

investigate the metabolic processes governing bacterial interactions within the host gut environment. This line of research has successfully been applied to study gut microbial symbiosis as well as pathogenesis, generating 15+ publications cited over 700 times in the past five years. As an Early Stage Investigator, I have been collaborating with basic and applied scientists and make discoveries, including the first evidence for gut microbiome in B vitamins provision to support *Drosophila* development (6), and a novel virulence mechanism of *Vibrio Cholerae* attributed to microbial consumption of a single metabolite (16). My latest research on the role of microbiome in *Drosophila* foraging behavior (10) has been featured by multiple news media including the **Phy.org** and **ScienceDaily**. The proposed research will extend my program in defining how the microbiome modulates insect behavioral and physiological responses to changing environment, translating into economically important insects and ultimately impactful applications.

EXPECTED IMPACT

According to the Entomological Society of American recent position statement, invasive pests incur control costs of over \$2.5 billion in the US and cause economic damage to crops, lawns, forests, and pastures totaling \$18 billion per year (17, 18). Invasive fruit flies are a major concern for growers because they can destroy crops and propagate rapidly once established in a new area. Zero-tolerance practices are in place where the detection of a single fly larva is sufficient to cause an entire fruit load to be rejected. As a result, chemical insecticides are being sprayed on crops prophylactically at frequent intervals. The excessive use of insecticides not only drives up production costs, including health-related expenses, it also runs against a strong public demand for sustainable agriculture and less chemical use in crop production. This project has the potential to change this practice by leveraging the natural microbiomes of insects and plants to develop novel pest management strategies. Discovery of insect microbiome taxa or genes that affect larval performance will serve as molecular targets to disrupt pests. Fruit microbiome-derived insect attractants and repellents can be implemented into applications such as fly baits and pest repellents.

The UF Entomology and Nematology Department has a track record of success in translating basic research into world changing inventions and technologies to enhance pest management, where invasive species is one of our signature programs. Support from the UF/IFAS early career seed grant will empower PI Wong to expand his expertise in insect microbiome research to invasive species, thus making direct contributions to safeguard agriculture and positively impact people's lives.

METHODOLOGY

Preliminary data from field-collected samples show that the microbiomes of SWD and one of its target crops, strawberries, were highly distinct. Foraging visits by SWD adults to strawberries rapidly altered the strawberry microbiome (Figure 1), and the strawberries became more attractive to SWD (Figure 2A). The observations lead to the two hypotheses in this proposal. The *first hypothesis* is that the insect microbiome facilitates larval development and fly establishment in host fruits. The *second hypothesis* is that the fruit microbiome can influence fruit susceptibility to SWD invasion.

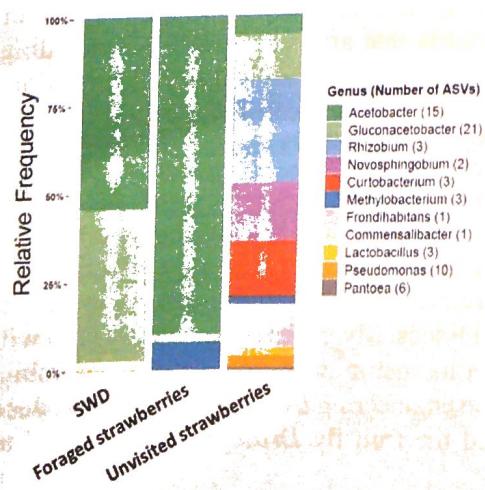


Figure 1. Strawberries that are foraged upon by SWD quickly shift from the original microbiome to being dominated by the microbes inoculated to the fruit by the fly (in 24 hours). This finding shows that SWD flies are manipulating the microbial composition of their potential host fruits. The ability to manipulate the host fruit microbiome may be important for these invasive flies. Fruits whose microbiomes are not as easily colonized by fly microbes may be less suitable hosts for SWD larval development. Similarly, fruits that resist fly microbes may be less attractive to flies and escape female egg laying. Microbiomes were revealed by highthroughput sequencing of the 16S rRNA gene amplicons. Community profile was resolved using the amplicon sequence variants (ASVs) approach (19).

Objective 1. Identify microbial taxa and gene activities associated with SWD larval performance across different host fruits.

Hypothesis: The insect microbiome facilitates larval development and fly establishment in host fruits.

Research timeline: 8 months (4 months: larval performance assays, 4 months: RNAseq experiment)

The ability to exploit new food resources is a critical initial step in the invasion process. Based on our preliminary data comparing the microbiomes between SWD-foraged and unvisited strawberries, microbes introduced by SWD promptly dominated the microbiome of the foraged strawberries. Our working model is that displacement of the native fruit microbiome by insect-associated microbes is important for larval colonization. To identify microbial taxa and gene activities that may affect colonization, larval performance on a range of host plants in response to the presence or absence of the maternally-transmitted microbiome will be compared. Field collected SWD will be expanded in the laboratory by rearing in strawberries (a primary host fruit) for multiple generations. Once a sufficient population size is reached (>1000 flies), eggs from mated females will be collected, half of them will be surface-sterilized to generate viable, axenic eggs using an established procedure developed by Wong (6), while the other half will remain untreated (conventional, bearing the SWD microbiome). Equal numbers of eggs will be transferred to a range of fruits, including, strawberries and blueberries (common SWD hosts), peaches and figs (less common hosts), and tomatoes and oranges (rare hosts) in at least triplicates. Survivorship and larval development time to pupae and adults will be recorded. Following assessment of larval performance in different host fruits, we will select fruit treatments and run them in five replicates to prepare larval samples for a subsequent RNAseq experiment. The two fruits that yield the biggest larval performance gap and the two with the least performance gap will be selected. RNA will be extracted from the larvae five days after egg inoculation, which will guide us the (microbial) gene activities in the larvae on the different host fruits. Larvae from conventional and axenic eggs will both be examined because the larvae from axenic eggs will likely be associated with a different suite of microbes from the environment, which will enable us to compare and contrast their microbiomes with the conventional larvae and link their differences to fly performance. The experimental design will result in 40 samples (4 diets x 2 egg treatments x 5 reps). Complementary DNA libraries with multiplex tags will be prepared for sequencing using the Illumina HiSEQ platform. Relative abundance of microbial taxa will be revealed by extracting the marker gene reads for bacteria (16S ribosomal RNA gene) and analyzed in QIIME 2 (20). A custom Simple Annotation of Metatranscriptomes by Sequence Analysis 2 (SAMSA2) pipeline (21) and the pathway analysis function in MicrobiomeAnalyst (22) will be used to analyze the RNAseq functional data. Gene activities of initial interest will be differentially expressed microbial transcripts that couple with larval performance. Additional bioinformatics support will be pursued from the Interdisciplinary Center for Biotechnology Research (ICBR) at UF if necessary.

Expected Results, Potential Pitfalls, and Contingencies

We speculate displacement of the fruit microbiome by SWD-introduced microbes is a precondition for larval development. If our working model is true, we expect to observe disproportionately low performance of larvae from axenic eggs than conventional eggs on a given host fruit, coupled with a positive association between abundance of specific SWD microbiome taxa and larval survival and/or development time. For a fruit that yields contrasting performance between larvae from the conventional eggs and from axenic eggs, we anticipate a pronounced meta-transcriptional difference between the larvae. Specifically, we expect to detect microbial metabolic activities or pathways in larvae from conventional eggs that are downregulated or absent in larvae from axenic eggs, and absent in fruits with the least larval performance gap. These genes will be potential signatures for fly establishment and molecular targets to disrupt the pest. False positives are a common issue with RNAseq experiments. We improve our chances of discovering true positive associations between microbial gene activities and SWD performance by selecting two fruits for each larvae performance categories and starting with five biological replicates. One potential pitfall is that there may be no survivors from axenic eggs across all the fruits. If this happens, we will use our collection of SWD-associated microbes to generate gnotobiotics flies (e.g. axenic eggs inoculated with single bacteria) to test for the impact of selective microbiome depletion on SWD larval performance.

Objective 2. Test and identify volatiles from the fruit microbiome that attract or repel SWD.

Hypothesis: The fruit microbiome can influence fruit susceptibility to SWD invasion.

Research timeline: 7 months (4 months: GC/MS experiment, 3 months: volatile testing in choice assays)

- Strawberries that had been foraged upon by SWD were found to be more attractive than unvisited strawberries to SWD flies (Figure 2A), indicating that specific microbes or community of microbes in the SWD microbiome can modify the volatile profiles of the strawberries to promote SWD attraction.
- Additionally, multiple bacteria isolated from berry crops have been tested for their attractiveness to SWD using a two-choice trap assay on a blueberry juice medium. Two bacteria were found to be attractive and four repellent to SWD (Figure 2B).

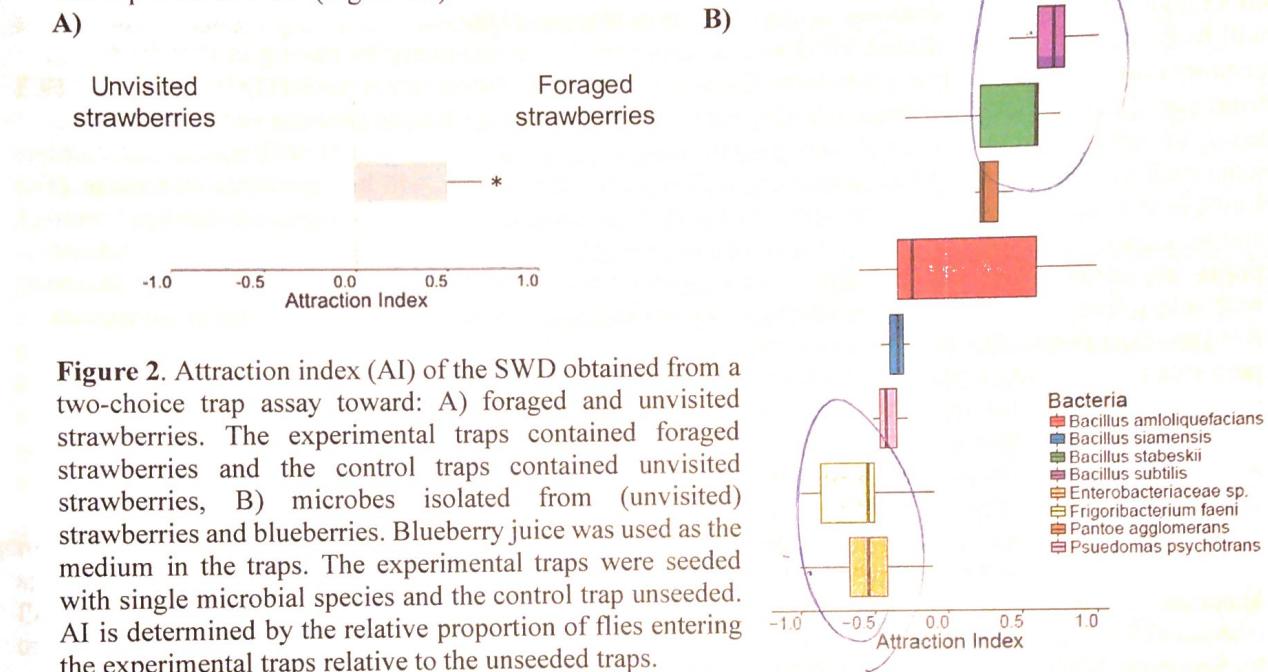


Figure 2. Attraction index (AI) of the SWD obtained from a two-choice trap assay toward: A) foraged and unvisited strawberries. The experimental traps contained foraged strawberries and the control traps contained unvisited strawberries, B) microbes isolated from (unvisited) strawberries and blueberries. Blueberry juice was used as the medium in the traps. The experimental traps were seeded with single microbial species and the control trap unseeded. AI is determined by the relative proportion of flies entering the experimental traps relative to the unseeded traps.

Two sets of gas chromatography–mass spectrometry (GC-MS) experiments and the two-choice trap assays will be performed to extract, test and identify distinct volatiles from the foraged strawberries and also from single microbial species that were showed to be attractive/repellent to SWD. First, volatiles from groups of foraged strawberries and unvisited fruits will be compared, in which volatiles that are distinct between the foraged and unvisited strawberries will be extracted. Second, volatiles from blueberry juice inoculated with single attractive/repellent microbial species will be collected. Volatile collection will be performed in five replicates of glass chambers using a push-pull system. Charcoal-filtered air will be pushed into the top of the closed volatile chamber and pulled through collector trap, containing adsorbent. Volatiles will be collected over 2h and eluted from the adsorbent by extraction under a gentle stream of nitrogen. The attractiveness/repellency of the extracted volatile compounds to SWD will be assessed using the two-choice trap assay as described. Volatile analysis and identification will be supported by the Liburd lab and also our collaborator in the USDA Chemistry Research Unit (John Beck), both labs have robust GC-MS setups.

Expected Results, Potential Pitfalls, and Contingencies

Many insects, including fruit flies, rely on chemical signals to locate their food sources. SWD is distinct from other *Drosophila* species in that it infests intact, ripe and ripening fruits. Yet, studies have demonstrated SWD responded strongly to fermentation products when they make feeding choices (23). We expect to discover fruit and insect microbiome-derived volatiles that are potent attractants and also repellents to the SWD. It is possible that the attractive/repellent volatiles are produced in a microbial community fashion. If our single microbial species experiments do not yield any attractive/repellent volatiles, we will test different mixtures of attractive and repellent microbes.

EXTRAMURAL FUNDING STRATEGY

The National Academies of Sciences, Engineering, and Medicine (NASEM) most recently released "Science Breakthroughs to Advance Food and Agricultural Research by 2030," to determine the greatest scientific opportunities in the next decade within the fields of food and agriculture. **Microbiome is listed as one of the top five priorities.** It is anticipated that ample funding opportunities in agriculture-related microbiome research will be launched in the next 5 years. Below are some existing opportunities:

1. The U.S. Department of Agriculture (USDA). Under National Institute of Food and Agriculture (NIFA), the Agriculture and Food Research Initiative (AFRI) Foundational Program has two priorities that are highly relevant: *Pests and Beneficial Species in Agricultural Production* supports basic research that is expected to lead to innovative, environmentally-sound strategies to manage agricultural pests. *Agricultural Microbiomes in Plant Systems and Natural Resources* is a new theme that funds studies on the multipartite interactions among the host, environment, and the microbiome. By identifying insect microbiome factors that facilitate invasive insect traits, our proposal will be well suited for this award. The applied side of the proposed project will appeal to the Crop Protection and Pest Management grant, which supports development of IPM tactics that are "economically viable, ecologically prudent, and safe for human health". **Timescale:** Proposal announcement for the Foundational Program is usually released in April/May, with submission due in July. Proposal for the Crop Protection and Pest Management grant will be announced in February and due in May. We are aiming for the 2019 submission for both funding opportunities.

2. National Science Foundation (NSF). The Division of Integrative Organismal Systems (IOS) is an appropriate target and we will pursue either Behavioral Systems or Physiological and Structural Systems as each study section is interested in integrative research with the goals of identifying fundamental principles for physiological or behavioral mechanisms and how they are integrated at the level of the whole organism. IOS has a long history of funding insect-microbe symbiosis research. Moreover, the NSF along with other agencies have recently joined to produce the *Interagency Strategic Plan for Microbiome Research*, which outlines the objectives, structure and principles for coordinated research to support transformative research in microbiome studies.

Timescale: Once we have the RNAseq data and gene candidates, we will be able to target a submission next year. It is important to note that NSF is implementing the "No-Deadline, Full-proposal Submission Process" to accept proposals in an ongoing basis. Our project is timely for the many new funding opportunities that are anticipated from Interagency Strategic Plan for Microbiome Research.

3. Industries and organizations. Some of the deliverables from this proposal include microbial-based insect attractants or repellents. With a rising demand for sustainable agricultural solutions, these symbiotic microbes and volatile compounds will appeal to the industry as eco-friendly products. Production of these microbes can be scaled up easily to meet industrial need. Once our microbes and metabolites are validated in the lab, we will seek funding from industries and organizations to run trials in the field. We will also work the UF Innovate team to devise a plan for commercialization and patenting.

BUDGET JUSTIFICATION

Funds are requested to subsidize a graduate student stipend, the costs for hightthroughput sequencing, consumable and materials for fly rearing, microbial culture, the trap assays and the GC/MS experiments.

	Cost (\$)
Graduate student stipend (0.5FTE)	
RNA extraction kit and reagents	
Illumina: HiSeq3000, 2x100 run	
Library construction (40 samples)	
Consumables and materials for fly and microbial work	
GC/MS	
Total	

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