  $F_{2,120} = 0.851$ ,  $P = 0.429$ ). Specifically, there were approximately equal microbe abundances present on the butterfly's proboscis, feet, and thorax. This contradicted my hypothesis.

Butterfly age significantly affected the total microbe abundance on the proboscis, thorax, and feet. As butterfly wing wear or age increased, the total microbe abundance also increased (Fig. 3; GLMM:  $F\text{-Ratio}_{3,27} = 4.4$ ,  $P = 0.0058$ ).

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There was a significant difference between species in the number of total microbes present on the proboscis, feet, and thorax (Fig. 3 & Fig. 4; Mann-Whitney U:  $W=83$ ,  $P=0.01496$ ). *Glaucopsyche lygdamus* had significantly fewer microbes present than *Speyeria mormonia*. There were not significant differences between species in the number of total microbes when we normalized the data using approximate body size ( $P=0.66$ ).

### Experiment 2

The donor floret microbe abundance significantly affected the recipient floret microbe abundance on the proboscis, thorax, and feet. As the donor floret microbe abundance increased, the recipient floret microbe abundance increased (Fig. 6; GLMM:  $df=21$ ,  $P<<0.001$ ).

There were significant differences in the quantity of microbes present in the recipient floret between floral organs (Fig. 7; ANOVA:  $F_{2,69}=19.71$ ,  $P<<0.001$ ). Specifically, there were more microbes dispersed to the recipient floret nectaries than to the anthers, which received significantly more microbes than the stigma.

### Discussion

Our results provide direct evidence that butterflies can contribute to differences in microbe abundance among and within flowers through dispersal. Specifically, we found that butterflies foraging on live flowers inoculated the most microbes in the nectaries. Since different floral organs fulfill certain roles in plant reproduction, our results provide evidence that butterfly-flower-microbe interactions could play a role in plant and flower pathogen transmission thus affecting plant reproductive success (Alekkett, Hart, & Shade, 2014; Steven, Huntley, & Zeng, 2018). Butterfly microbe dispersal could also affect plant reproduction through pollinator preference. Since microbes change nectar composition and quality, pollinators may avoid certain flowers due to microbe colony composition (Herrera, García, & Pérez, 2008; Good, Gauthier,

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Vannette, & Fukami, 2014). These changes in pollinator visitation could impact reproductive success. Future work is needed to determine if butterfly foraging behavior such as proboscis probing could impact microbe dispersal rate as well as if there are differences in butterfly dispersal ability between different microbes.

We demonstrate that microbial transmission among and within flowers by generalist butterflies can depend on floret microbial abundance. The nectaries received significantly more microbes than the anthers, which received significantly more microbes than the stigma. Assuming that our results are representative of butterfly-flower-microbe interactions, butterflies could contribute to the maintenance of a core microbiome among floral organs (Albright & Martiny, 2018). Microbial community structure can differ between floral organs and parts (Herrera, Canto, Pozo, & Bazaga, 2010; Junker & Keller, 2015). These differences have previously been attributed to environmental variation (Pozo, Lachance, & Herrera, 2012). These differences have also been attributed to differences in foraging behavior affecting microbe dispersal and acquisition in bees (Russell, Rebolleda-Gómez, Shaible, & Ashman, 2019).

If our results apply across epiphytic microbial species, butterfly-mediated dispersal could affect plant microbial diversity (Allard, Ottesen, Brown, & Micallef, 2018). Given that all butterflies disperse microbes to the nectaries, anthers, and stigmas, our results are consistent with studies that show that pollinator foraging affects microbial communities (Russell, Rebolleda-Gómez, Shaible, & Ashman, 2019). It should be noted, however, that floral organ size may contribute to microbe abundance since the larger organs acquired more microbes. Since flower organ size varies among plant species, patterns of microbial community abundance in nature are likely more complex than this study suggests (Laverly, 1980). Additionally, it should be noted that sugar content in the nectaries could also play a role in microbe abundance in nature. In this

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study, the samples were plated immediately after feeding. In nature, however, nutrient availability could allow differences in microbe abundance between floral organs to compound over time.

Our results also show that butterfly foraging and microbe dispersal could affect the transmission of pathogens and diseases to other pollinators. Many butterflies are generalists and feed on a variety of flowers that other pollinators also frequent (Scott, 1946). Flowers act as vectors of pollinator diseases and pathogens (Graystock, Goulson, & Hughes, 2015). Since butterflies successfully disperse microbes within flowers, these microbes could be transferred to other pollinators. Given that only one flower species was used, future work will be required to characterize the extent to which butterflies disperse microbes among and within different flower species.

There were not significant differences in microbe abundance on different butterfly parts including the proboscis, feet, and thorax. Further studies should be done on which body parts are instrumental in microbe dispersal via foraging. There was a significant difference between species in the number of total microbes present on the proboscis, feet, and thorax. These differences could be attributed to size differences or other characteristic differences between these species. *Glaucopsyche lygdamus* is found to have a wingspan of 2.2 to 3.2 centimeters (Doubleday, 1841). *Speyeria mormonia* has a larger wingspan of 3.8 to 6.1 centimeters (Boisduval, 1869). Further studies are needed to explore species differences in microbe abundance and dispersal ability.

Our results showed that butterfly age significantly affected the total microbe abundance on the proboscis, thorax, and feet. As butterfly wing wear or age increased, the total microbe

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abundance also increased. This finding is important because it shows that butterflies acquire microbes from the environment over time.

Our study lays the foundation for exploring conditions that affect microbe dispersal among flowers and pollinators. By quantifying microbial acquisition and inoculation by butterflies, we show the potential impact that butterflies have on the plant microbial community. Even if butterflies acquire few microbes during each flower visit, butterflies visit many flowers during foraging trips and flowers receive many visits over the season (Williams & Thomson, 1998). Butterfly dispersal ability could also depend on microbe adaptation for dispersal by pollinators, and future work should be conducted to see if such adaptations exist. As awareness increases of the importance of plant-pollinator-microbe interactions and of flower role in microbe transmission, consequences of pollinator behavior extend beyond plant reproduction.

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### Figure Legends

*Figure 1.* Illustrated depiction of methods for Experiment 2.

*Figure 2.* Mean number of microbe colonies ( $\pm$ standard error [SE]) found on *Glaucopsyche lygdamus* and *Speyeria mormonia* body parts including the feet, thorax, and proboscis. N=41 butterflies.

*Figure 3.* Log-transformed mean number of microbe colonies ( $\pm$ standard error [SE]) versus butterfly wing wear as a proxy for age. N=40 butterflies.

*Figure 4.* Total number of microbe colonies on the proboscis, feet, and thorax of different butterfly species. Data are not normally distributed. N=41 butterflies.

*Figure 5.* The log-transformed total number of microbe colonies on the proboscis, feet, and thorax of different butterfly species. Data are not normally distributed. N=41 butterflies.

*Figure 6.* The donor floret microbe abundance versus the recipient floret (microbe abundance on the proboscis, thorax, and feet. The donor floret was the floret that was inoculated with yellow bacteria. The recipient floret was the floret that butterflies dispersed yellow bacteria to. N=26 butterflies.

*Figure 7.* Mean number of microbe colonies ( $\pm$ standard error [SE]) found on different floral organs including the stigma, anthers, and nectaries. N=41 butterflies.

*Figure 8.* Map of beaver pond perimeters in long-term *Euphydryas gillettii* site. Not relevant to the findings in this study.

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Figures

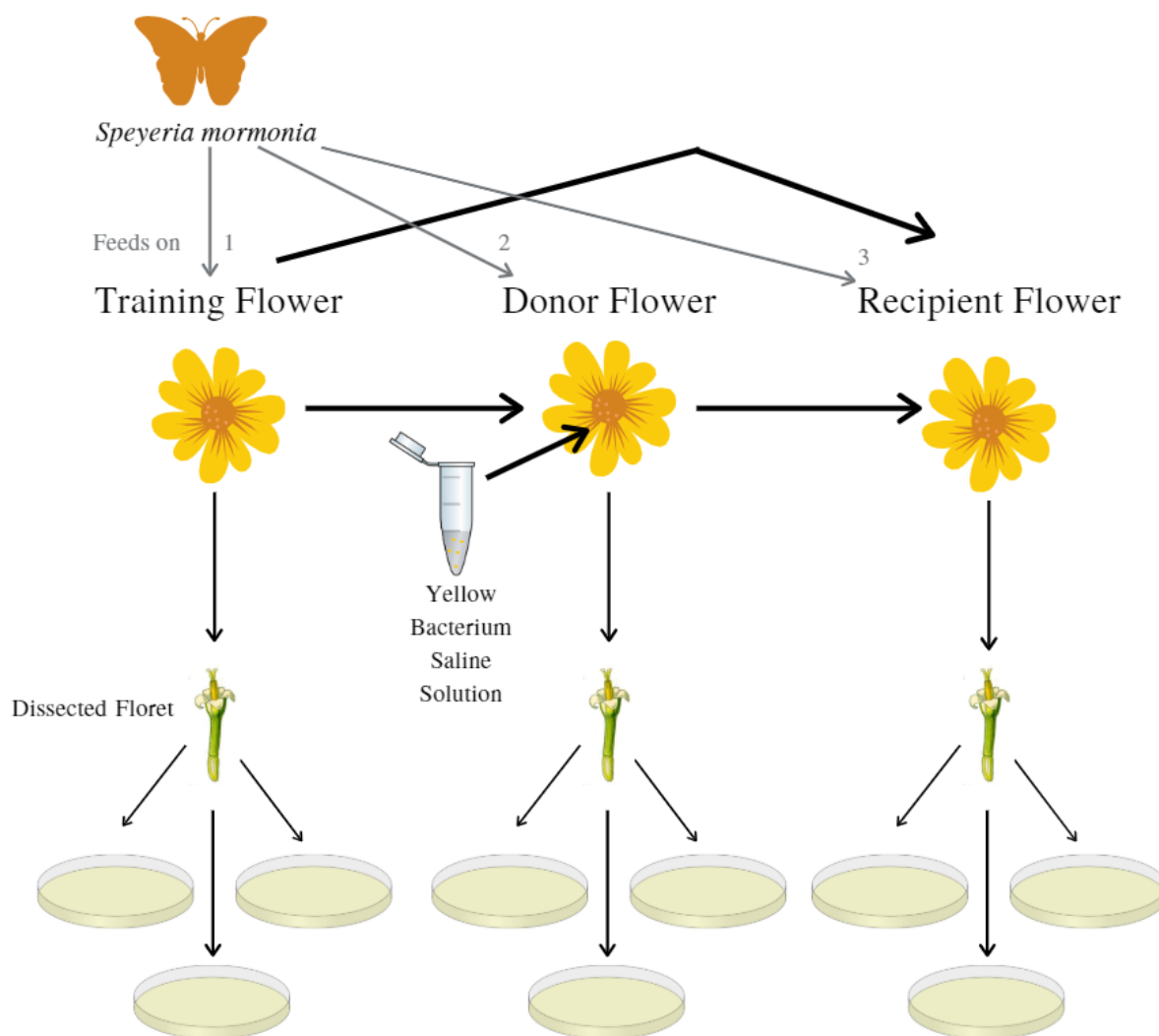
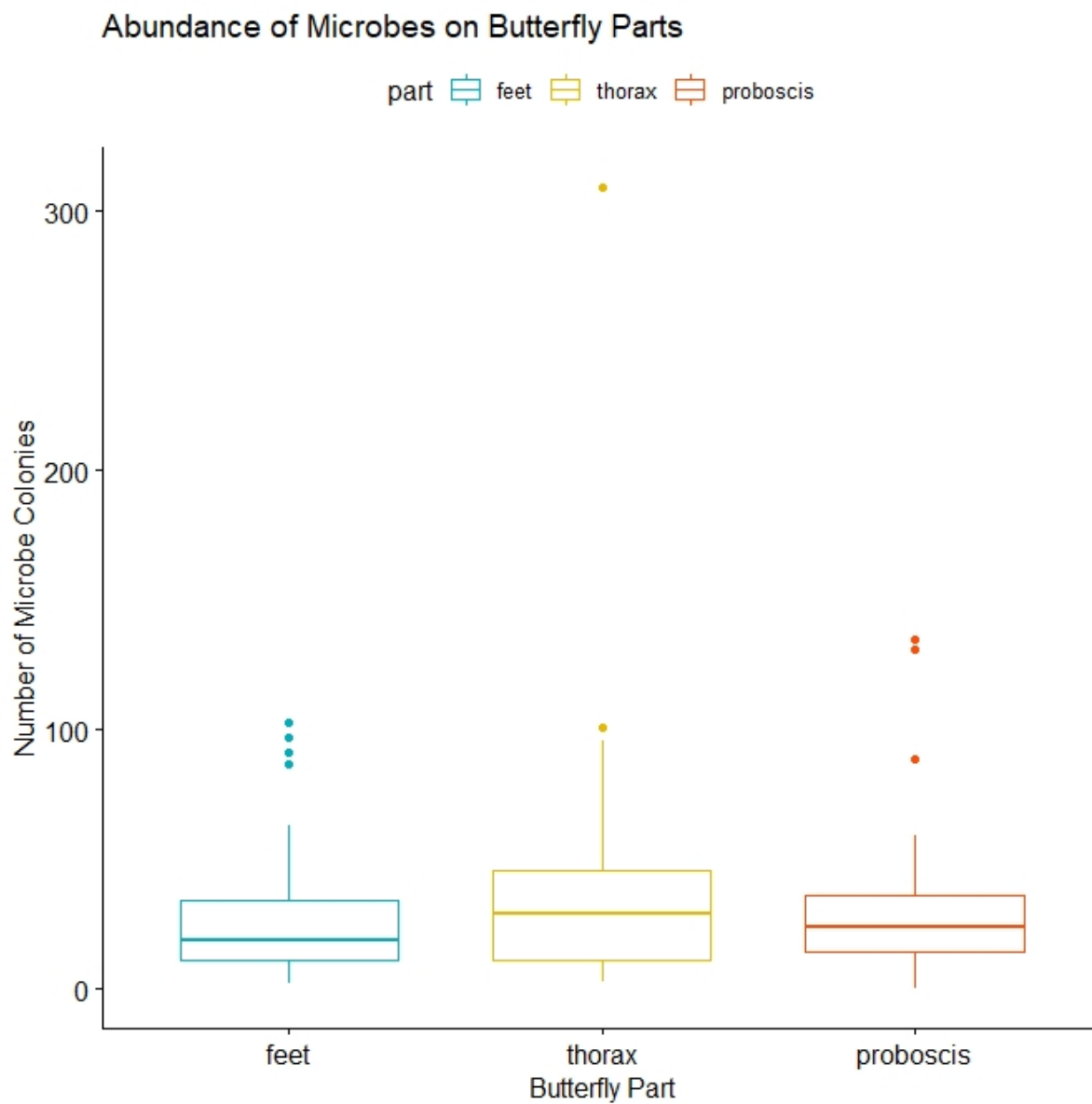


Figure 1

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*Figure 2*

## Least Squares Means

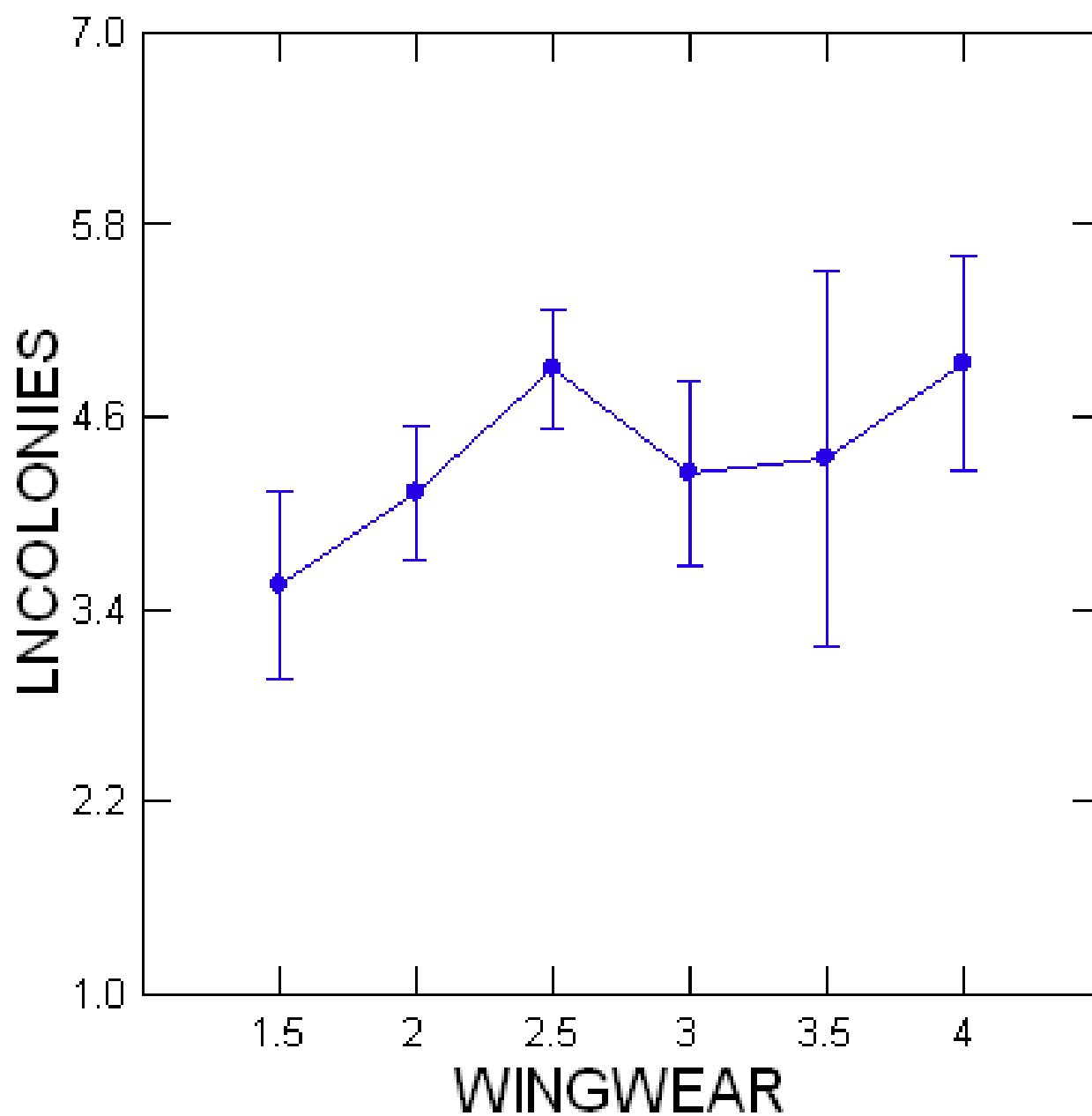
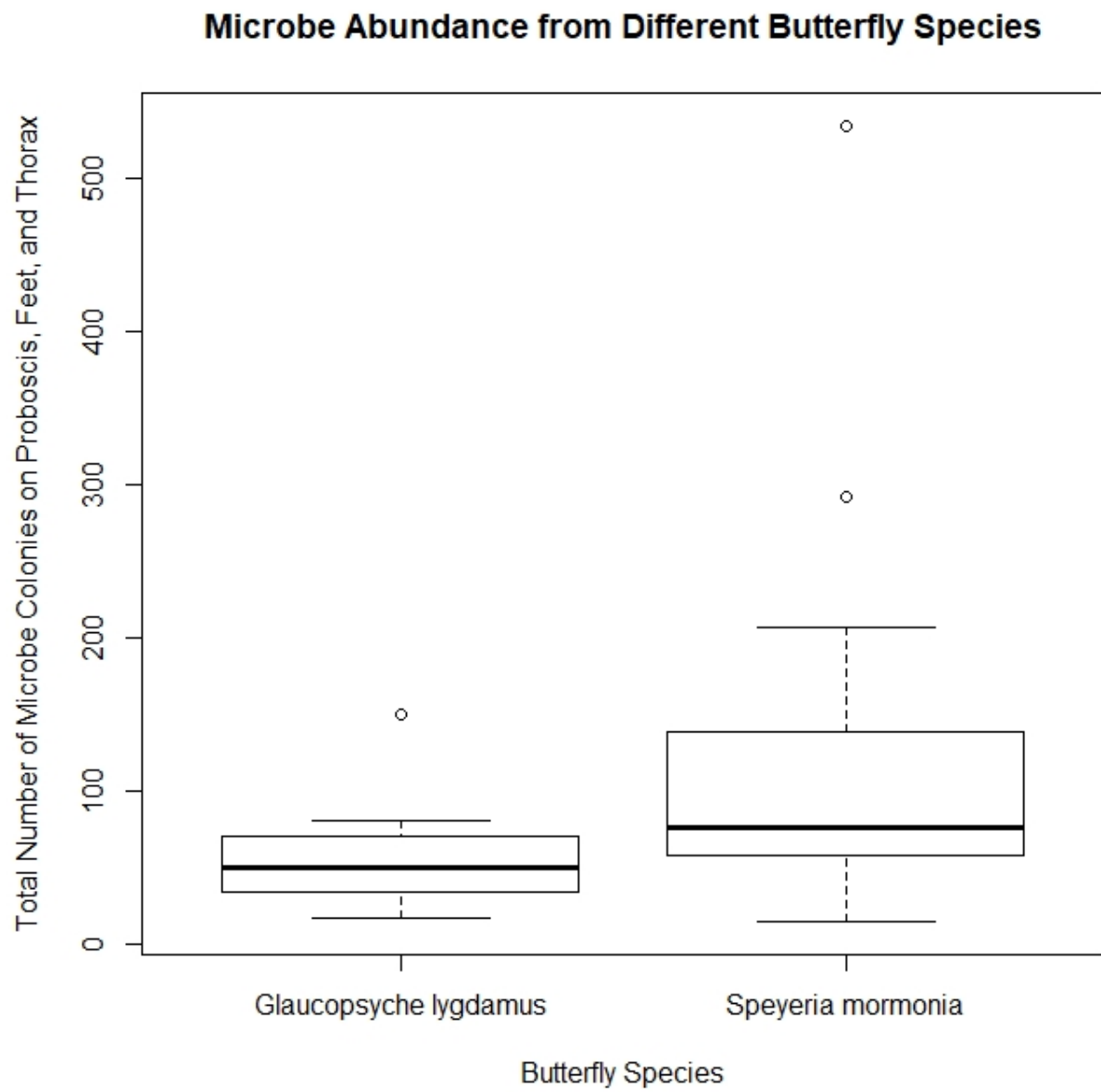
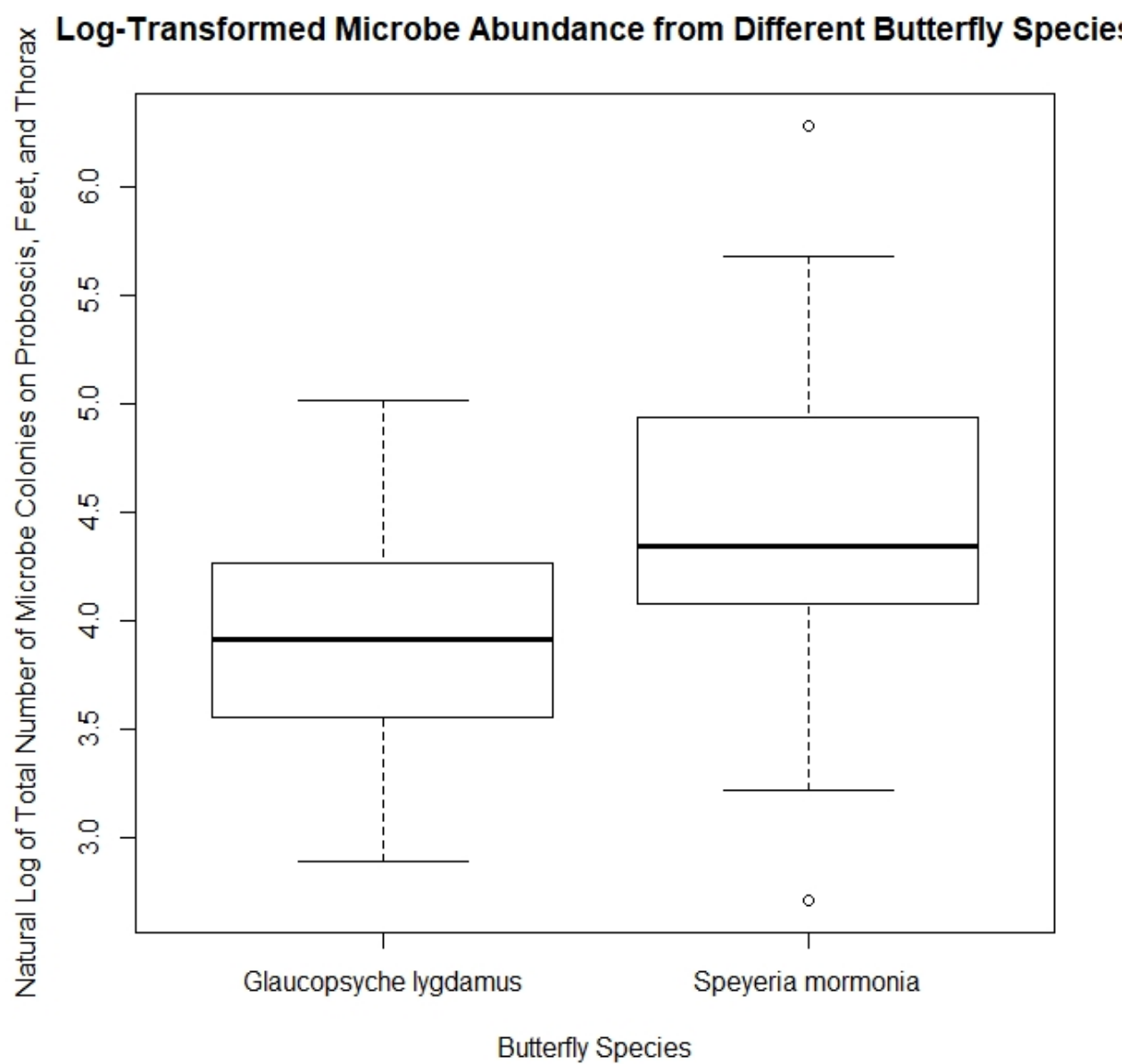
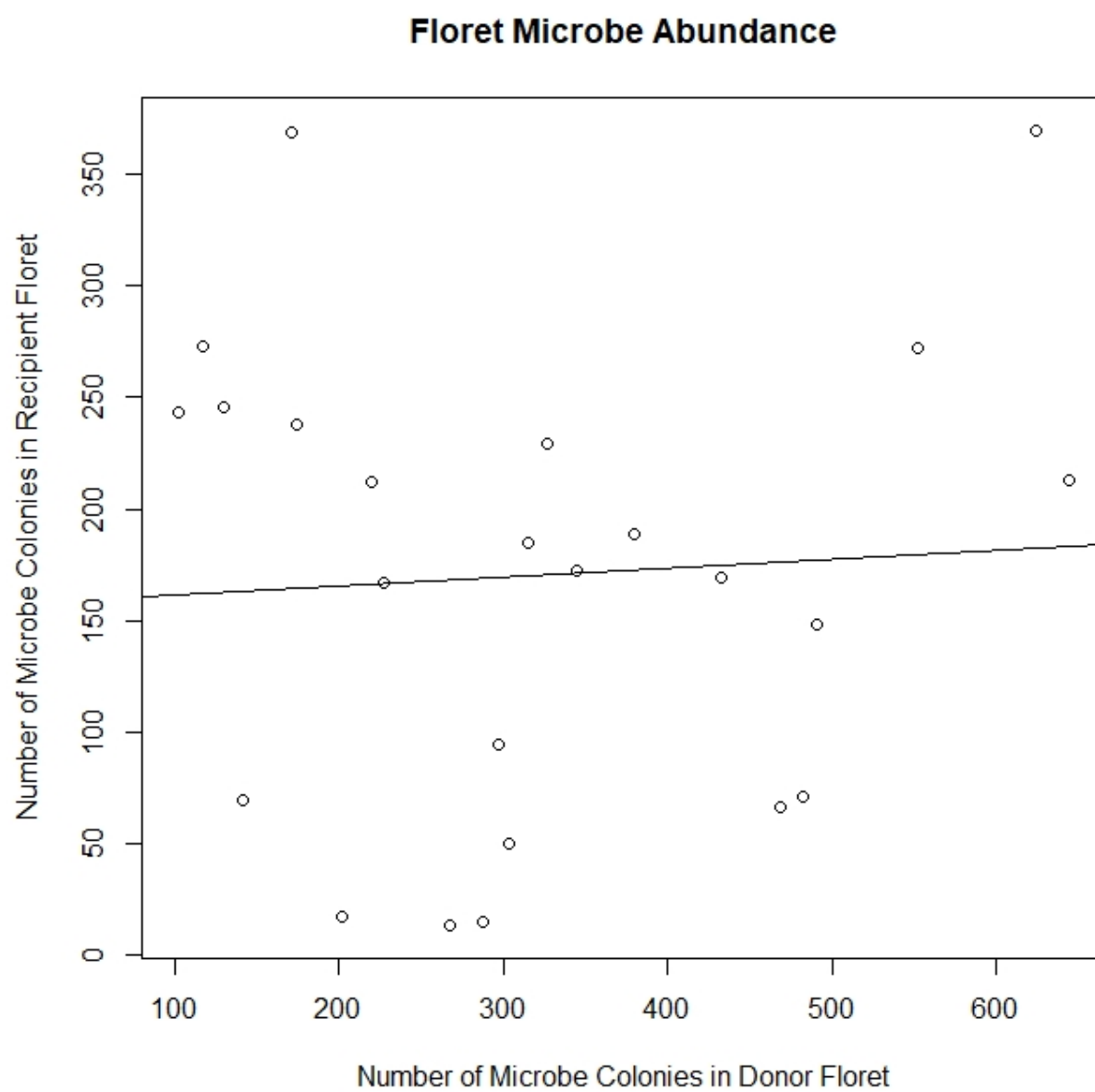


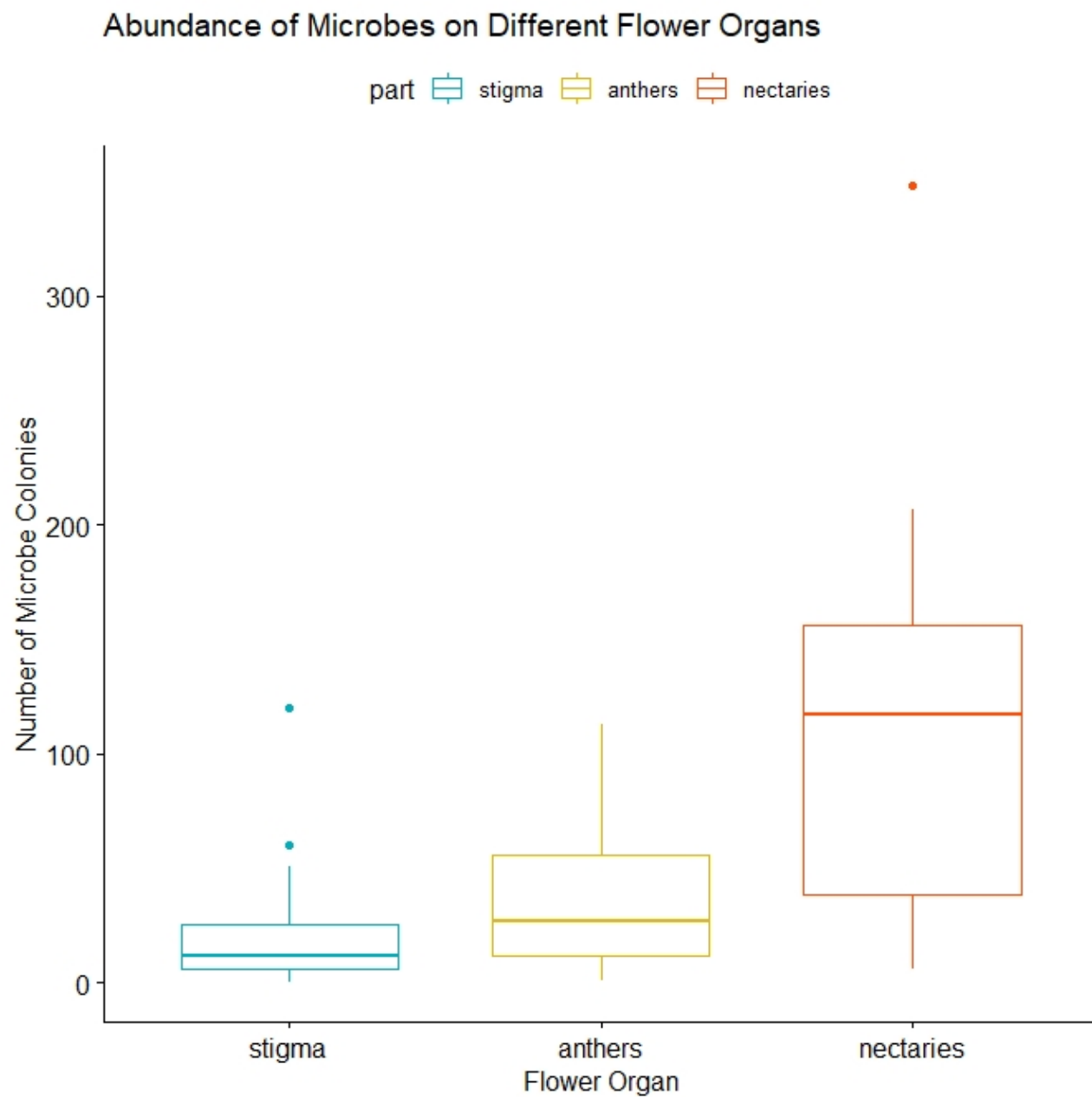
Figure 3

*Figure 4*

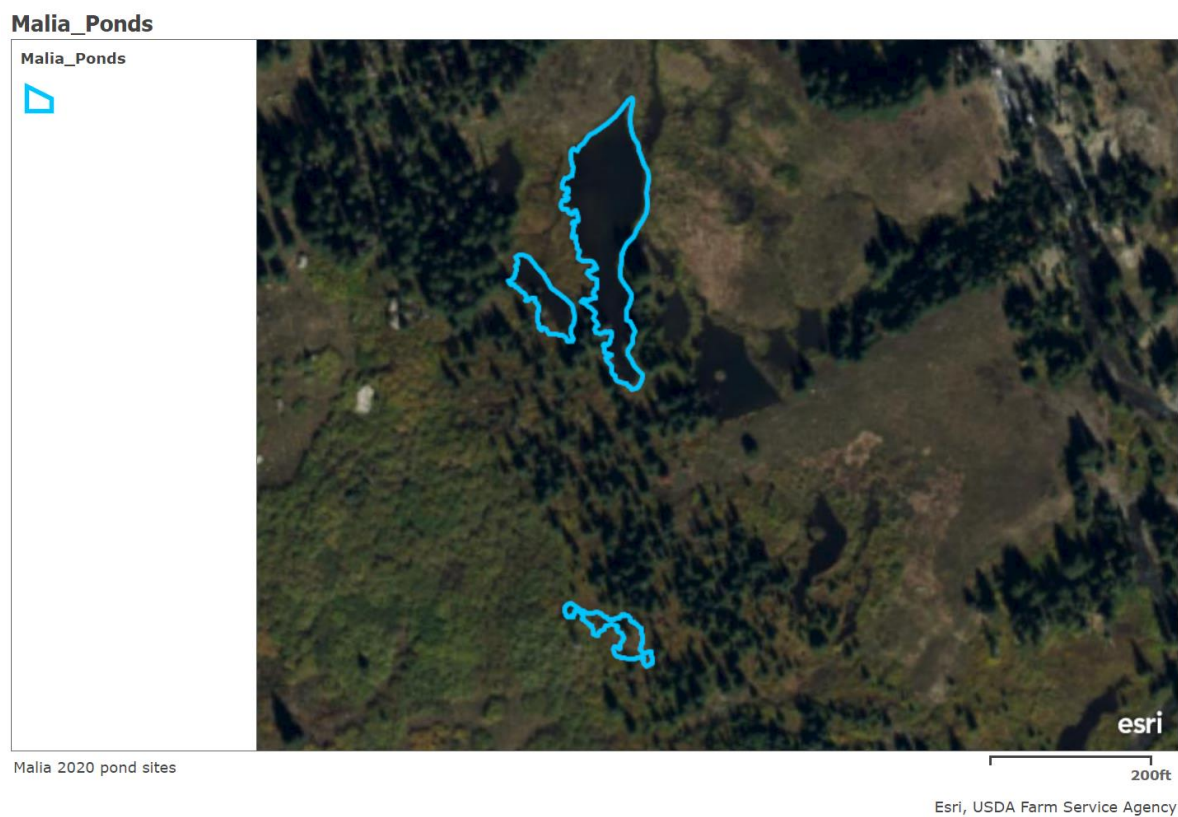
*Figure 5*

*Figure 6*

## BUTTERFLY FORAGING &amp; MICROBE DISPERSAL

*Figure 7*

## BUTTERFLY FORAGING &amp; MICROBE DISPERSAL

*Figure 8*