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Emmalee Schlager April 8, 2019

Effect of the Removal of Microbial Communities by Antibiotics on *Callosobruchus macultus* During Various Life Cycle Stages

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Abstract

Effect of the Removal of Microbial Communities by Antibiotics on *Callosobruchus macultus* During Various Life Cycle Stages

By Emmalee Schlager

Commensal microbial organisms have a profound impact on their hosts. While the host supplies an environment for the microorganisms to live, the microbes also interact with their host, often assisting them in various ways. Many microbes provide essential functions for their host organisms. One of the ways microbial communities benefit their hosts is by contributing to the nutrition. *Callosobruchus maculatus* (the bean beetle) is a species of beetle whose larval life cycle occurs within stored legumes. They lack the genes to produce cellulase, which is essential for cellulose digestion. Therefore, bean beetles are likely assisted by their gut microbial community in the digestion of the plant material they consume. In order to test the impact of the microbial communities on the fitness of *C. maculatus,* antibiotics were added to their environment at various life cycle stages. Extended exposure to antibiotics was hypothesized to alter the gut microbial communities, ultimately having a negative impact on the beetles' fitness. I found that exposure to antibiotics during early development resulted in fewer beetles emerging from beans. Similarly, treatment of artificial beans with antibiotics throughout the bean showed that continued exposure to antibiotics during the beetles' larval stages prevented successful emergence. Furthermore, adult beetles with access to liquid antibiotic solutions had significantly lower densities of gut bacteria, but no impact on their overall lifespan was observed. Overall, the presence of antibiotics was shown to have a negative impact on the fitness of the beetles, particularly during larval development.

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Introduction

A microbial community is a community of microorganisms that live in a particular environment, such as the body of another organism. A single organism can host multiple different microbial communities, such as in their gut, in their mouths, or on their skin. Gut microbiota are the microbial community living within the gut of another organism. These communities begin developing from birth and continue to form as the host matures (e.g., Koenig et al., 2011). The microorganisms interact with their host, often benefitting the host in various ways, while the host supplies the environment for the microbial communities to live. Ultimately, a combination of where in the host the microbial community exists and the environment it encounters determines the structure and function of the microbial community (e.g., Bahrndorff et al., 2016).

While animals naturally host microbial communities, the types of bacteria found varies between species and even among members of the same species (Bahrndorff et al., 2016). Gut microbial communities are influenced by factors such as the host's diet, the host's environment, and developmental processes of the host (Koenig et al., 2011). For example, the microbial community associated with insects' changes with developmental stage. Many of the gut microbiota in herbivorous insects are inherited maternally (Hansen and Moran, 2014). Therefore, the initial structure of the insect's gut microbiota is derived predominantly from their mother's gut microbiome. Yet, after an organism is born, its gut microbial community continues to develop in response to the environment. For example, in an experiment on the gut microbiome in *Leptocorisa chinensis,* it was discovered that *Burkholderia* symbionts found in the gut microbiome of the alydid bugs matched isolates obtained from the soil environment, suggesting horizontal transfer of the bacteria occurred from the environment (Kikuchi et al., 2005). Furthermore, gut microbial communities also have been found to be influenced by the diet of

their host. In a study on *Lymantria dispar*, the makeup of the caterpillars gut microbial community was highly dependent on their diet (Broderick et al., 2004). For example, caterpillars that fed on larch (*Larix* sp.) had the highest diversity of gut bacteria, while the highest proportion of uncultivated bacteria was found in the guts of caterpillars that fed on aspen (*Populus* sp.) Furthermore, the caterpillars that fed on white oak (*Quercas alba*) had the lowest diversity of gut bacteria (Broderick et al., 2004).

Due to the wide range of environments in which insects can be found, many insects are reliant on their microbial communities to assist them in their survival. Generally, insect microbial communities are smaller and more specialized than those found in mammalian guts (Engel and Moran, 2013). In addition, insects with more complicated digestive tracts have more complicated microbial communities than insects with simpler digestive tracts (Lehman et al., 2009). The roles of gut bacteria in insects are diverse and are known to contribute to nutrition, protection from pathogens and parasites, communication, and even modulation of immune responses (Engel and Moran, 2013). For example, in *Drosophila neotestacea*, the gut bacterium *Spiroplasma* helps protect the host from a parasitic nematode, *Howardula aoronymphium* (Jaenike et al., 2010). Similarly, in *Anopheles stephensi*, Gram-negative bacteria inhibit oocyst formation of *Plasmodium falciparum,* allowing the mosquito to carry the pathogen without being susceptible to it (Pumpuni et al.,1993). Furthermore, as the mosquito's midgut bacteria encounters *Plasmodium* sp., and senses the infected blood constituents, the mosquito's immune system is able to induce anti-plasmodium immune responses (Dong et al., 2006). While the gut microbial community can help to fight off parasites and pathogens for its host, it can also provide communication for its host. In locusts (*Schistocerca gregaria*), the gut microbiota has been found to help them communicate with other locusts by producing pheromones (Dillon et al., 2002).

Since insects inhabit a vast range of ecological niches, including surviving on nutrient-poor diets, their microbial symbionts often assist them in processing the nutrients (Engel and Moran, 2013). Many herbivorous insects, such as *Cryptocercus sp.,* are unable to digest compounds such as cellulose on their own; however, a symbiotic interaction with their gut microbiota breaks down cellulose for the host to consume (Varma et al., 1994). Similarly, the leaf beetle, *Cassids rubiginosa,* is unable to digest pectin on its own, and as a result, the beetle is dependent on its microbial community for pectinase production (Salem et al., 2017). In other insects, the gut microbial community plays a specialized role in nutrition. For example, the pea aphid, *Acyrthosiphon pisum,* has formed a symbiotic relationship with the bacteria *Buchnera aphidicola*, which provides them with amino acids (Wilson et al., 2010).

The bean beetle, *Callosobruchus maculatus,* is a widespread insect found on all continents with the exception of Antarctica. The beetle is thought to have originated from West Africa, but through trade became an invasive pest around the world (Tran and Credland, 1995). The bean beetle inhabits a variety of stored legumes, including mung beans, (*Vigna radiata*), adzuki beans (*Vigna angularis*), and black-eyed peas (*Vigna unguiculata*). The adult female beetle will lay its eggs on the outside of the legumes, and from the eggs, the larvae develops inside the legume, emerging as a fully formed adult. All feeding is done when the beetle is in its larval stage, and once the mature adult emerges, the beetles' mate and lay their eggs (Fox, 1993). The adult beetle still possesses a fully formed digestive system and even some leftover plant material in its gut when it emerges, as evidenced by chloroplast sequences in MiSeq analysis of microbial communities (Gerardo lab, unpublished data). When given access, the adult beetle will consume sugar water that will increase its lifespan (Fox, 1993).

Although bean beetles acquire all of their nutrition from the endosperm of beans during their larval stage, they do not possess the genes required for digesting cellulose at any stage of

development (Pauchet et al., 2010). As a result, gut microbial communities are likely essential to the digestion of cellulose in bean beetles. However, little is known about the gut microbial community in bean beetles and its function.

Preliminary research on the microbial community in bean beetles using culture-based approaches have identified several key genera of bacteria. In an experiment to determine the types of bacterial communities in stored product pests, Sevim et al. (2015) discovered that of the eleven isolates found in bean beetles, the bacterial flora included *Bacillus primus, Staphylococcus* sp. and *Pantoea* sp. When using culture-based methods, the most common genera of bacteria were found to be *Enterobacter, Enterococcus*, and *Staphylococcus* (https://www.beanbeetles.org/microbiome/).

Recent research using next-generation sequencing techniques have found 11 genera of bacteria in bean beetles (Gerardo lab, unpublished data). Of these 11 genera, the core bacteria in the community are Staphylococcus and Enterobacter (Gerardo lab, unpublished data). While Staphylococcus and Enterobacter are the most common genera, many strains of each genus are found within *C. maculatus.* Depending on the type of bean from which the beetle emerges, the main genus can fluctuate from either Enterobacter or Staphylococcus (Gerardo lab, unpublished data). For beetles who emerge from black-eyed peas, the most abundant genus is Staphylococcus, with its abundance ranging from 70-80% of the beetles' gut microbiome (Gerardo lab, unpublished data). Furthermore, a cellulase sequenced in the metagenome is derived from a Staphylococcus strain (Gerardo lab, unpublished data). Since the beetles themselves do not possess the ability to digest cellulose (Pauchet et al., 2010), yet a significant portion of their gut microbiome does, these findings help support the hypothesis that the bean beetles' gut microbiome aids in digestion.

To determine the impact that microbial communities have on their host, antibiotics are used to alter the microbial community. For example, the inclusion of antibiotics in the food of omnivorous beetles, *Harpalus pensylvanicus,* successfully altered the gut microbial communities in this beetle (Lundgren and Lehman, 2010). Altering bacterial communities has different effects on different hosts. Depending on which bacterial taxa are affected by the antibiotics, some hosts can survive asymptomatically while others experience significant symptoms as a result of their gut microbiome has being altered (Lundgren and Lehman, 2010). For instance, the absence of gut microbiota increases the susceptibility of mosquitoes to plasmodium and other pathogens to which they are often exposed (Dong et al., 2009). In bean beetles, some of their bacterial symbionts have been shown to be susceptible to the antibiotic's rifampicin, streptomycin, and tetracycline among others (Sevim et al., 2015). Therefore, these antibiotics could be used to alter the makeup of the beetles' gut microbial communities.

The purpose of my experiments was to determine the fitness impact of an altered microbial community on bean beetles at different life cycle stages. To examine the impact of removing the microbiome of *Callosobruchus maculatus* on their fitness, I examined the impact of the antibiotics on the beetles at various stages of their life: when they emerge from their egg, as they develop in the bean, and after they have emerged as adults (Figure 1). To test the effect

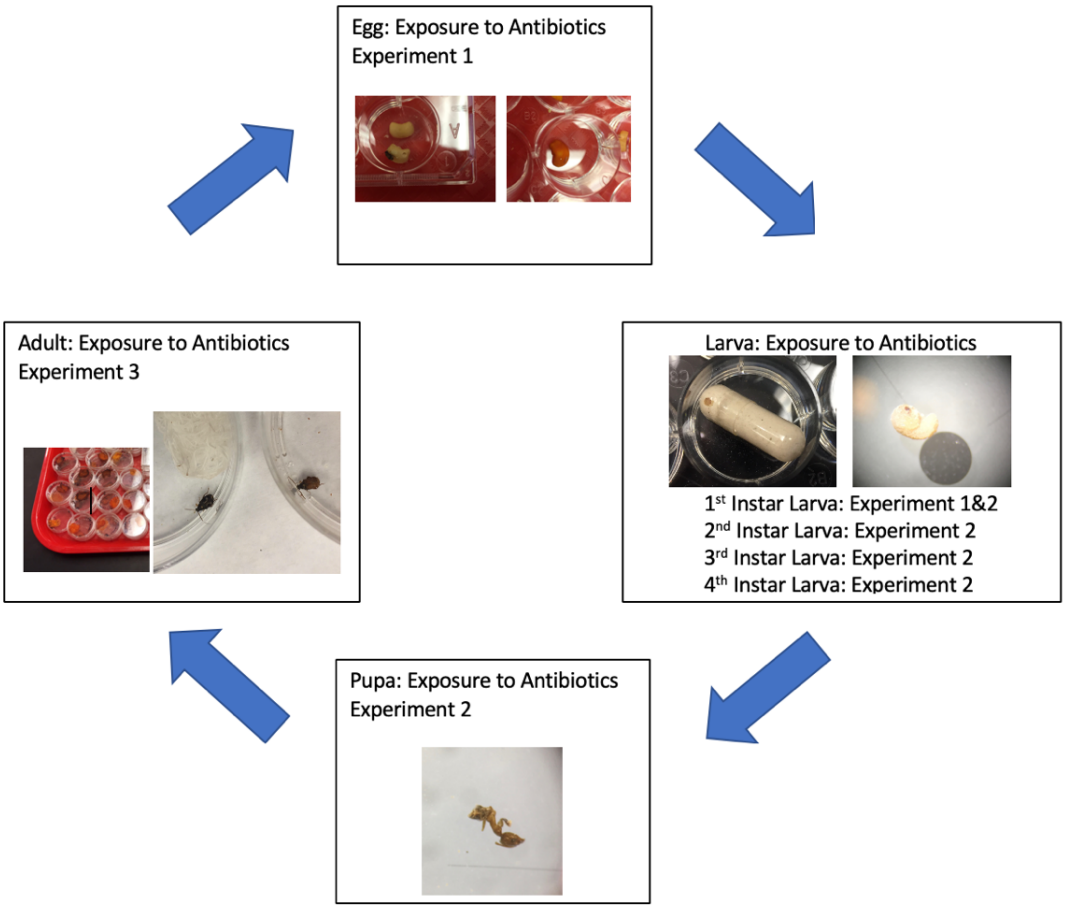

Figure 1: Life cycle of *Callosobruchus maculatus.* Experiments tested antibiotic exposure to beetles within the various life cycle stages.

of antibiotics on the beetles at each of these life cycle stages, I used three different experimental approaches. My first approach was to examine the development of the beetles during their embryonic and first instar larval stage by testing them in normal beans with artificial antibiotic seed coats. My second approach was to measure larval developmental of the beetles as they developed in artificial beans with antibiotics throughout. Finally, my third approach was to examine the lifespan of adult beetles by giving them access to liquid solutions of antibiotics immediately after their emergence. For each experiment, the beetles were exposed to a control

with no antibiotics, 0.3% rifampicin, 0.15% rifampicin, antibiotic cocktail containing 0.3% rifampicin, 0.075% tetracycline and 0.15% streptomycin, or an antibiotic cocktail containing 0.15% rifampicin, 0.075% tetracycline and 0.15% streptomycin**.**

Rifampicin, streptomycin, and tetracycline are all antibiotics that target Gram-positive bacteria, such as *Enterococcus* and *Staphylococcus* (Sevim et al., 2016). Since the majority of the beetles' microbial community is made up of either *Enterococcus* or *Staphylococcus*, targeting these types of bacteria should significantly alter the make-up of the gut microbial community. Furthermore, since *Staphylococcus* seems to produce the cellulase required for the beetles to digest the plant material (Gerardo lab, unpublished data), should *Staphylococcus* be eliminated the beetles should no longer be able to digest nutrients. Due to the predicted role of the bacterial community in cellulose digestion in bean beetles, I predicted that altering the microbial community with antibiotics would lead to a decrease in beetle fitness. I expected that the exposure to antibiotics during larval development would be the most influential, because it gives the beetles consistent exposure to antibiotics. Additionally, larval development is when the beetles feed and digest the plant material and would be most vulnerable to any changes that would impact their ability to digest the legumes. Should the antibiotics target *Staphylococcus* and wipe it out, the larvae would then no longer have it to assist them while they try to consume the plant material. The antibiotic seed coat only impacts the beetles in their embryonic and early instar larval stage, and thus is only present at the beginning or their development. Also, the drinking experiment occurs once the beetles have completed development and become adults; therefore, while the beetles still have plant material in their guts as adults, the effects of the exposure to antibiotics once they are fully developed is expected to be minimal.

Methods

Experiment 1: Effects of antibiotics on beetle development when exposed during early developmental stages

To test how an antibiotic outer coat would affect the beetles during their early developmental stages, dried black-eyed peas were coated with antibiotics after their seed coat was removed. In order to ensure only the embryo and first instar larvae get exposed to the antibiotics, a layer of antibiotics was added to the outermost layer of the beans, leaving the insides mostly unimpacted.

To coat black-eyed peas with antibiotics, they were soaked in water for 15 minutes and their seed coat was removed. While removing the seed coat, many of the beans separated and split in half. However, bean beetles can develop successfully in split beans (Harris et al. 2013). After removing the seed coat, the beans were soaked in either sterile water, a 0.3% rifampicin liquid solution, or a liquid antibiotic cocktail solution containing 0.3% rifampicin, 0.15% streptomycin and 0.075% tetracycline, making a 1:2:4 ratio, as performed in a similar experiment previously by Hammer et al., (2017) for 15 minutes. After soaking, the liquid was drained, and the beans were left to dry for 24 hours. Once dry, 10-15 beans were added to 10 35mm petri dishes, per treatment. A mature female from a black-eyed pea stock culture was added to each dish and observed after a few days to confirm she had laid eggs. If the beetle failed to lay any eggs, she was replaced by a new mature female. Isolated beans with eggs laid on them were then separated into individual wells within 12-well tissue culture plates. The beans were then maintained at room temperature so as to prevent moisture in the incubator from leading to mold growth.

The beans were observed every day in order to determine the day each adult emerged. Once the adults emerged, I recorded their time to emergence, sex, and mass. I calculated percent

emergence for each treatment as the percentage of eggs laid that resulted in an emerged adult. Once the data were recorded, the beetles were placed individually in 1.5mL microtubes in a freezer at -80C to preserve them for future culturing of the bacterial community. I collected data on the density of culturable bacteria in each of the beetles through the methods described below (see "Estimating density of culturable bacteria").

Experiment 2: Effects of antibiotics on beetle development when exposed during total larval development

To test how exposure to antibiotics would impact the larval development of *C. maculatus,* artificial beans with antibiotics mixed in throughout were created. Dried black-eyed peas were soaked in deionized water for approximately 15 minutes before removing the seed coat. Once the seed coat was removed, the beans were dried at room temperature for at least 24h, and then ground into a flour using an electric flour mill. The rifampicin flour was made by taking 100 grams of the flour and adding 0.3 grams of rifampicin to the flour (0.3% w/w). The antibiotic cocktail flour was made by adding 0.3 grams of rifampicin, 0.075 grams of tetracycline and 0.15 grams of streptomycin to 100 grams of flour and thoroughly mixing it up (0.3%, 0.075%, and 0.15% w/w, respectively). For each flour type, 100 "0" gelatin capsules then were filled with the flour and separated into ten standard petri dishes of 10 capsules. A mature female beetle from a black-eyed pea stock culture was placed in each of the petri dishes to lay eggs on the artificial beans. If no eggs were laid after a few days, the female was replaced with a new beetle until eggs were found on the beans. Isolated beans with eggs laid on them were then separated into individual wells within 12-well tissue culture plates and kept in an incubator at (26C)

The beans were checked on every day in order to establish the day each adult emerged. Once the adults emerged, I recorded their time to emergence, sex, and mass. I calculated percent emergence for each treatment as the percentage of eggs laid that resulted in an emerged adult. Once the data were recorded, the beetles were placed individually in 1.5mL microtubes in a freezer at -80C to preserve them for future culturing of the bacterial community. I collected data on the density of culturable bacteria in each of the beetles through the methods described below (see "Estimating density of culturable bacteria").

For treatments in which no adults emerged after 100 days, the beans were dissected and the level of development in which the beetles achieved was analyzed. Level of development was measured by first looking at the beetles' physical traits and determining whether they were a larva, a pupa, or an adult. From there, an image of the beetle larva was taken nest to a 2 mm circle in order to determine which stage of larval development it had reached. According to Devereau et al. (2002), for *C. maculatus,* the size of the larva head capsule was approximately 0.13-0.15mm for first instar larva, 0.24-0.26mm for second instar larva, 0.36-0.38mm for third instar larva, and 0.56-0.64mm for fourth instar larva.

In order to see how a lower dose of rifampicin would impact the beetle development, a follow-up experiment was performed with lower concentrations of rifampicin. From the flour, I made 30 artificial control beans, 50 artificial rifampicin beans, and 50 antibiotic cocktail beans. The rifampicin flour was made by taking 50 grams of the flour and adding 0.075 grams of the antibiotic rifampicin to the flour (0.15% w/w). The antibiotic cocktail flour was made by adding 0.075 grams of rifampicin, 0.0375 grams of tetracycline and 0.075 grams of streptomycin to 50 grams of flour and thoroughly mixing it up (0.15%, 0.075%, and 0.15% w/w, respectively). For each flour type, "0" gelatin capsules then were filled with the flour and separated into standard petri dishes of 10 capsules. A mature female beetle from a black-eyed pea stock culture was

placed in each of the petri dishes to lay eggs on the artificial beans. If no eggs were laid after a few days, the female was replaced with a new beetle until eggs were found on the beans. Isolated beans with eggs laid on them were then separated into individual wells within 12-well tissue culture plates and kept in an incubator at (26C).

The beans were observed every day in order to determine the day each adult emerged. Once the adults emerged, I recorded their time to emergence, sex, and mass. I calculated percent emergence for each treatment as the percentage of eggs laid that resulted in an emerged adult. . Once the data were recorded, the beetles were placed individually in 1.5mL microtubes in a freezer at -80C to preserve them for future culturing of the bacterial community. I collected data on the density of culturable bacteria in each of the beetles through the methods described below (see "Estimating density of culturable bacteria").

Experiment 3: Effects of antibiotics on adult gut microbial communities

To test how exposure to antibiotics would impact *C. maculatus,* after emerging as a fully formed adult, the adults were given access to water containing antibiotic treatments. Although adult beetles do not require food or water once they have emerged from their legume, previous research shows that the beetles will drink when provided with water or aqueous solutions (Butcher and Chirhart, 2013). Newly emerged beetles were taken and added to a standard petri dish containing a 35mm dish with cheese cloth soaked in either sterile water, a 0.3% rifampicin liquid solution, or a liquid antibiotic cocktail solution containing 0.3% rifampicin, 0.075% tetracycline and 0.15 % streptomycin. To determine if adult beetles were consuming sufficient antibiotic to reduce the density of gut bacteria, the living beetles were frozen in individual 1.5ul microtubes at -80C after 10 days. Then, I collected data on the density of culturable bacteria in

each of the beetles using the methods described below (see “Plating out gut microbial communities”).

To determine the effect of antibiotics on adult lifespan, I conducted a follow up experiment. Newly emerged adult beetles were initially sexed and massed since both sex and weight contribute to the expected lifespan of the adult beetle (Tran and Credland 1995). The beetles were then added individually to 35mm petri dishes with cheesecloth soaked in either sterile water, a 0.3% rifampicin liquid solution, or a liquid antibiotic cocktail solution containing 0.3% rifampicin, 0.075% tetracycline and 0.15 % streptomycin. The cheesecloth was checked regularly to make sure it still contained a substantial amount of solution for the beetle to drink as well as to change it out should mold begin growing on it. The beetles were observed for their entire adult lifespan. Beetles that died from natural means (i.e., didn’t escape or drown in their liquid solution) were in individual 1.5ul microtubes at -80C for subsequent estimation of density of culturable bacteria.

Estimating density of culturable bacteria

Since the microbial communities present in the whole beetle and the gut of the beetle are effectively the same, as long as the immediate outside is sterilized (Gerardo lab, unpublished data), no special procedures are required to isolate the gut before the microbial communities can be plated out. The gut bacteria of bean beetles were plated out by removing the beetles from the freezer and surface sterilizing them by submerging them in bleach for 3 seconds, sterile water for 10 seconds, 70% ethanol solution for 5 seconds, and then sterile water once more for 10 seconds before being placed in a sterile 1.5ul microtube. I added 450ul of 0.9% saline solution to the microtubes with the beetles and released the interior bacteria by using a sterile pestle to crush the beetle. Then, I centrifuged the microtubes for 5 seconds to separate the large debris. Finally, 100

microliters of the solution were spread onto nutrient agar plates and left to incubate for 24-48 hours at 37C before counting the colonies. To facilitate counting colonies, I also plated 10-fold and 100-fold serial dilutions. I ended up analyzing the 10-fold serial dilution as it had a moderate number of colonies present without forming a lawn making it difficult to distinguish one colony from another.

Results

Experiment 1: Effects of antibiotics on beetle development when exposed during early developmental stages

When analyzing the impact of antibiotics on the development of embryos and first instar larvae, significantly fewer eggs were laid on the beans with antibiotic-cocktail coating than on the control beans (Kruskal-Wallis Test: X^2=4.94, df=1, P=0.026; Figure 2). However, there was not a significant difference between the number of eggs laid on the rifampicin coated beans compared to the control (Kruskal-Wallis Test: X^2=0.72, df=1, P=0.40; Figure 2). The beans were observed for 156 days, with the experiment getting terminated after 21 days without emergence. However, after the experiment was terminated, two subsequent beetles emerged, one from a control bean and one from a rifampicin coated bean. Data from these beetles was included in all trials except for time to emergence since the exact date of emergence was unknown.

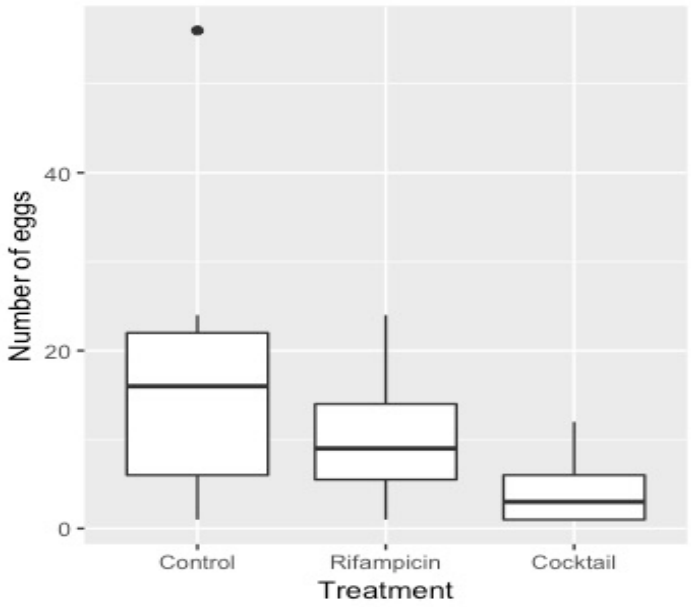

Figure 2: Experiment 1, average number of eggs laid per female on black-eyed peas coated in either sterile water, 0.3% liquid rifampicin solution or a liquid antibiotic cocktail solution (0.3% rifampicin, 0.15% streptomycin and 0.075% tetracycline). Eggs were also found on the dishes of the rifampicin treatment as well as the cocktail treatment and were not included in this analysis. N=9 for control treatment, N=7 for rifampicin treatment, and N=9 for antibiotic cocktail treatment

Following exposure to antibiotics during their embryonic and first instar larval stage, percent emergence was significantly lower from beans coated with rifampicin (9.6%, 7 out of 73 beetles total) compared to the control (22%, 35 out of 158 beetles total) (Fisher's Exact Tests: Control versus Rifampicin, one-tailed P=0.014). However, percent emergence was not significantly different between the control beans and antibiotic cocktail coated beans (13.5%, 5 out of 37 beetles total) (Fisher's Exact Tests: Control versus Cocktail, one-tailed P=0.17).

For all treatments testing the impact of artificial seed coat on beetle emergence, the sex ratio was consistently biased towards female emergence. Out of the 35 beetles which emerged from control beans, 21 were female, making the sex ratio 61.8% female and 38.2% male, Of the beetles emerging from the rifampicin coated beans, the first six were female and one male emerged much later, making the sex ratio 85.7% female and 14.3% male. Furthermore, for the

beans coated in the antibiotic cocktail, four were female and one was male, making the sex ratio 80% female, and 20% male.

Over 3 weeks after the final beetle seemed to emerge, three more emerged. Since the actual day of emergence was not collected, they were not included in the data on development time. The average time to adult emergence for beetles collected within the experiment time frame showed marginally significantly shorter time of emergence for beetles that emerged from control beans than from rifampicin beans (Mann-Whitney U Test: X^2=3.58, P=0.059; Figure 3). In contrast, average time to emergence was not significantly different between control beans and antibiotic cocktail coated beans (Mann-Whitney U Test: X^2=2.16, P=0.14; Figure 3). The average weight for female beetles upon initial emergence showed no significant difference between treatment and the control (Mann-Whitney U Test: Control vs Rifampicin, X^2=0.050, P=0.95; Control vs Rifampicin, X^2=0.032, P=0.82: Figure 4). For male beetles, the pattern appeared to show greater mass for control beetles than for treatment beetles; however, due to low male emergence, no statistical tests were able to be performed.

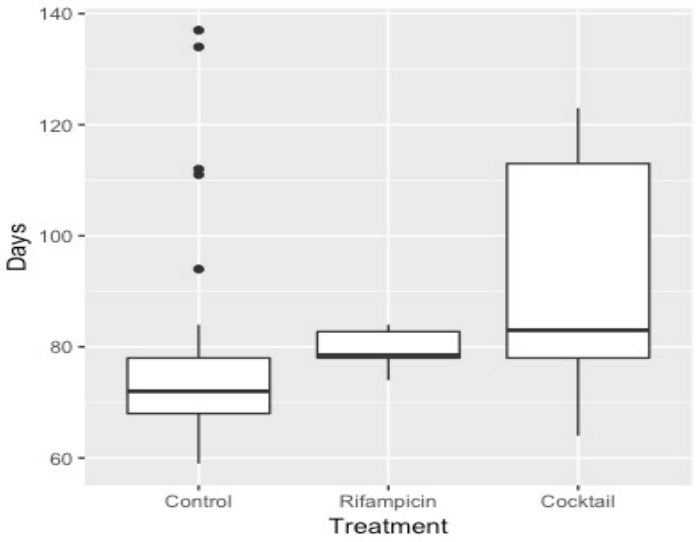

Figure 3: Experiment 1, average time to emergence for *C. maculatus* reared on beans coated in either sterile water, 0.3% rifampicin solution or an antibiotic cocktail solution (0.3% rifampicin, 0.15% streptomycin and 0.075% tetracycline). N=35 for control treatment, N=7 for rifampicin treatment, and N=5 for antibiotic-cocktail treatment.

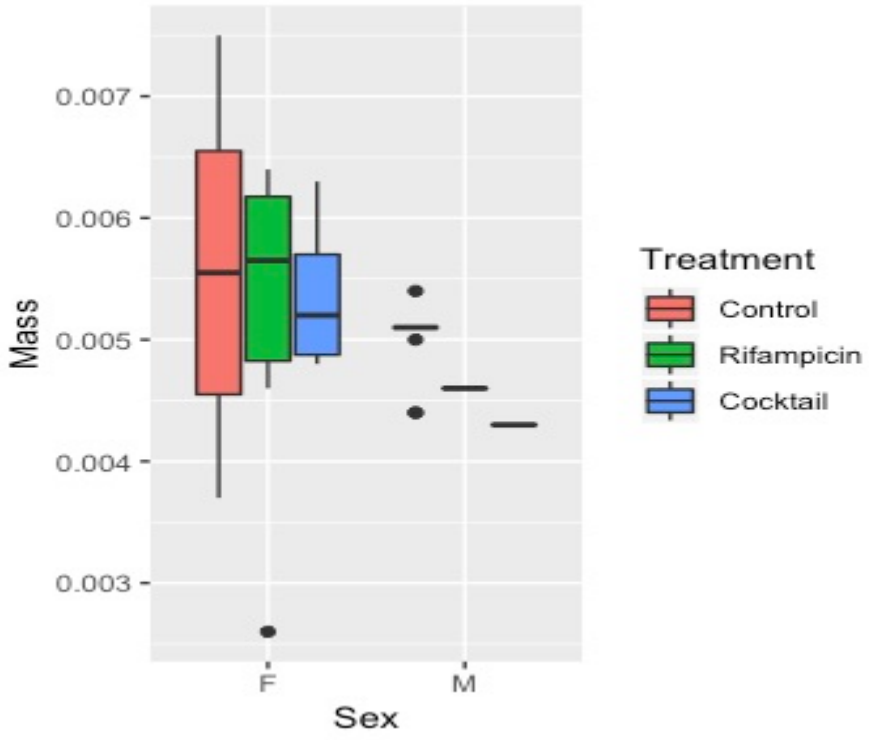

Figure 4: Experiment 1, average weight (grams) of beetles upon emergence based on sex and treatment. For the beetles that emerged from control beans, N=22 for females and N=17 for males. For the beetles that emerged from rifampicin-coated beans, N=6 for females and N=1 for males. For the beetles that emerged from antibiotic-cocktail-coated beans, N=4 for females and N=1 for males.

When culturing the bacterial communities from the beetles in this experiment, potential contamination resulted in almost no bacterial colonies developing in any of the trials. Since there were only a few replicates of each of the beetles from antibiotic beans and no issues had arisen previously, they were all plated out at once. As a result, the effect of antibiotic coating on the abundance of bacteria could not be determined.

Experiment 2: Effects of antibiotics on beetle development when exposed during total larval development

Trial 1

When analyzing the impact of antibiotics on the development of the beetles from larva until adult emergence, the number of eggs laid varied substantially between artificial control beans and artificial rifampicin beans (Kruskal-Wallis Test: X^2=5.86 df=1, P=0.02; Figure 5). There was also shown to be a marginally significant difference between eggs laid on control beans compared to artificial rifampicin beans (Kruskal-Wallis Test: X^2=3.43 df=1, P=0.06; Figure 5). Of the three treatments, the rifampicin treatment was found to have the most eggs laid per female while the control treatment had the fewest. The total emergence from the control beans was 58 beetles, resulting in a 30% emergence rate. In contrast, no adults emerged from either the artificial rifampicin beans or from the artificial antibiotic cocktail beans after 100 days.

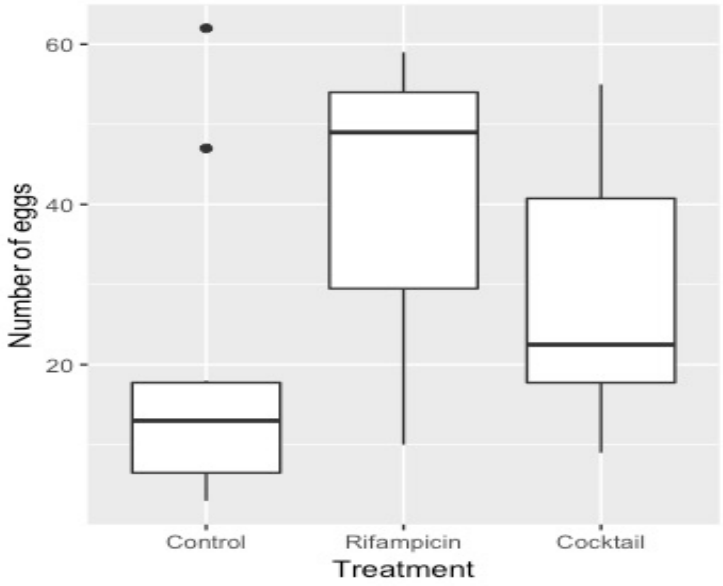

Figure 5: Experiment 2 trial 1, average number of eggs laid per female on artificial beans containing ground up black-eyed peas with either no antibiotics, 0.3% rifampicin or an antibiotic cocktail mixture (0.3% rifampicin, 0.15% streptomycin and 0.075% tetracycline). N=10 for each treatment.

Since no beetles emerged from any of the antibiotic treatments, yet there were signs of development, the artificial beans were separated and dissected to collect data on the developmental stage of the larvae. Fifty-six (56) of the artificial rifampicin beans were dissected and out of 284 potential eggs on those beans, 40 beetles (15% of the eggs) were found to have developed to a point where they could be seen. However, as a result of dissecting the beans after many of the larvae had perished and shriveled up, it was unclear what stage of development 26 of the beetles had achieved. Twenty-eight (28) of the beetles discovered were confirmed to be at some point of larval development, while three were pupa, and four were too desiccated to determine. Of the 28 confirmed larvae, only 13 showed clear development stages, eight were first instar larva, three were second instar larva, one was a third instar larva, and one was a fourth instar larva. Furthermore, three of the beetles had developed to their pupal stage, showing evidence of wing development as well as a hard exoskeleton. Forty-four (44) of the artificial antibiotic cocktail beans were dissected and out of 111 potential eggs on those beans, three beetles were found, so 3% of the eggs developed to a point where they could be seen. These beetles were too dried up to determine what level of development they were able to achieve.

The average time of development for beetles from the control beans was 40 days. Immediately after emergence, the average weight for the beetles from the artificial control beans was 4.20 mg. Of the beetles which emerged, 37 out of 58 beetles were female, this means that the sex ratio was 64% female and 36% male.

Due to desiccation of the beetles found within the antibiotic treatments, we were unable to gather data on the bacterial communities within the larva

Trial 2

In contrast to Trial 1, the number of eggs laid on capsules did not vary substantially between treatments (X^2=0.60, df=2, P=0.742; Figure 6). After 78 days, five beetles have emerged from control treatments while one beetle has emerged from the rifampicin treatment and one from the antibiotic cocktail treatment. Time to emergence was shorter for beetles from the control treatment (51.4 ± 3.88 days) as compared to beetles from the rifampicin (64 days) and cocktail (78 days) treatments. Female beetles from the control treatment were larger (N=2, mean=0.00365 g) than those from rifampicin (0.0035 g) and cocktail (0.0027 g) treatments.

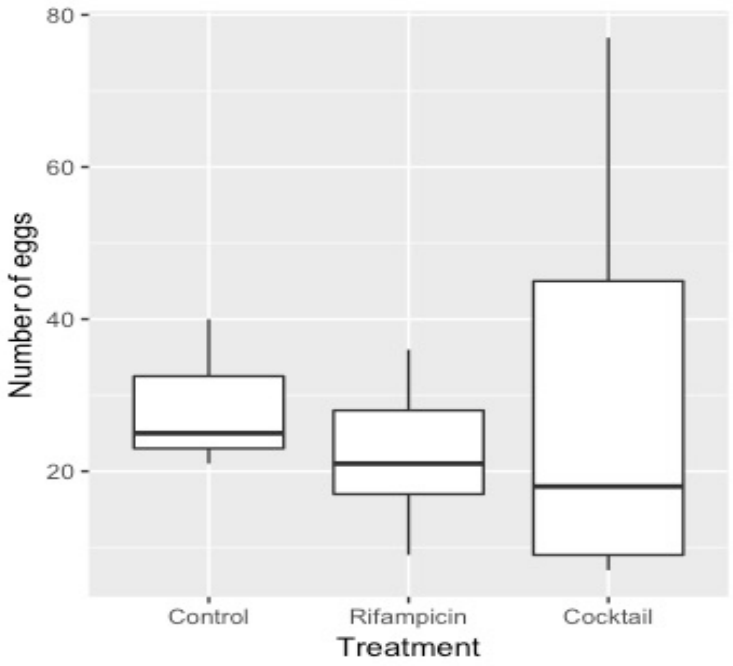

Figure 6: Experiment 2 trial 2, average number of eggs laid per female on artificial beans containing ground up black-eyed peas made up of either no antibiotics, 0.3% rifampicin or an antibiotic cocktail mixture (0.3% rifampicin, 0.15% streptomycin and 0.075% tetracycline). N=3 for control treatment, N=5 for rifampicin and cocktail treatments.

Experiment 3: Effects of antibiotics on adult gut microbial communities

An initial experiment testing the impact of antibiotics on the adult microbial communities showed that after 10 days of continuous access to liquid antibiotic solutions, the average density of gut bacteria of the beetles was significantly less for beetles who drank the liquid antibiotic cocktail solution (0.3% rifampicin, 0.15% streptomycin and 0.075% tetracycline) than for either the sterile water or the liquid 0.3% rifampicin solution (Kruskal-Wallis test X^2=10.2, df=2, P=0.0064; Figure 7). In the follow-up experiment on the effect of liquid antibiotic solutions on the lifespan of adult beetles, no significant impact was observed (Kruskal-Wallis test X^2=2.61, df=2, P=0.27; Figure 8)

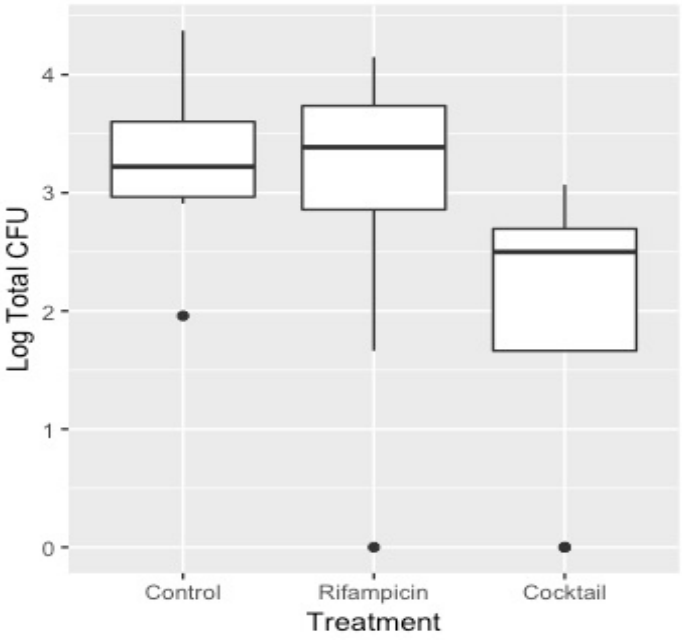

Figure 7: Experiment 3, average total density of gut bacteria after 10 days exposure to liquid solution. Densities were estimated based on 10-fold dilution. N=11 for control, N=17 for the liquid rifampicin solution, and N=9 for the liquid cocktail solution.

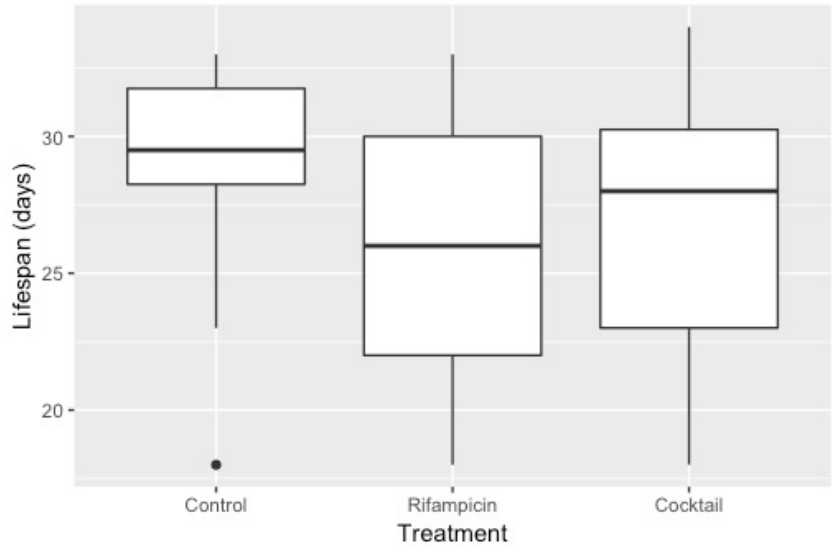

Figure 8: Lifespan of adult beetles after exposure to liquid solutions. N=10 for control solution, N=20 for liquid rifampicin solution and N=16 for liquid antibiotic cocktail solution.

Discussion

Although the entire larval life cycle of *Callosobruchus maculatus* occurs within a bean, they do not possess the gene to produce cellulase (Pauchet et al., 2010). Therefore, the beetles likely rely on their gut microbial communities to perform functions such as digesting the plant material as they grow and develop within the legume. As a result, I hypothesized that using antibiotics to alter the beetles' gut microbial community would have a negative effect on their fitness. Overall, consumption of antibiotics had a definite impact on the fitness level of the beetles, particularly during larval development. Exposure to antibiotics during their embryonic and early larval stage showed a negative impact on beetle development for the rifampicin treatment, it did not for the antibiotic-cocktail treatment, therefore data on the impact of antibiotics during early development for bean beetles was inconclusive. However, antibiotic exposure during larval development was shown to have a negative impact on the beetles' fitness.

Furthermore, exposure to liquid antibiotics during adulthood significantly altered the beetles' gut microbial communities; however, no significant impact on the adult lifespan was observed.

When analyzing the impact of antibiotics on the development of embryos and first instar larvae, significantly fewer eggs were laid on the beans with antibiotic-cocktail coating than on the control beans, while there was no significant difference between the amount of eggs laid on. The variation in number of eggs female beetles laid on each bean type could have many explanations. For example, Credland et al. (1986) showed that larger females who have just mated for the first time lay more eggs in comparison to females who are older or smaller. Since female beetles were chosen at random from a stock culture, factors such as their weight or age were unaccounted for, and therefore the potential to lay eggs was quite variable among females chosen. Moreover, *C. maculatus* are known to have a preference for laying eggs on the same type of bean from which they emerged (Wasserman, 1981). For example, beetles reared on adzuki beans will lay more eggs on adzuki beans than beetles reared on pigeon peas (Wasserman, 1981). Since antibiotics coated the outer layers of the beans, it is possible that the females were more resistant to laying eggs on those beans, particularly the antibiotic-cocktail-coated beans which were coated in rifampicin, streptomycin, and tetracycline. Female *C. maculatus* are capable of distinguishing beans through chemical markers (Giga and Smith, 1984). For example, beans with egg previously laid on them, even if the eggs were removed, contain pheromones that keeps the female from laying as many eggs on those beans as she would on a fresh bean (Giga and Smith, 1984). Therefore, females may avoid the antibiotic-cocktail-covered beans as a result of chemical signals produced by the antibiotics. Finally, *C. maculatus* prefer to lay eggs on smooth surfaces rather than rough surfaces (Nwanze and Horber, 1976). The antibiotic-cocktail outercoat may have dried, making the surface of the beans rougher and thus less favorable for egg laying. The hypothesis that female beetles have an aversion to the

antibiotic coating, whether due to a chemical signal or texture, is supported by the presence of many eggs laid on the smooth surface of the plastic petri dishes in which held the rifampicin coated beans as well as the antibiotic-cocktail-coated beans. Furthermore, no eggs were found on the petri dished that held the control beans when the female was added. Since multiple factors impact beetle egg laying preferences, it is difficult to determine the exact cause of the differences in the number of eggs laid on different bean types and whether the differences are biologically important.

The percent emergence following exposure to antibiotics during their embryonic and first instar larval stage was significantly lower from beans coated in rifampicin. However, beans coated in the antibiotic cocktail solution did not possess a significantly lower rate of emergence than the control beans. These results show that rifampicin outer coat does hinder the development of the bean beetles while the antibiotic cocktail does not. The lower rate of emergence for beetles from the rifampicin treatment could be a result of added roughness on the beans. *C. maculatus* larvae have more success penetrating beans with smooth surfaces than rough (Nwanze and Horber, 1976). Furthermore, the reduced emergence could be the result of antibiotic presence immediately killing off the inherited microbial communities, hindering the beetles' ability to penetrate the bean and begin development. However, since the antibiotic cocktail contained the same amount of rifampicin as the rifampicin-coated beans, in addition to extra antibiotics, any change in texture caused by the rifampicin-coated beans also would occur on the antibiotic-cocktail-coated beans. Additionally, any impact the antibiotics from the antibiotic cocktail have on the beetles' gut microbial community would be equal or greater than that of the rifampicin-coated beans. However, while the percent emergence from beans coated in antibiotic cocktail was not significantly different from the control due to the low sample size, it did show a trend for decreased emergence.

The average time of development was marginally shorter for beetles that emerged from control beans than for beetles that emerged from the beans with rifampicin outer coats. Since the antibiotic-cocktail-coated beans has the same amount of rifampicin as the rifampicin-coated beans, it would be assumed that any difference caused by the rifampicin would be present in both treatments. Moreover, since the average time of development was only marginally significant for rifampicin-coated beans and not significant for antibiotic-cocktail-coated beans, it is unlikely that the presence of antibiotics during early development had a significant impact on the beetles' time to development. However, since three beetles were found to have emerged after the experiment was terminated, it is quite possible development is ongoing.

In contrast to percent emergence and time to emergence, average weight at emergence did not differ for beetles that emerged from beans with or without the antibiotic coating. Therefore, while the short-term exposure to the antibiotics does impact the beetles' survival, it ultimately does not impact their overall growth once they enter the unimpacted part of the bean. Should exposure to antibiotics impact the time to emergence, yet the beetles emerged at roughly the same size, it can be assumed that the antibiotics result in a reduced growth rate per day.

The sex ratio for beetles favored the females for all treatments in this experiment. However, since very few beetles emerged from the antibiotic treatments, the ability to detect non-random changes in the sex ratio is very low for those treatments. Particularly for the antibiotic experiments, due to the fact that each one had one male emergence in total, and the male from the rifampicin coated bean emerged over two months after the other beetles from that treatment, it is difficult to determine whether that was due to chance or as a result of the treatments. Since a female emergence bias is present for all treatments, it is unlikely that the antibiotics had any significant impact on the sex of the beetle that emerged. Since act of removing the seed coat prior to coating with water or antibiotics caused the black-eyed peas to

split in half, the beans were made much smaller. Furthermore, most of the beans only had one or two eggs laid on them. Similarly, Paukku and Kotiaho (2008) found female-biased emergence when a limited number of eggs were laid on small mung beans**.**

When culturing the gut microbial communities from beetles from the seed-coat experiment, contamination in the materials resulted in almost no bacterial colonies developing in any of the trials. Therefore, the impact of antibiotics on the bacterial communities of embryos and first instar larvae was undetermined. As a result, I was unable to determine whether effects of beetle development were a result of antibiotics altering the beetles' gut microbial community or due to other factors, such as modified texture of the beans.

While the exposure to antibiotic treatments during the beetles' embryonic and early instar larval stages produced minimal impact on beetle development, I predicted that continual exposure to antibiotics during larval development would have a significant impact on the beetles' fitness. Since the beetles' larval stage is when most of the feeding occur, the larval microbial communities should be most susceptible to antibiotic exposure, thereby reducing beetle fitness. Indeed, the continued presence of antibiotics during larval development significantly impaired the beetles' ability to develop and reach adulthood.

Although each of the three treatments used gelatin capsules and therefore no differences should be detectable based on the outside of the bean, the female beetles laid significantly more eggs on rifampicin beans than on either the antibiotic beans or the control beans. Overall, the number of eggs females laid on artificial beans was much greater than for beans coated with antibiotics. This could be due to the fact that the artificial beans were much larger and therefore had more room for eggs to be laid. Additionally, since there should be no apparent differences based on the outside of the beans, variation among the treatments in the number of eggs laid was most likely a result of variability in female fecundity rather than characteristics of the beans

themselves. Since females were collected randomly, factors that could impact the number of eggs she could lay, such as age and weight (Credland et al. 1986), were unaccounted for.

Due to the fact that the control beans resulted in a 30% emergence rate, yet no adults emerged from the antibiotic beans, exposure to antibiotics during larval development seems to inhibit successful development of the beetles. However, the presence of larval tracks inside the beans suggests that some larval development was able to take place. The stage of development for the beetles recovered from the antibiotic capsules were mostly ambiguous due to desiccation, however, of the ones that were intact, the majority were found to be first instar larva. The furthest stage of development in which the beetles within the antibiotic beans were able to achieve was to their pupal stage.

It is unknown whether the reason the beetles were unable to reach adulthood was because the antibiotics created a toxic environment or because the antibiotics successfully altered the beetles gut microbial communities to a point where the beetles were unable to grow. In insects, depending what functions the gut microbial communities perform, their absence can either have a relatively minor or significant impact. Since the microbial community of bean beetles is assumed to assist with the beetles' ability to process the plant material within the bean, the absence of the gut microbial community should inhibit the beetle from receiving proper nutrients needed for development. However, since the initial gut microbiome is inherited maternally (Gerardo lab, unpublished data), it is possible that the beetles would be able to initiate development. However, continued exposure to antibiotics could result in susceptible microbes getting cleared from the beetles' gut until their populations become too diminished to perform the necessary functions for beetle development. Due to desiccation of the beetles found within the antibiotic treatments, we were unable to gather data on the bacterial communities within the larva.

In order to make sure the stunted development of the beetles from the earlier trial was not due to the antibiotics creating a toxic environment, I conducted a follow up study using artificial beans with 0.15% rifampicin, reducing the concentration of rifampicin by half while maintaining the concentration of tetracycline (0.075%) and streptomycin (0.015%). While still ongoing, it has been determined that beetles reared on beans composed of 0.15% rifampicin as well and the antibiotic cocktail beans (0.15% rifampicin, 0.15% streptomycin, 0.075% tetracycline) were both capable of reaching adulthood. After 78 days, it has been shown that beetles reared in the control developed quicker and are larger at emergence. This suggests that while development can be completed with 0.15% rifampicin treatment as well as the antibiotic cocktail treatment, development and growth appear to occur at a slower rate. Further development of the beetles should produce more beetles to plate out and compare microbial colonies, thereby getting a more definitive sample of the impact of rifampicin on gut microbial communities.

Finally, when observing the impact of antibiotic exposure on adult beetles, consumption of the liquid antibiotic cocktail reduced the density of gut bacteria present in the beetle. However, since the beetles that were exposed to the liquid rifampicin solution did not show a significant reduction in the abundance of their gut bacteria, it can be assumed that either the streptomycin or the tetracycline had more significant impacts on the beetles' gut microbial communities than the rifampicin alone. Assuming rifampicin does target certain bacteria within the beetles' gut microbiome, it is possible that the other bacteria within the gut microbiome were able to take its place, thereby showing no significant difference in the total bacterial density despite the elimination of certain bacterial types. Since the antibiotic cocktail solution had multiple antibiotics, they all could have targeted different bacteria, preventing any bacteria from increasing in density.

Currently, ongoing research on the impact of antibiotic solutions on adult beetles is testing how continuous exposure to the antibiotic solutions throughout the beetles' life impacts their lifespan as well as the makeup of their gut microbial communities once they eventually die. While a few beetles are still alive, preliminary analysis shows no impact of liquid antibiotic solutions on the overall lifespan adult beetles, consistent with my hypothesis that altering the microbial communities of adult beetles would have little impact on their overall fitness.

Conclusions and Future Directions

Overall, antibiotics were shown to impact both the fitness as well as the microbial communities of *C. maculatus.* Of the three life cycle stages observed, antibiotic exposure during larval development showed the greatest impact on beetle fitness. However, due to methodological issues, I was only able to show definitive impact of antibiotics on the beetles' microbial community in adults. Additional experiments are being conducted currently to gather further data on the impact of antibiotics on both the microbial communities of the beetles as well as beetle fitness. The ongoing experiments include the long-term impact of liquid antibiotic solutions on the lifespan of adult beetles, as well as the effect of continuous antibiotic exposure, at reduced levels of rifampicin (0.15%), during larval development on the beetles' gut microbial communities.

To gather further data on the impact of antibiotics on the early stage of beetle development, a repeat of the artificial seed-coat experiment should be performed with a larger sample pool in order to get more definitive results. Also, the effect of continuous antibiotic exposure on the make-up of the beetles' gut microbial communities could be detected by next-

generation sequencing of the gut bacterial community from larvae collected from artificial antibiotic beans. Next-generation sequencing would allow examination of change in the structure of microbial communities based on antibiotic exposure, including bacterial taxa that cannot be cultured. To continue exploring the influence of antibiotics on the fitness and gut microbial make-up of bean beetles, it would be interesting to examine the transmission gut microbial communities in more detail. It is assumed that the bacteria are maternally derived, since eggs laid on sterile glass beads were shown to have microbial communities (Gerardo lab, unpublished data). By using the liquid antibiotic treatment on adults for a few days before granting them access to the opposite sex in order to lay eggs, whether the altered gut microbial community of the parents impacts the offspring could be determined. Male bean beetles produce large spermatophores, and it is unknown whether bacteria could be transferred through these spermatophores to either the egg or the female. In order to test the transmission of bacteria from parents to offspring, the eggs and offspring of all crosses between individuals with and without modified gut microbial communities would be tested. The focus of this experiment would be on the initial microbial communities of eggs laid on glass beads, as well as the long-term impact on development into adulthood. Together, the results from my current experiments and the proposed future experiments will help us to better understand the role of bacterial communities in insect nutrition for insect species that are stored product pests. Through the understanding of the beetles' reliance on their gut microbiome, and the various methods of manipulating their microbial communities with antibiotics, it becomes possible to develop various strategies to control the population of the stored product pests.

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