

Biotic Factors Influencing Feeding Preferences of *Scaptomyza nigrita* (Drosophilidae) on its Host Plant,
Cardamine cordifolia (Brassicaceae)

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Introduction

Most lineages of multicellular life on earth are either flowering plants or insects that feed on them. The interaction between plants and herbivorous insects plays a major role in shaping the functioning of ecological communities. However, interactions between an insect and its host plant do not occur in isolation. Instead, plants must simultaneously cope with a variety of biotic stressors, including insects or microbial pathogens, which may negatively impact the host plant's growth and reproductive success. In order to defend themselves against these diverse biotic stresses, plants have evolved a large range of inducible defense mechanisms towards specific invasive attacks. Due to this large array of natural enemies, plants have specific distinct defense pathways that are triggered for different natural enemy attacks (Koornneef and Pieterse, 2008). Although there are distinct pathways it is difficult to coordinate these defenses from the host's perspective. The consideration of tradeoffs must be taken into account to acknowledge whether they influence preferences. Studying the preferences and performance of herbivores on their host, under the conditions resulting from previous attack by other organisms on the host plant (cross-resistance), is key to understanding the outcome of plant-herbivore interactions in an ecologically-relevant context.

Two distinct plant defense pathways plants trigger due to certain biotic stressors are the jasmonic acid (JA) and salicylic acid (SA) pathways. Although both JA and SA are plant hormonal defense pathways, they differ in important ways, with respect to factors that activate them. JA-mediated defenses are induced by wounding, such as herbivore attack (Snoeren, Broekgaarden, and Dicke, 2011), whereas SA-mediated defenses are induced by the presence of pathogenic bacteria

(Verhage, Wees, and Pieterse, 2010). This can result in plants becoming vulnerable to attack by insects if previously attacked by bacteria, and to attack by bacteria if previously attacked by insects, because induction can last for long period of time. Plants that have recently encountered attack by bacteria are expected to be resistant to future attack by bacterial infections and less resistant to herbivory, many studies have identified the interaction between JA and SA as being antagonistic to each other (Koornneef and Pieterse, 2008). Similarly, plants may be able to defend themselves more effectively from future herbivory if JA is induced from a previous herbivore attack, but will be less resistant to bacterial attack due to downregulation of SA by JA.

Furthermore, plants that grow in the sun and in the shade may exhibit different levels of inducible defenses, due to the trade-offs plants must make as a result of living in a low-light environment in the shade (stress avoidance syndrome, typically means that shade plants are less well defended than plants growing in the sun because they instead must invest in harvesting light). Past studies on differences between light environments, reflect an abundance of herbivores insects in sun habitats than those shade habitats (Collinge and Louda, 1989). Here at Rocky Mountain Biological Laboratory in 1980's experiments were carried on *Scaptomyza nigrita* and it's host plant *Cardamine cordifolia* in relation to sun vs. shade habitats, from this study it was found that plants in the sun habitats were heavily attacked than those in the shade (Collinge and Louda, 1989).

Scaptomyza nigrita (Drosophilidae), an herbivorous, drosophilid fly that specializes on the mustard plant *Cardamine cordifolia* (Brassicaceae) in western North America, has been studied extensively at RMBL by Svata Louda and colleagues (Collinge and Louda 1998). Adult female flies pierce leaves using their ovipositor and feed on the exudates, and their larvae are leafminers. Preliminary experiments conducted by Andy Gloss showed that pre-treating *C. cordifolia* with JA induces resistance to *S. nigrita* larvae suggesting that these flies likely trigger this pathway and that it is necessary for resistance to *S. nigrita* (A. Gloss, Unpublished data). Research has shown that *S. nigrita* activity and damage are higher on *C. cordifolia* located in the sun than those under the shade of

adjacent willows (Collinge and Louda, 1988), although it is not entirely clear whether this is due to thermal ecology of *S. nigrita* and/or improved larval fitness in the sun or the earlier availability of plants in the sun following snowmelt. Plants growing in the shade are known to exhibit the shade-avoidance syndrome, which is due to the ratio of red and far red light perceived by the plants (Moreno et. al. 2009, Agrawal et. Al, 1999). As a result, plants in the shade have longer petioles, longer internode lengths, larger leaves, and are less sensitive to JA. This suggests that plants from the shade, even after induction by JA, will be less well defended from herbivores than plants from the sun (Moreno et. al. 2009, Agrawal et. Al, 1999). Previous research also showed that female *S. nigrita* flies prefer to feed on and lay eggs in plants that were grown in the shade than plants grown in the sun (H. Affeldt, Unpublished data). Separately, it is known that most *C. cordifolia* plants are infected with *Pseudomonas syringae* bacteria, which is Gram-negative inter-cellular pathogen dispersed via the water cycle (Whiteman, Groen, Chevasco, Bear, Beckwith, Gregory, Denoux, Mamarella, Ausubel, Pierce. 2010). Because these bacteria typically induce the SA pathway, they are likely to render plants more susceptible to feeding by *S. nigrita* flies because the JA pathway is antagonized (downregulated) by an induced SA pathway. However, how this antagonism plays out in plants from the sun vs. shade, which are likely to have different sensitivities (although likely not different levels of JA production) to JA, is not known. Importantly, *P. syringae* could also cause plants to be resistant to herbivory, if they possess of the virulence gene coronatine, which is a JA-mimic. Thus, pre-treating with this bacterium could reasonably result in qualitatively different effects on herbivores, depending on bacterial genotype. Also a bioassay looking at the effects of co-infection and herbivory may reflect whether the presence of one affects the plant more than the other. Previous studies have shown that chlorophyll content and fluorescence are either reduced or increased with the presence of herbivory. Herbivory on host plants may indirectly influence its host's photosynthesis negatively (Retuerto, Ferná'ndez-Lema, Obesoy, 2006). No experiment has been conducted on whether differences in herbivore-or pathogen-induced defenses between sun vs shade plants results in different feeding or oviposition preferences in adult

female *S. nigrita*, yet this experiment is important in understanding how natural enemies of *C. cordifolia* interact directly and indirectly. Furthermore, experiments performed to date have not tested if adult female feeding preferences benefits larval performance under differential induction of JA and SA pathways. To test my previous hypotheses of there being variance in preferences of adult feeding in relation to hormone/bacteria pre-treatment to replicate previous damage that the flies may face as a common occurrence. Here, I conducted experiments using adult female *S. nigrita* feeding and oviposition preference assays to determine if they varied their behavior in response to: (1) JA or SA induction, (2) pre-infection with two natural occurring plant-pathogenic bacterial strains and (3) whether these preferences varied in relation to whether the plants came from sun or shade habitats. Finally, I tested for female preference co-variance with larval performance, which is a common prediction in plant-herbivore studies.

Methods

Plant Collection

C. cordifolia ramets used for manipulation experiments were reared from Copper Creek and 401 trail. From each site sun and shade plants were collected for laboratory experiments. With the exception of the 401 trail, only shade plants were collected from the site for use. From Copper Creek, sun and shade plants were collected. Sun and shade plants were defined as shade being those under dense cover of willows, and sun being fully open areas. Fully-grown, relatively undamaged non-flowering *C. cordifolia* were collected replanted in Johnson Lab. A total of 120 shade plants used for treatments, and 48 sun plants were used, resulting in a total of 168 plants. I recorded the amount of previous damage from each plant; plant height, total number of leaves per plant and the number of previous herbivory attack per leaf per plant, prior to conducting the experiments below.

Treatment for differences in preferences toward induction of plant defense hormones (JA and SA):

To test the hypothesis that female fly feeding and oviposition preference behavior varied between JA, SA, and bacteria, and mock treatments, a choice test was used to expose the female *S. nigrita*. The experiments for JA and SA treatment test consisted of having a total of twenty four mesh cages containing four plants each. Out of the twenty-four cages used, twelve were for JA and the other twelve for SA. Plants were placed into cages based on similar plant height (cm). In each cage only two plants were infiltrated with JA or SA, the other two plants were infiltrated with a mock, which acted as the control for the experimental treatment. To designate treatments, plants were labeled one through twenty-four for each treatment JA and SA in the context of sun and shade plants, and I used the analytical program R to randomize within treatments. After being labeled and grouped into cages, leaf number was counted starting from the bottom to the top, the number of individual feeding punctures (stipples) and damage on each leaf was recorded to avoid bias after experiments. Infiltration of either JA, SA, bacteria, or mock for each treatment was carried out at the same time to ensure they experienced the same amount of time exposed to the hormones. The infiltration procedure itself was conducted by using a blunt syringe (Terumo syringe, Terumo Medical Corporation,, Tokyo) to infiltrate the plant with either JA and Mock or SA and Mock, concentration of 10ml. The volume infiltrated remained the same throughout, being 0.3cc/mL. JA solution was produced by dissolving 10ul 1mM MgSO₄ and 41.65ul JA with 9.9ml of water (pure), added with. The SA infiltration was produced from 9.0ml water (pure), 10ul 1mM MgSO₄, 41.65ul MeOH, and 71.4ul SA. The mock mixture was the same as JA, except substituting the JA with 41.65ul MeOH. The first two bottom leaves were the only ones infiltrated and are referred to as the **local** leaves, and the remaining leaves that are on the same plant, but untreated are called **systemic**. Once all plants had been infiltrated they were placed into designated cages under 32W fluorescent lights downstairs in Johnson Laboratory. The plants were left there for a period of 48 hours to be effectively induced, before the introduction of four adult female *S. nigrita* per cage.

S. nigrita were collected from Copper Creek and Emerald Lake for experiments. Flies were

either collected on the same day as placement in cages or a day before and placed into a cage. Only four females were placed into each cage at the same time and left to feed on treated plants for 48 hours, before being removed. Once flies were removed, plants were also removed from cages and placed on trays on the racks above the lights. Stipples were counted on each plant per leaf and recorded the same day the flies were removed. The eggs planted on each plant by the flies were allowed to hatch, and the larvae were allowed to feed for an entire week from when the female flies were removed. After the week, the larvae from each plant were removed from leaves and weighted to test for differences in performance (growth rate, a measure of fitness). Also leaves with mines were photographed to determine leaf area mined, which is a good proxy for herbivore performance (growth rates) in *Scaptomyza* flies (Whiteman et al. 2011). The same procedure was repeated for sun plants, with the exception of only six total cages of JA (JA and mock) and six cages of SA (SA and mock).

Treatments Bacteria strains 8 and 12:

To test whether a pre-infection influences feeding, two isolates of *Pseudomonas* bacteria isolated by other members of the Whiteman lab from *C. cordifolia* leaves were used to determine if pre-infection influenced fly feeding/oviposition behavior, before increasing the sample sizes for trials with each strain. The two strains that were used are called strains 8 and 12. In plants, strain 12 was more virulent than strain 8 (Ortiz and Humphrey, unpublished data). Similar to the JA and SA treatments, two basal leaves of each plant were infiltrated with a blunt syringe containing either strain 8 or 12, at OD 600 of 0.002 and mocks were buffered water as above. A total of eighteen cages were used between the two strains; plants in nine cages were treated with strain 8 or mock, and nine cages were strain 12 or mock. Instead of allowing a period of 48 hours before placing flies in the cages with the plants, flies were placed after 96 hours to allow the bacteria to infect and induce the plant. Chlorophyll content and fluorescence were measured for each plant before flies were added and after having removed the flies to determine how each bacterial strain influenced leaf photosynthetic ability and how

the interaction between bacterial infection and herbivory later influenced photosynthetic ability. To measure chlorophyll content a SPAD meter was used (SPAD-502, Konica Monolta, Japan) the two local leaves (infiltrated) were measured and two systemic leaves (uninfiltrated) above the locals were measured. Three points were taken on each leaf, than averaged. The same procedure was conducted for chlorophyll fluorescence measurement (FluorPen, Photon System Instruments, Czech Republic); plants were placed in a dark environment for at least three hours before taking measurements

Results

JA and SA Treatment:

The number of stipples (feeding punctures) on local shade JA leaves (infiltrated) varied significantly from those on local mock leaves ($p\text{-value} < 0.04408$). Adult female *S. nigrita* favored plants not infiltrated with JA (Figure 1A). The opposite was shown when flies were placed into a cage containing pre-infiltrated SA and Mock. The females favored the pre-infiltrated SA leaves than the mock (Figure 1B). Sun plants infiltrated with JA and mock, showed a local and systemic response to avoidance of JA induced plants (Figure 1C: $p\text{-value}(\text{local}) < 0.043042228$ and $p\text{-value}(\text{systemic}) < 0.047751801$).

Chlorophyll Content and Fluorescence (Fo):

Chlorophyll content and fluorescence varied significantly before and after adding adult female *S. nigrita*. The amount of chlorophyll content between leaves infiltrated with either bacteria strains 8 or 12 and mock all decreased after herbivory by the flies. Bacteria strain 12 had the greatest reduction between bacteria 8 and no bacteria leaves (Figure 2). Overall, there was significant difference in chlorophyll content between plants without herbivory and those with herbivory (F ratio=12.9081 and $P < 0.0001$). The opposite pattern was seen in minimum chlorophyll fluorescence (F_o), leaves infiltrated with either no bacteria or bacteria 8 increased, where as strain 12 decreased dramatically (Figure 3). There was significance between non-herbivory and herbivory on fluorescence content present after the removal of flies (F ratio=28.1538 and $P < 0.0001$).

Bacteria Treatments:

The adult female flies preferred plants infiltrated with bacteria strain than mock. Between the two strains of bacteria infiltrated into shade plants, there was a preference toward strain 8 more than strain 12 (Figure 5) although these differences were not significant statistically.

Discussion:

My data support the conclusion that pre-induction with either plant defense pathway precursors (SA or JA) or natural bacterial pathogens, influences feeding choices made by herbivores. In this case, previous research showed that pre-treatment with JA reduced larval growth in planta. I also found that this effect (for JA in particular) varied in relation to light environment. After induction of the JA pathway, *S. nigrita* strongly preferred to feed on plants that had not been infiltrated with JA (mock) relative to those that had been treated with JA. However, whereas I found avoidance of local (treated) leaves in shade plants, I found local and systemic avoidance in sun plants. This is consistent with the hypothesis that plants in the shade are less sensitive to JA than plants in the sun (Moreno et al. 2009). On the other hand, there was a trend that plants infiltrated with SA were more susceptible to *S. nigrita* feeding. This is consistent with the hypothesis that SA downregulates JA, thereby reducing the ability of the plant to produce anti-herbivore compounds. Bacterial strains acted as a model of co-infection, reflecting that females preferred infected bacterial plants than the control. Between the two strains, there was a stronger preference towards strain 8 than 12. These data are consistent with the general idea that bacterial pathogens often induce the SA pathway, but we did not measure this in planta, so this is speculative. Also from the bioassay on chlorophyll, the presence of co-infection and herbivory had a negative affect on the plants; chlorophyll decreased and fluorescence increased for strain 8 plants and decreased for strain 12. This shows that these bacteria are likely to actually negatively affect fitness of the plants.

Biotic factors do affect feeding preferences of adult female *S. nigrita* on their host plant *C. cordifolia*. Three plant defense elicitors were introduced (JA, SA, bacteria) to replicate previous attack

and in order to create an induced defense response by the plants. From this project we are able to learn that plants induced under the JA pathway are less threatened by herbivores, than plants induced with SA. *S. nigrita* has a preference towards attacking plants that are induced by SA and had an avoidance toward JA induced plants. Future experiments should (1) expand the sun vs. shade treatments to the bacterial assays, (2) these experiments should be conducted in the field and (3) plant hormone levels should be measured after bacterial infiltration.

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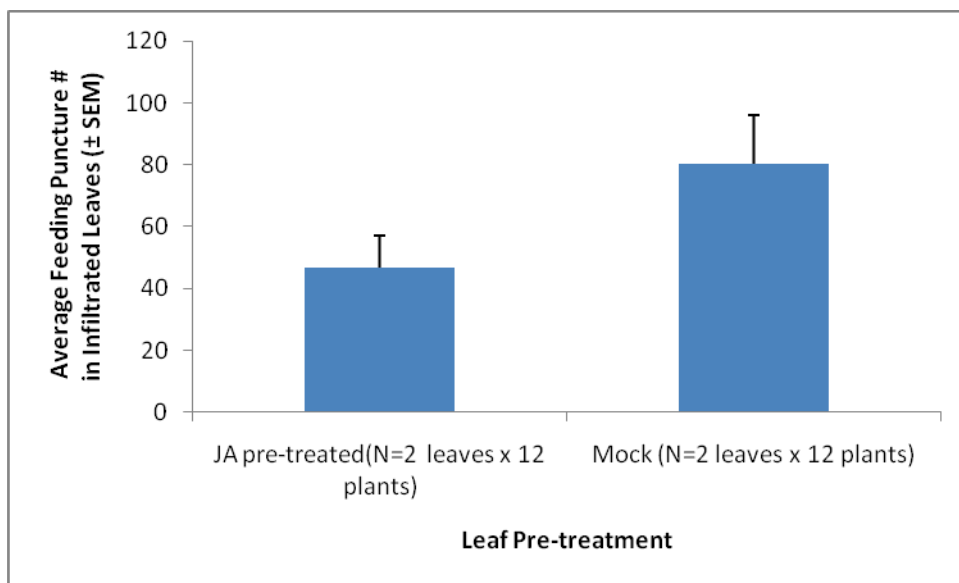


Figure 1A: Average Feeding Stipples of Infiltrated Leaves with JA/Mock, p -value < 0.04408. Shade plants collected from 401 trail. Infiltrated with a concentration of 10ml, using a blunt syringe at 0.3cc/ml

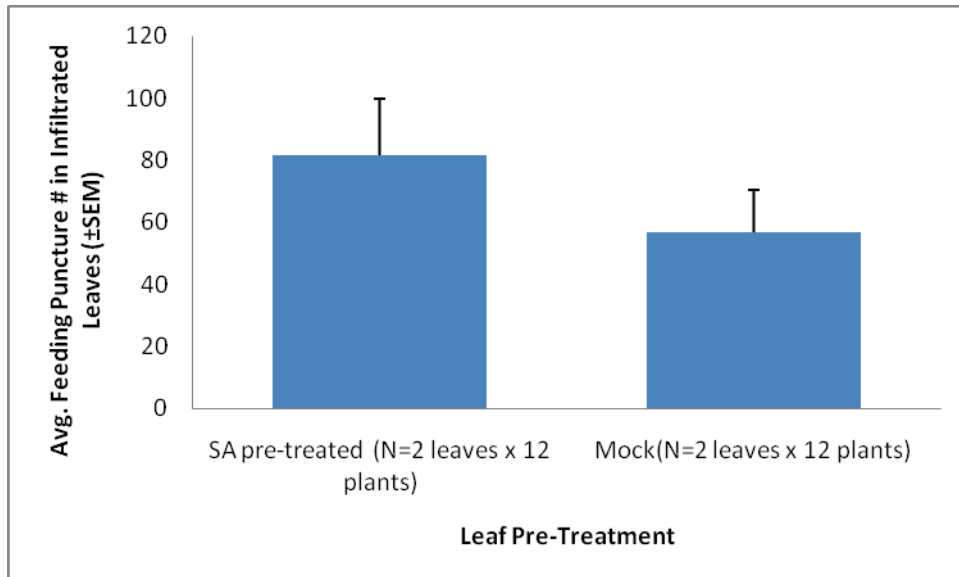


Figure 1B: Average Feeding Stipples of Infiltrated Leaves with SA/Mock. Shade plants collected from 401 trail. Infiltrated with a concentration of 10ml, using a blunt syringe at 0.3cc/ml.

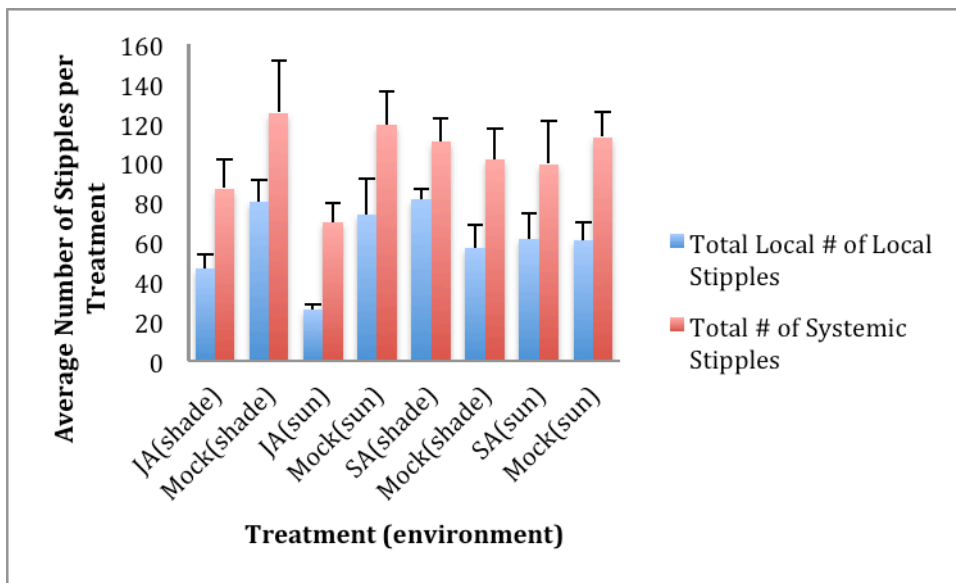


Figure 1C: Average number of stipples per treatment and local and systemic level of JA/SA/mock.(p-value(local)< 0.043042228 and p-value(systemic) < 0.047751801). Plants collected from Copper Creek and 401 trail, bith sun and shade habitats.

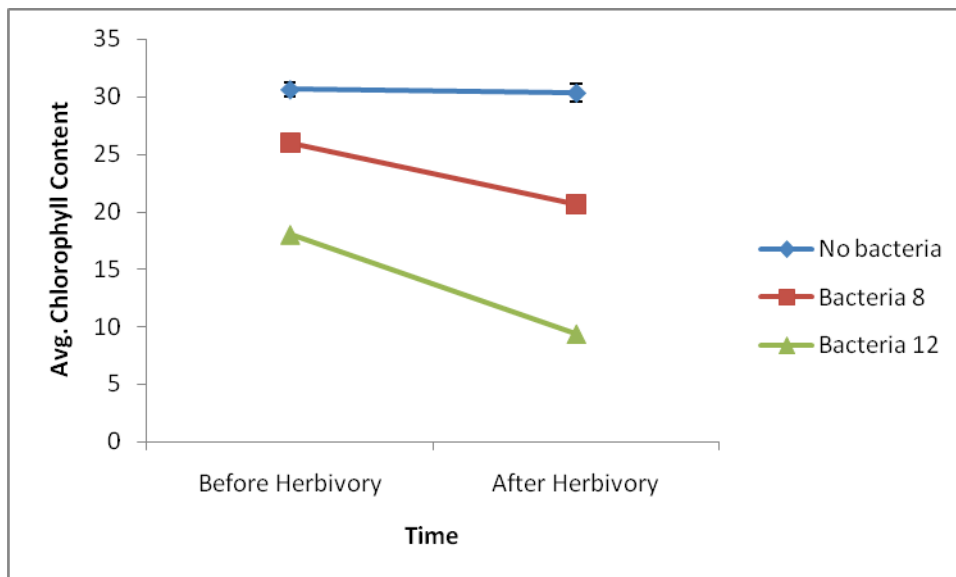


Figure 2:Average Chlorophyll Content over time, F ratio=12.9081 and P<0.0001. Chlorophyll content measured for shade bacteria strain plants (8 &12). Plants infiltrated with bacteria at 0.3cc/ml with a

blunt syringe. Measured after 96 hours of being infected, before placing four adult female *S. nigrita* flies in cages. After 48 hours flies were removed and content was measures again.

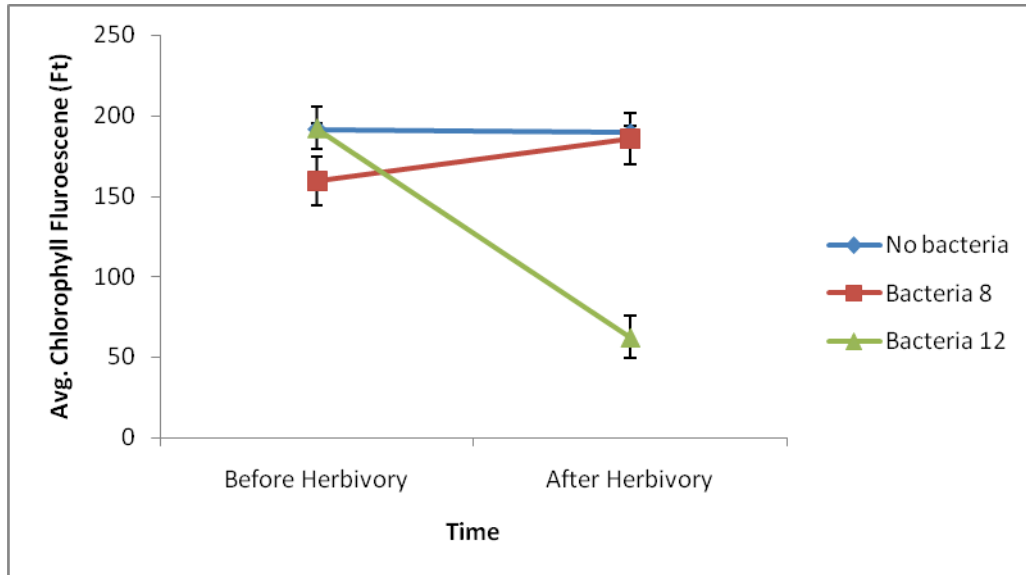


Figure 3: Average Fluorescence (Ft) over time, F ratio=28.1538 and $P < 0.0001$. Chlorophyll fluorescence measured for shade bacteria strain plants (8 & 12). Plants infiltrated with bacteria at 0.3cc/ml with a blunt syringe. Measured after 96 hours of being infected, before placing four adult female *S. nigrita* flies in cages. After 48 hours flies were removed and content was measured again.

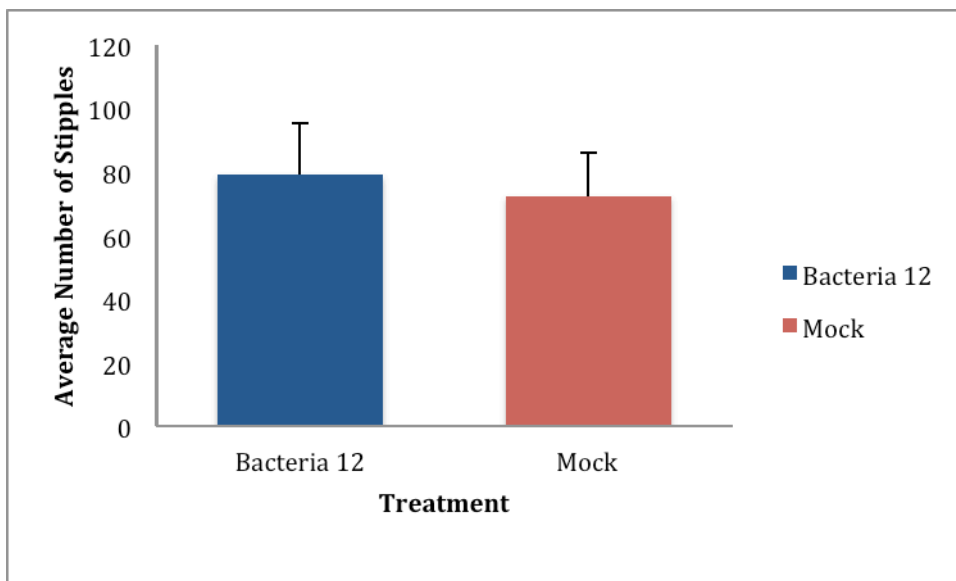


Figure 4: Average number of stipules of Bacteria 12 and mock infiltrated plants after herbivory by

female flies.

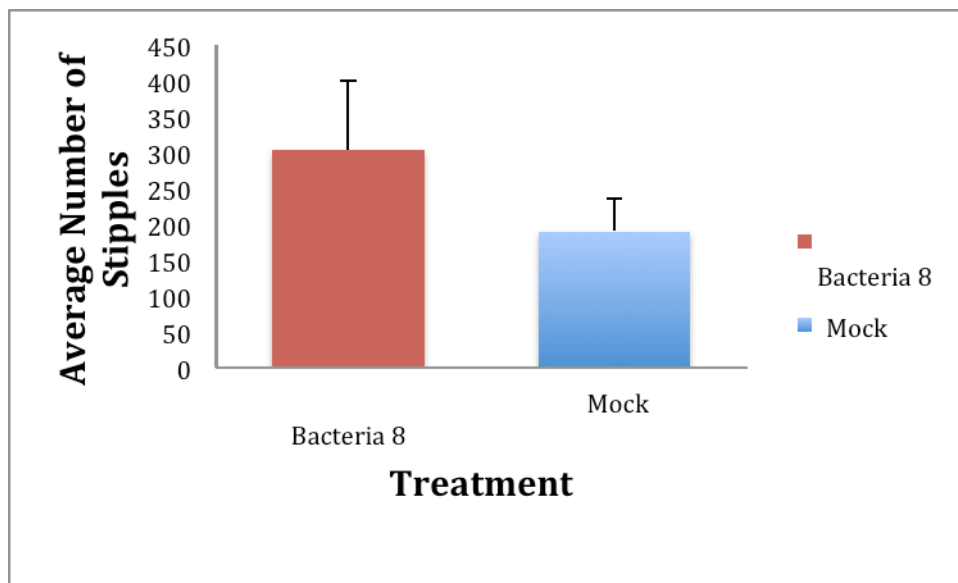


Figure 5: Average number of stipules of Bacteria 8 and mock infiltrated plants after herbivory by female flies.