Development of Methods for Testing Alterations in Flight Behavior of Cry1Fa-Resistant and Susceptible *Spodoptera frugiperda* in Response to Feeding on Cry1Fa Protoxin

A Thesis Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

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May 2018

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Dedication

This work is dedicated to:

My best friend and fiancée Catherine, for her unwavering support

My parents Leif and Littlebird, for their exceptional advice

My brother Nickholas, for his selfless assistance

My many friends, for their good humor

My family, for their guidance

The Future

Acknowledgements

I want to offer many thanks to all those who assisted during and before this project, and who will undoubtedly continue to help me in my scientific career. Thanks to Dr. Juan Luis Jurat-Fuentes, my major professor. He has been a strong supporter of my project from the beginning, and a great advocate of personal independent growth throughout my upper education. Thanks to my committee members; Dr. Scott Stewart who provided the seeds for the plant materials used in the project and instruction in field crop pests. Thanks to Dr. William Klingeman, the first person to show me entomology as a viable career. It is also thanks to him that I have confidence in my future, and is a great reason for my optimism for the future. Thanks to Dr. Kimberly Gwinn who was there for the chemical parts of my project. She was more than willing to train me in lipid extraction techniques and spectrographic analysis. Thanks to Eric Tague for his assistance with the analysis of lipids. Thanks to Dr. Xiaocun Sun, who showed good humor in the face of statistical analysis and helped to interpret the mountain of data collected in every instance, including those where the data should have been better in the first place. She has been wonderfully supportive and encouraging, available and amicable at every turn. Thanks to my past and present fellow lab members: Dr. Heba Abdelgaffar, Dr. Rahul Banerjee, Dr. Jerreme Jackson, Amy Michael, Dylan Parker, and James Dee. Thanks to all of the faculty and staff of the University of Tennessee Department of Entomology and Plant Pathology for their support, advice, and comradery.

I want to thank my family and friends without whom I would not have been able to complete this project. I would like to thank my parents, Littlebird and Leif Hietala, for their support and assistance, and for their ability to offer advice and suggestions in troubleshooting problems. My brother, Nickholas Hietala, has been the programming wizard for this project, and beyond helping to design and write the program for the flight mill data collection has continually tweaked the visualization program, making it ever more useful and readable as time goes on. My best friend and fiancée, Catherine Toennisson, has offered encouragement through the entirety of the project, and was the reason that I settled on the moth adhesive method I did. My friends in distant places deserve mention as well for their support, ideas, and questions that led to improvements to flight mill designs or the way that insects or issues were handled over time: Aurora Toennisson, Jennifer Chandler, William Borgers, Courtney Borgers, Krista Taylor, Jennifer Lones, Mae Smith, Wes Spain, Alexander Hill, Ellalyn Crossno, Jerry Crossno, Lee Borgers, and Matthew Borgers.

Finally, the most important, I thank the insects that were used in this project. Without the sacrifice of thousands, not a word of this may have been written.

Abstract

The fall armyworm (*Spodoptera frugiperda*, Lepidoptera: Noctuidae) is an important agricultural pest of corn, cotton, soybean, and a number of other American specialty and row crops. The annual life cycle of this insect in the United States involves a seasonal migration during the growing season from overwintering locations in Florida and Puerto Rico, and Texas and Mexico, to northern regions of the United States and southern Canada. Control of this insect in corn is generally achieved through the use of transgenic varieties producing a transgene coding for the Cry1Fa insecticidal protein derived from the bacterium *Bacillus thuringiensis* (Bt). The exposure of populations of *S. frugiperda* to Cry1Fa toxin has led to several instances of field-evolved resistance to corn producing this toxin and lack of appropriate control. In a related species, *Spodoptera exigua* (Lepidoptera: Noctuidae), exposure to the marginally effective toxin Cry1Ac has been shown to reduce pupal weight, but increase flight behavior. This study is intended to determine the relationship between exposure of Cry1Fa-resistant *S. frugiperda* to that toxin and flight behavior as compared to unexposed and susceptible insects. In addition, in order to determine physiological effects of resistance and exposure to sublethal toxin doses, the phospholipid profiles of susceptible and Cry1Fa-resistant *S. frugiperda* exposed to control of Cry1Fa-containing diet. Determining the effect on flight behavior and capacity of these insects will further the understanding of resistant insect interactions with Cry toxin pesticides, and particularly the effects that these toxins have on resistant insect behavior and physiology. This study shows that Cry1Fa-resistant *S. frugiperda* larvae feeding on diet containing Cry1Fa do not have a significantly different flight behavior than those feeding on untreated diet. Flight behavior in this study was positively affected by pupal weight (p=0.0081). Analysis of phospholipids in *S. frugiperda* abdomens determined that the Cry1Fa-resistant strain did not display a significantly different phospholipid profile (p=0.1089), and that those insects reared on corn (*Zea mays*) had greater detected phospholipid levels (p=0.0002), while Cry1Fa-resistant insects exposed to Cry1Fa toxin did not express altered phospholipid levels (p=0.3738).

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Chapter I

A Review of Flight Mills and Their Use to Study Flight of Lepidoptera

This chapter to be submitted to Insect Science

Hietala, L. and J.L. Jurat-Fuentes. (2018). A review of flight mills and their use to study flight of Lepidoptera

My contributions included: (1) data collection and analysis, (2) writing the manuscript and making figures. Juan Luis Jurat-Fuentes assisted with (2).

Abstract

Many adults of lepidopteran species (moths and butterflies) display long distance migration during their life cycle. Apart from its ecological and behavioral relevance, lepidopteran migration is also relevant given that larvae of many lepidopteran species are notable pests of human food crops. Considering this potential for extensive crop damage by lepidopteran larvae, it is important to understand the ability of moths and butterflies to locate and travel to new hosts. The study of local and migratory movement of moths has been traditionally performed using metabolic studies on energy storage and its use by flying moths and butterflies, and tests of flight capacity using flight mills. The purpose of this chapter is to review the designs and use of flight mills to study flight propensity of lepidopteran adults in relation to their metabolism, migration habits, and reactions to stress.

1.1 | Introduction

Insect flight has long been a topic of interest to scientists given its importance to the spread of insect pests and vectors of disease. In addition, the aerodynamics of insect bodies and wings have been studied at length, both for the information gleaned about biological flight systems and for the design of mechanical flight systems, particularly on a small scale [1-3]. These tests on aerodynamics and air movement are mostly conducted in wind tunnels or flight chambers that use an affixed insect provoked to flight, often through the use of forced air currents [4]. Smoke or vapor released upstream of the insect is used to create a visual representation of air currents during flight. Studies that do not directly rely on wind tunnels may consist only of a constrained insect that is allowed or provoked to flight, which allows researchers to study the relative movement of body parts [1, 2, 5].

In order to record the movement of an organism over time, a category of devices called actographs are used. Actographs that are used in studies to determine flight variables are commonly called flight mills. From this most basic premise, the exact purpose of a given flight mill may vary from the study of metabolic rates of insects to determining flight periodicity [6, 7]. Many studies that use a flight mill are interested in only one or two factors, or only specific parts of flight, as seen in Table 1.1. For instance, it may be unnecessary for the purposes of an experiment to record the distance flown by an insect, while remaining absolutely necessary to capture all gasses emitted by that insect. In contrast, for other studies it is not needed to keep the insect in a known atmosphere, but require that they are able to obtain vertical height [8].

The moving insect flight mill was first introduced in the early 1950's as a large metal hoop affixed to the ceiling of a room [9]. Along the rim of the wheel were a large number of spokes onto which migratory locusts were attached after having been prepared for long-distance migration stimulation via removal of their tarsal extremities. From that initial project, there was a rapid increase in new designs. In some cases, only visual observation was used for recording, while other protocols took advantage of relatively new computing systems to create a more accurate representation of data collected [7, 10]. One of the first electronically recording flight mills used magnetic tapes to store data. Included in the paper was a cost estimate for time used on a communal computer for data retrieval [4]. Considering the number of flight mill designs and functions that exist, this review will be organized into sections explaining the use and apparent origins of rotational, untethered, static, and lever flight mills, the basic uses of which are shown in Table 1.1.

1.1.1 | Rotational Flight Mills

Rotational flight mills consist of an insect attached to the end of an arm, and record flight data by rotation of the arm as the insect flies. These mills are particularly valuable in studies that possess either large numbers of insects tested, due to the relatively low cost for their construction, or those that require accurate measurement of distances traveled. Rotational flight mills have been recorded as being used for several different families of lepidopterans, including torticids and noctuids [11, 12]. Often, these mills involve a central axis, which suspends an arm of known length that the insect may turn freely either through the use of low-friction fittings or bearings, or magnetic levitation [13].

Table 1.1- An abbreviated table of flight mill varieties

Flight Mill Type	Test For	Sensor Type	Families Generally Tested
Rotational	Flight distance, speed, duration, and/or periodicity	Electrical, optical, or physical	Coleoptera, Diptera, Lepidoptera, Orthoptera
Lever	Flight take off, duration, and/or periodicity	Electrical or optical	Coleoptera, Hemiptera, Lepidoptera
Static	Flight aerodynamics, reaction to visual factors	Optical	Diptera, Hymenoptera, Lepidoptera, Orthoptera
Untethered	Flight periodicity, metabolism, environmental reaction study	Chemical or optical	Diptera, Hemiptera, Hymenoptera, Lepidoptera

Table 1.1 shows the general flight mill varieties, tests that they are generally used for, and insects of interest for each mill type

There are two main sub-varieties of rotational flight mill depending on the recording method for collected data: photometric and magnetic. Those that rely on photometric recording involve a marker that creates an interruption in the signal of a laser or other light source, or a clear point in a disc that allows light to penetrate through to reach a sensor [14]. Magnetic recording involves the use of a Hall effect sensor or similar device that is triggered by a magnet placed on the rotating head of the flight mill, leading to collection of a unit of data [12].

Some of the issues with the use of rotational flight mills include machining, levelness, and uniformity of the resulting design. Machining rotational flight mills is simple, but requires exact measurements to maintain uniformity among mills used in one experiment. Levelness is necessary for rotational flight mills both to reduce potential sources of friction and to ensure minimum interference of flight path for the insect attached to the rotational arm. Apart from a squared mill, it is also necessary to create a leveled platform on which the insect will be attached, which may be achieved through the alteration of the angle of the mill base or the head of the mill.

Benefits of the rotational design include the number of functional tested designs that are readily available and low device costs. Many rotational flight mills cost less than $15 per unit when assembled by the researcher. It is also relatively easy to make one of these devices, although appropriate tools are highly desirable. While it is possible to create one with hand tools only, some larger devices such as drill presses and power saws significantly increase the speed of production and the uniformity of the results.

The first rotational flight mill was used to test the flight of locusts in 1952 [9], with later designs scaled down sharply to meet the demands of insects too small to meet the physical requirements of turning a larger device [10, 15]. Flight mills are still in common use in entomology, particularly within the orders Lepidoptera and Coleoptera. These designs have taken on a number of different forms through time, and possess different recording methods that have been implemented. Some of the most common varieties of flight mill are detailed in this paper.

1.1.2 | Untethered Flight Mills

In untethered flight mills, monitoring does not rely on direct contact with the insect being studied.

which is then attached to a sensor or sensors that record any of several different factors that may be associated with the flight of the insect. These studies generally involve fewer individuals in a time period

than those that use rotational mills, with the possible exception of those that record flight periodicity. Untethered flight mills have been used to study flight of dipteran, lepidopteran, hymenopteran, and hemipteran insects [16, 17]. These experiments mainly focused on the study of phototaxis, metabolism, or social behavior. In these cases, due to the size of the insect or the nature of the experiment, it was necessary or beneficial to leave the insect free from restraint, as the insects are generally too small to attach to a tether without causing physiological damage or restricting the movement of the insect in question. For example, alate aphids are often tested in untethered flight mills due to their soft body and small size, which makes them unsuitable for a rotational flight mill or lever flight mill [17].

There are two typical sub-varieties of untethered flight mills based on the type of data obtained: metabolic and photometric. Experiments that require metabolic data often involve placing an insect inside a closed mill with a known quantity of gas inside, which is then attached to a collection apparatus. After insect flight, the gases from inside the mill chamber are collected and analyzed to extrapolate the metabolic rate and energy used during insect flight [18, 19]. Photometric studies are more often run in a larger or taller container that is attached to a light interference sensor or other photometric device, which records data when the insect moves above or through a certain level in the mill. This data is potentially important due to the periodicity and height of the insect flight, which depend on a number of factors, including light, temperature, number of individuals in a given space, and the developmental or mated stage of the insect in question [20-22].

Some issues with untethered flight experiments include machining, expense, and precision. The machining of metabolic flight mills must be airtight and create a good seal in order to obtain accurate data for the purpose of the experiment. This issue is not as prevalent with photometric studies. The expense of the collection apparatus and substance analysis device for the metabolic flight mill, such as a gas chromatograph, may be cost prohibitive, while this is not likely to be an issue with photometric studies or those that utilize common laboratory materials, such as calcium sulfate (Drierite) [18]. In these metabolic studies using Drierite, the amount of CO_2 produced by a given insect may be measured, though this information has limited utility in advanced research. Sensor device sensitivity is a more relevant issue in photometric studies, as the insect may fly below the sensor, may trip or block the sensor accidentally or may display altered flight patterns due to limited or excessive space. Most importantly, some of these studies are monitored throughout the life of the insect. In absence of outside influence, insects may remain on the floor of the enclosure in order to feed instead of flying, hindering flight data collection. Thus, metabolic studies may require a researcher to agitate an adult

insect repeatedly, causing it to fly until it lacks the required energy to continue flight and even continue to agitate the insect until it is clearly dead or a given length of time has passed in order to collect as much of a given metabolite as possible. Insect stress may alter results obtained.

1.1.3 | Static Flight Mills

Unlike other flight mills, static mills do not allow for the relative movement of an insect, yet are used in the recording of insect flight patterns, wing movement, and other factors. The basic design of static mills is a stable platform on which an insect is affixed, which may be a pin or rod. These devices function by providing a stable region to which an insect is affixed, which may then be monitored via optical recording devices in order to determine either the behavior of the insect in response to environmental cues or the body of the insect and its movements in flight. One primary example of the use of these devices is the study of insect wing movements as they affect air currents. This type of study has been particularly used successfully with the *Manduca sexta* (Lepidoptera: Sphingidae) in studying the vortices caused by the motion of insect wings, potentially explaining the possibility of insect flight in the face of the formerly held belief that some insects should be physically incapable of flight, such as the bumblebee [1]. In this example, mills are generally created in the form of a wind tunnel, which provides a constant current of air over the body of the immobilized insect, stimulating and simulating flight [23]. Thus, while these studies may not be necessarily useful in terms of distance flown, speed of travel, or periodicity, they are still quite valuable. Through data obtained from static flight mill studies involving the use of flies and moths, researchers have been able to develop minute robots capable of sustained flight [3]. Further research on the motion of insect wings in flight and the movement of air currents as they are affected by the wing movements across time, is also of great potential importance not only for human flying machines but for greater understanding of fluid dynamics and aeronautics.

1.1.4 | Lever Flight Mills

The rarity of lever flight mills makes it difficult to compare the data collected with these devices to data obtained from other types of mills. Lever mills may come in two basic categories: simple and complex. A simple lever mill involves an arm attached to a pivot point, one end of which is connected to the insect and the other completes a circuit or activates a sensor when it makes contact. This method allows the insect a degree of vertical movement, but disallows horizontal motion. A mill used in this manner is best

suited for quantifying the tendency of the insect in question to make attempted flights, and to measure the amount of time that would be spent in flight [24]. In contrast, complex lever mills involve a high degree of machining and require a significant amount of calibration based on the size of the insect intended for study. These complex systems consist of a lever arm that hovers over a rotating barrel, both of which are attached to a counterweight system that is suspended in a liquid, often mineral oil, to increase lubrication and decrease friction. In these systems, when the insect takes flight, it lifts against the counterweight, which in turn pulls the rotating barrel away and allows the insect unimpeded vertical flight, while restricting horizontal motion [8].

The benefits of lever mills are largely in the ease of data collection. Data collection for simple lever mills is exceptionally easy, as a current or signal can easily be recorded into a digital format, and for complex lever mills, optical sensor suites are commonly available for use in laboratory settings. Both systems are robust, require little to no maintenance once constructed, and take little space overall. Additionally, a simple lever mill system is very easy and inexpensive to make with a high degree of accuracy, even with few available resources.

Drawbacks to lever mills largely involve the limited movement of the attached insect. While potentially useful for determining tendency and duration of flight, this is all that can be determined from the movement of the insect [24]. In a complex lever mill system, it may be possible to record the rotation of the barrel the insect rests on, thereby determining the distance traveled terrestrially, but it would be highly difficult to even qualitatively determine how far or fast the insect would have traveled in flight. The complex lever mill is also difficult to construct, and involves a great number of moving parts and careful measurements to mitigate the potential dual issues of insect weight and the counterweight that allows for flight and return to resting position. Finally, both systems are limited in the angles that they can achieve, which may create an unnatural angle at which the insect is required to fly for the duration of the experiment.

1.2 | Flight Mills in studies of Lepidoptera

In agricultural pests, the ability to disperse is important to avoid predators, move out of crowded living conditions, or seek out potential mates. While flight mills have been used with diverse groups of insects from aphids to honeybees [20, 25, 26], we would like to emphasize in this review their use with Lepidoptera (butterflies and moths) as key insect pests of diverse crops [27, 28]. While crop damage by

lepidopteran insects occurs by larval feeding, adults are the mobile stage responsible for spread of the pest. Adult lepidopteran insects are usually monitored by trapping using pheromones or other methods of bait or lure, and through radar monitoring of adult insects [29-31]. Data from monitoring allows forecast models [32], which are valuable in predicting infestations and allow proactive control measures. In the case of Lepidoptera, adult moths in the families Plutellidae, Noctuidae, and Torticidae have been of interest in flight studies [33-35]. These insects include meal moths and armyworms, which contribute to damage in dozens of different agricultural crops.

Although the first insect flight mill was the large 'roundabout' design for migratory locusts, it rapidly became apparent that similar apparatus would work with other insects [9]. Three years after the introduction of the first of the insect roundabouts, a short letter was published in Nature detailing the use of a much smaller and more simplified flight mill for use in Diptera, Hymenoptera, and potentially other insects [10]. This mill design was specifically intended for use in studying the metabolic rates and energy consumption of flying insects. In addition, since few insects engage in flight when stimulated on a static surface or otherwise held immobile, it was proposed advantageous to allow them some degree of movement to stimulate flight [10]. This small-scale flight mill appears to be the first of its kind published, and led quickly to the development of more complex and useful mills [4]. In presenting this small mill, it became obvious that metabolic and even extended flight studies could be performed in a controlled, laboratory setting.

From these basic designs, the insect flight mill branched quickly into its main four types, as detailed above. Each of these designs tends to be preferentially used in a distinct aspect of insect flight research, as determined by limitations of the design. For example, barring the use of sophisticated recording software, an untethered flight mill will prove inferior to a rotational flight mill for determining total distances flown. The primary two subjects studied with flight mills are flight behavior and metabolism.

1.3 | Discussion

Insect flight mills facilitate the understanding of the flight mechanics of insects. From their applications in the metabolic rates and energy consumption research, they are useful for nutritionists, geneticists, and agricultural experts. Studies regarding the flight period, distance, and speed are invaluable to agricultural producers and to ecologists. With this in mind, it becomes important to develop these devices in simple, robust platforms that may be used by researchers in low-income or geographically

isolated locations. Additionally, with the large number of designs that exist, it would be beneficial to have a single multi-faceted design that would allow a single researcher to perform any of the functions listed above, be it lever, static, or rotational in nature. A single design of simple construction, using readily available materials, would be beneficial in that it would reduce the amount of variability in the results provided by insect flight mills.

Chapter II

A Novel Software and Hardware Package for Recording and Visualizing Rotational Insect Flight Mill Data

This chapter to be submitted to Insect Behavior

Hietala, L., Hietala, N. and J.L. Jurat-Fuentes. (2018). A Novel Software and Hardware Package for Recording and Visualizing Rotational Insect Flight Mill Data.

My contributions included: (1) data collection and analysis, (2) writing the manuscript and making figures. Nickholas Hietala assisted with (1) and (2), Juan Luis Jurat-Fuentes assisted with (2).

Abstract

Rotational flight mills are commonly used to study flight behavior in insects. However, available software to record and manage flight mill data is outdated or has high commercial cost, and lacks portability or analytical capacity. In this study, we describe the development of hardware and software for a rotational flight mill design to study the flight activity of moths. A Raspberry Pi computer was chosen as the hardware platform due to its small size, minimal power requirements, and low cost. A Data collection software (MillCollector) was designed in Python programming language to record data from multiple flight mills and logging on a flash drive. The resulting data files were then analyzed through a new customized program named MothMill. Freeware availability of these software platforms at GitHub (https://github.com/nchietala/rpi-sensor-controller) should facilitate the recording and analysis of flight mill data, contributing to our understanding of insect flight behavior.

2.1 | Introduction

The use of rotational flight mills (RFMs) to record the dynamics of insect movement is well documented [6, 7, 36]. There are several methods to record the motion of an RFM which include light, electrical interruption or magnetic sensors [12, 23]. Currently used flight mill methods differ in the sensors, data collection and analysis programs, and differences in flight mill design (see Chapter I), make it difficult to directly compare data collected from diverse experiments. In addition, freely available software for flight mill data recording is severely outdated. To overcome this limitation and increase accuracy of data comparisons, we report development of a user-friendly platform and software to monitor insect flight and prepare automated preliminary data reports.

The RFM based on unipolar magnetic Hall-effect sensor [23, 37] originally described by Naranjo and Jones [12] was used as prototype. In this design, insect flight is monitored by the change in magnetic polarity induced by rotation of the RFM. By selecting this flight mill model, much of the necessary hardware design was already completed and therefore required no modifications. We focused on developing a method of determining the number of activations of the sensor, along with storage and analysis software that would create a visual or textual representation of the data collected.

2.2 | Materials and Methods

2.2.1 | Sensor computer

The sensor control computer selected for this project was the Raspberry Pi 3 (Raspberry Pi Foundation, UK), because the number of general-purpose input/output (GPIO) pins available for use and the ease of platform configuration. Of the 40 available GPIO pins, several perform alternate functions in the computer and were therefore unavailable for use. Consequently, 20 pins were selected as optimal for data collection and were programmed for this purpose.

2.2.2 | Data collection and computational hardware

The first sensor controller (prototype) constructed was a Raspberry Pi 2 B+. This module was housed in a commercial case designed to protect the Pi. A 62 by 12 port breadboard with 0.25cm (0.1”) pin pitch was attached to the top of this case (Figure 2.1). This was connected with jumper wires from the SPs to correspondingly labeled positions on the breadboard (Figure 2.2). GPIO pins 14 and 15 were attached to jumper wires leading to a switch and LED light with a 330Ω resistor, as well as wires leading from the 5 V and ground rails on the breadboard. This arrangement allowed the circuit to be assembled and for connection to the sensor controller for data collection. When the MillCollector software detected that GPIO pin 14 (physical pin 8) was in a low-voltage state when the switch is ‘off’, the software paused the recording of data and the USB drive used for data storage was unmounted so that it may be removed without corrupting data. GPIO pin 15 (physical pin 10) led to an LED that indicated whether the drive was mounted, writing, safe to remove, or missing with distinct patterns of blinking.

The original environment proposed for the duration of the flight mill experiment included high humidity, leading to an apparent drawback for the prototype design. Additionally, the prototype design proved

delicate due to the large number of exposed wires and parts. Consequently, a second version of the sensor controller (v1.0) was developed with a custom printed circuit board (PCB) cut to fit directly onto the GPIO header of the module (Figure 2.3). This design works correctly for the Raspberry Pi 2 B+ and Pi 3 models, and was secured in place using 0.32 cm (0.125 in) stainless steel bolts after enlarging the existing Pi module mounting holes, and held in place with tapped nylon spacers (Figure 2.4).

Aluminum risers (0.025-gauge aluminum sheeting) were adhered to the on-board processor and the smaller Ethernet controller. A 2.54cm (1 in) wide section of 0.025-gauge aluminum sheeting was folded to fit the Raspberry Pi and adhered to the aluminum risers. Approximately 4.44cm (1.75 in) of this aluminum sheeting was left protruding beyond the PCB on the side opposite the USB ports. Low-temperature hot-melt glue was used to increase the rigidity of the aluminum sheeting. Small sections of aluminum heat sink were adhered to the protruding aluminum to reduce heat buildup inside the v1.0 (Figure 2.5).

To each of the bare SPs (sensor pins), a hard attachment point was added to allow the three-wire connectors from the magnetic Hall-effect sensors access to the pins and data collection. The female attachment points for the wires (Alpha Wire, product no. 80009) allowed for a male jumper wire terminal or bare wire, and were arranged as 1: sensor, 2: 5 volt, and 3: ground.

Anhydrous calcium sulfate (Drierite, WA Hammond Drierite Co Ltd) was placed into the module, and the entire device excluding the heat sink and switch was shrink-wrapped. This was done to prevent moisture infiltration and to absorb as much native moisture in the area as possible (Figure 2.6). Holes were cut and taped down to allow access to a single USB port, micro-USB port, and RJ45 port. The USB port is necessary for the data collection process, and holds the data that results from attachment to RFMs. The micro-USB port provides power to the Pi module, and when unplugged resets the module for the next replicate or data run. An empty male RJ45 terminator is plugged into the RJ45 port to keep it clean and dry. This allows the module to be accessed via Ethernet for maintenance or to modify the software if necessary. Pilot holes were punched in the top of the shrink-wrapping in order to allow the male wire terminals easy access to the SP connection ports. It was determined through preliminary testing that the addition of the Drierite was not detrimental to the function of the device.

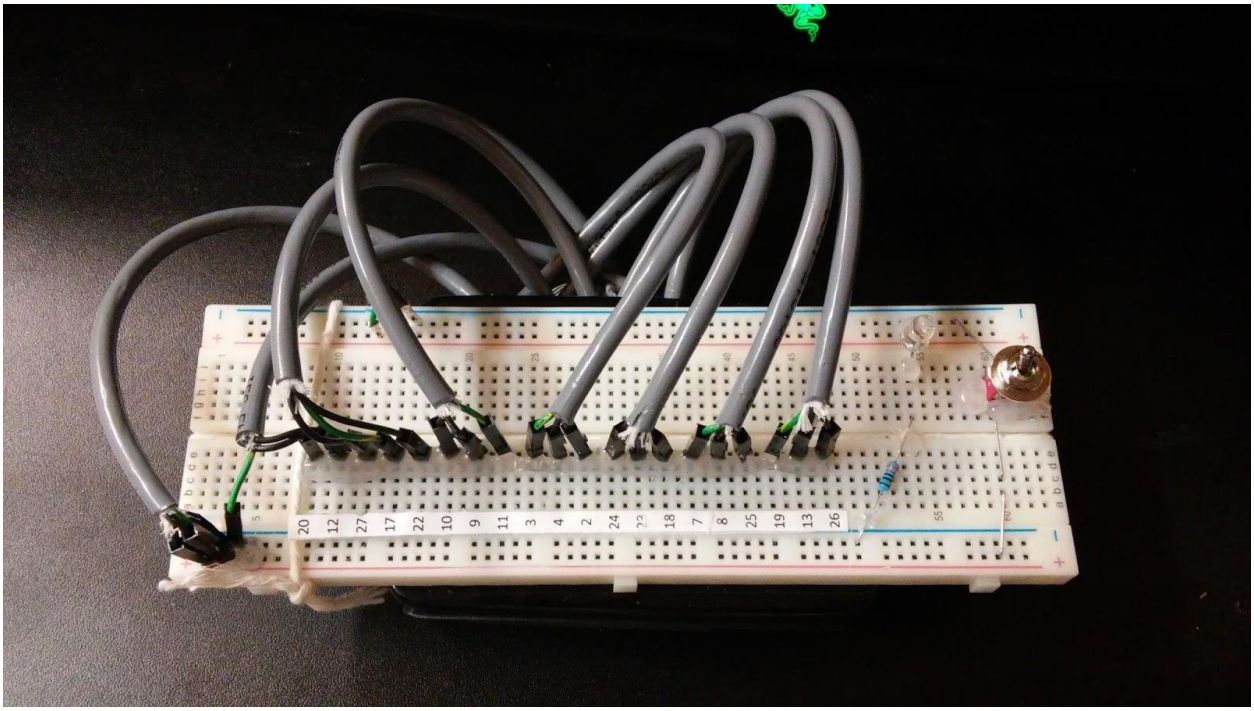


Figure 2.1- Raspberry Pi hardware v.0. A Raspberry Pi computer with a commercial breadboard serving as the hardware interface with the mill sensors.

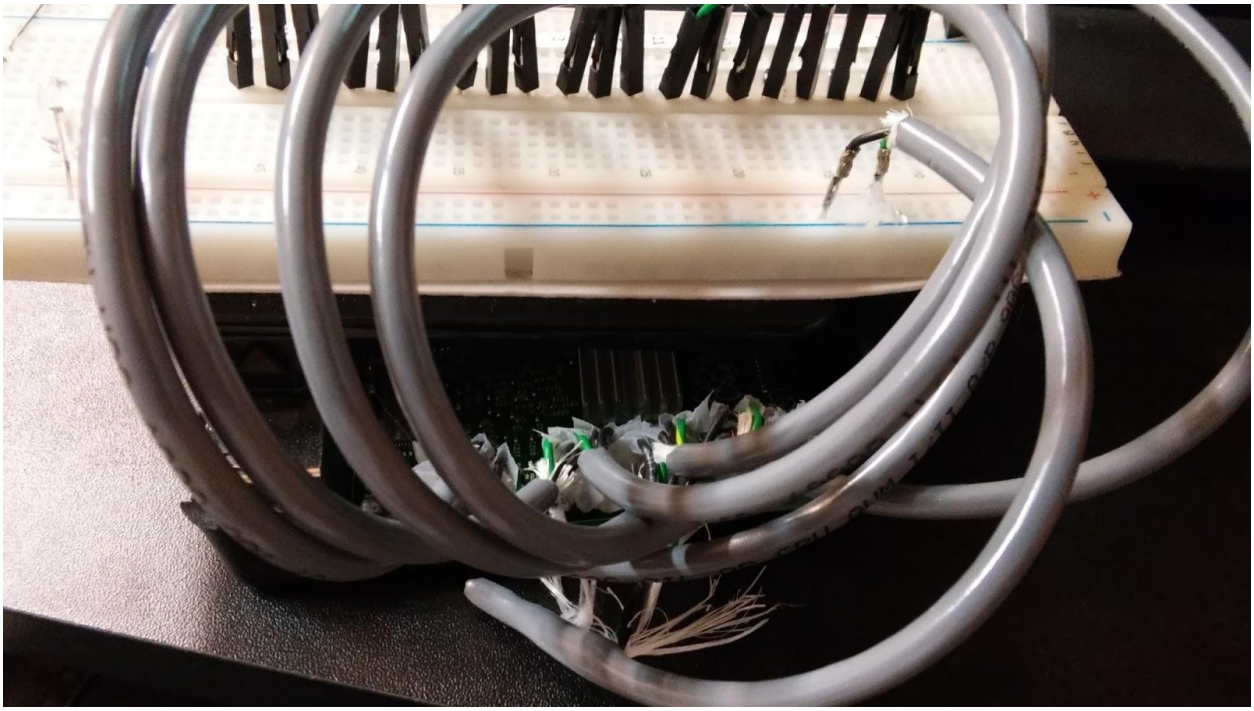


Figure 2.2- Raspberry Pi hardware v.0, back view. This view of the Raspberry Pi hardware version 0 details the connections between Pi circuitry and the breadboard.

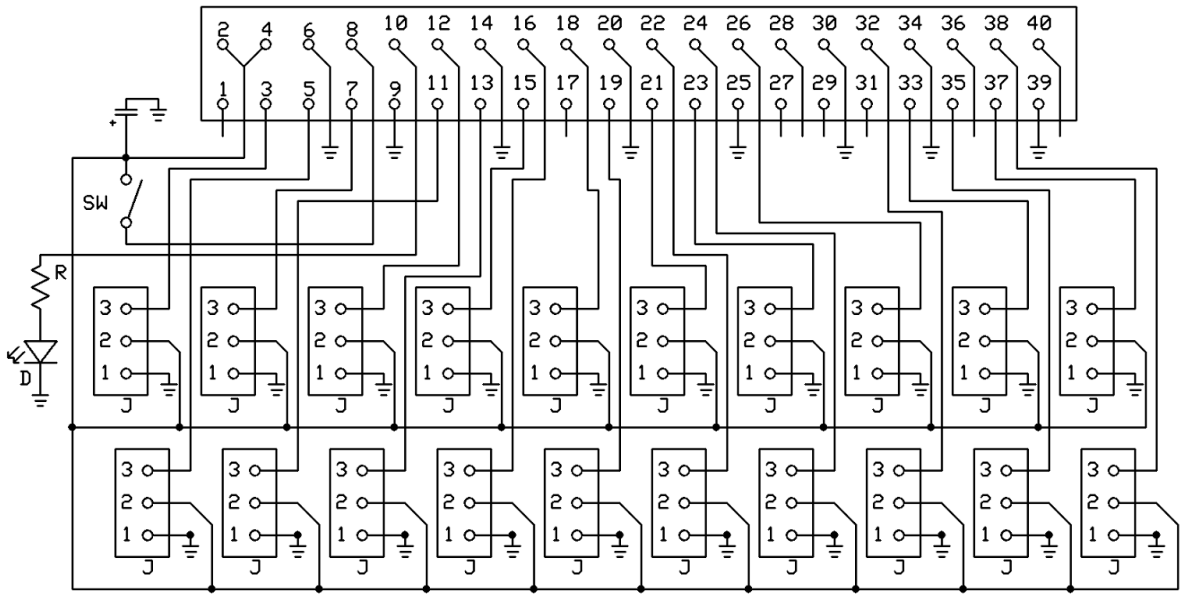


Figure 2.3- MillCollector circuit board diagram. Circuit board diagram of the top plate of the Raspberry Pi computer module, including channel pathway to each wire port.

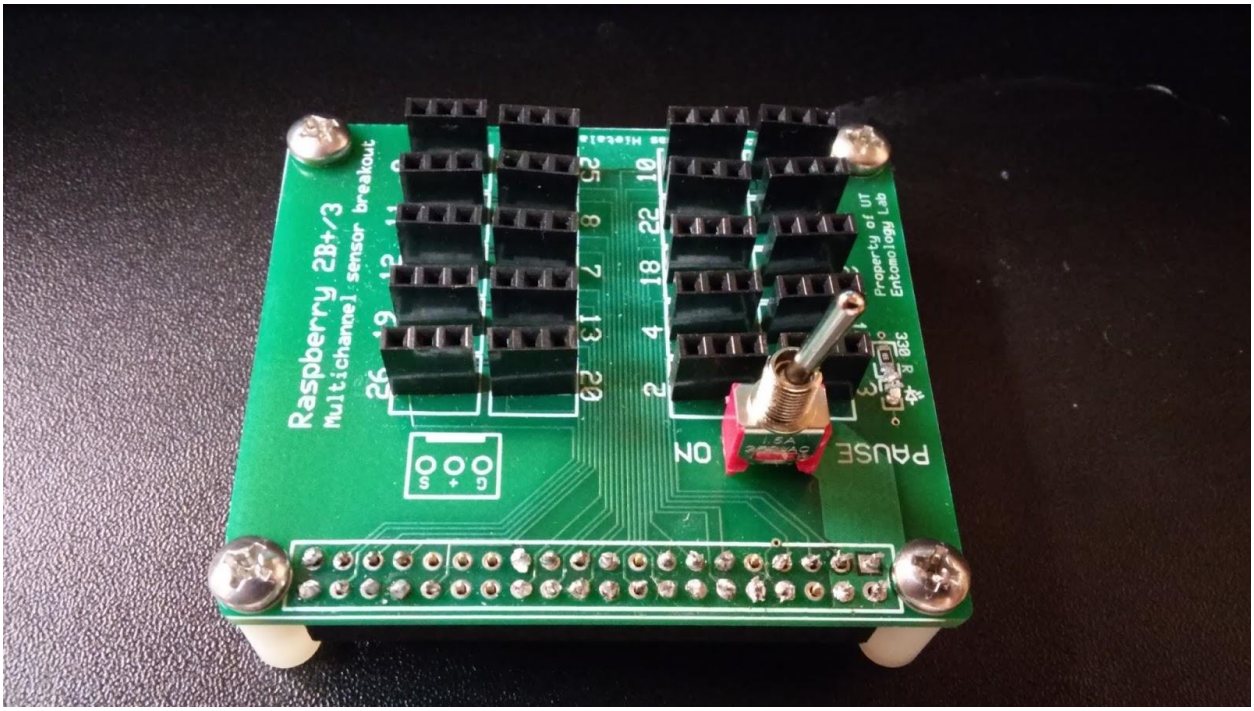


Figure 2.4- MillCollector circuit board, assembled. Raspberry Pi custom circuit board assembled, showing female connection ports, switch, LED, and printed notation to show pin configuration.



Figure 2.5- MillCollector heat sink and internals. This image displays the internal configuration of the MillCollector unit, including the aluminum heat sink affixed to the motherboard.



Figure 2.6- MillCollector Raspberry Pi computer, wrapped for use. The above image displays the MillCollector computer unit within a protective wrapping layer. Holes are pre-punctured in the wrapping to accept wiring from the flight mill units to the computer.

2.2.3 | Data collection software: MillCollector

The MillCollector software was programmed to respond to GPIO pin numbers 2, 3, 4, 7, 8, 9, 10, 11, 12, 13, 17, 18, 19, 20, 22, 23, 24, 25, 26, and 27, which corresponded to physical pin numbers 3, 5, 7, 24, 26,21, 19, 23, 32, 33, 11, 12, 35, 38, 15, 16, 18, 22, 37, and 13, respectively. These pins are hereafter referred to as the sensor pins (SP or SPs). When a SP is activated for the first time, the MillCollector program writes the current Unix Epoch Time to an output text file. Unix Epoch Time (or Unix time, or epoch time) is a timestamp commonly used in computing and defined as the number of seconds that have passed since midnight on January 1, 1970 UTC [38]. Each subsequent SP activation results in a recording in the output text file of the number of seconds (within 0.02 seconds) since the last activation of that same pin. Thus, if at the beginning of an experiment, a moth activated a SP at 2:30:15.76 pm on January 1 2017, the resulting timestamp would be 1483281015.76 (±0.02). If it took 1.2 seconds to complete one revolution, the next note in the document would be 1.2 (±0.02) and be separated from its previous number by a semicolon (;). This is the format required by the MothMill program in order to analyze data collected. The ±0.02 second margin of error is a non-compounding error that occurs when multiple SPs activate less than 0.02 seconds from one another. These errors therefore do not aggregate over time, and if a single record is off its subsequent record will compensate by being off by an opposite amount. This timestamp recording method is highly accurate and uses a 64-bit integer that should function correctly for almost three billion years.

2.2.4 | Data analysis software: MothMill

The program written to analyze the data that is presented by the magnetic Hall-effect sensor output (hereafter referred to as MothMill) was written in Java, due to the availability of tools that are present with that system. By creating a .jar file and a .batch file, it becomes extremely user friendly to activate and run this program, while reducing the ability of a researcher to accidently alter the results of the experiment. The Java program is activated by a batch file using the command: java- jar “MothAnalyzer.jar”.

The MothMill program provides the researcher the option of running a “discrete” or an “aggregate” data analysis. In the discrete data analysis, the program searches for any channel data files produced by the module, performs a series of mathematical operations to refine that data into readable information, formats it into text, draws a graph of how moth speed changed during test time, draws a frequency distribution of how long the insect flew, saves that text and those graphs to a PDF using an open-source

Java library called Apache PDFbox, and serializes and writes the information used to make those graphs to an ".aggr file" that can be used to perform an aggregate data analysis. Both the PDF and the .aggr files are saved to the directory containing the software itself in the memory drive. The .aggr files are renamed from the initial channel naming scheme produced by the Raspberry Pi based on the epoch start time of the channel file that each .aggr files is associated with. While multiple data runs using the same channel cannot be processed in the same discrete analysis due to file name limitations, they can be processed in the same aggregate data analysis.

When the researcher performs an aggregate data run, the software will search for any .aggr files from discrete data runs. The data contained in the .aggr files is mathematically processed to calculate means for flight parameters (number of insects, experiment duration, distance, speed, speed in flight, percent time in flight, longest flight duration, longest rest duration, and longest flight leg distance, and uses these data to build a PDF file similar to the ones made for a discrete analysis. This PDF is saved to the location that contains the software itself. Specific steps in this process for each individual dataset include analyzing MillCollector data using MothMill through the discrete function, and deleting all data in the MothMillDataProcess folder except .pdf channel outputs and .aggr files. Once this is performed for all datasets the generated files are analyzed using MothMill- aggregate.

The resulting aggregate file will contain data from all of the channels from MillCollector that were used, but will only have a maximum of one discrete file per channel, using the last set of data that was run through the program. Thus, it is recommended that individual discrete files be generated for each data run, and copies of the MillCollector output files be used for these purposes, as such files may be deleted as necessary.

2.3 | Results and Discussion

We provide new software tools for the collection an analysis of flight mill data. Previous alternative RFM methods required the use of separate hardware units to convert motion into data and for data collection [39]. In that type of setup, a separate computer was required to act as a sensor controller and data storage unit. The ability of the Raspberry Pi in the current design to remove this excess hardware and software interface results in a compact device that directly collects Hall-effect sensor output. Initially, the Raspberry Pi computer module presented several hardware issues in interfacing with the magnetic Hall effect sensor, likely the result of the connectors being placed in close proximity to each

other. Hardware issues of this variety were resolved through the use of insulation around all electrical contact points around the sensor. The interface was further improved through the individual calibration of sensors in distance from each magnet. The connection between each sensor, sensor wire, and Raspberry Pi was ensured through physical testing and then all wires were secured in order to ensure that movement did not detach sensor wires from the sensor or Raspberry Pi.

The software program is easy to modify by the programmer and easy to apply by the end-user. The sensor pins (SPs) in the Raspberry Pi were selected for use as a result of extensive physical testing, which concluded that GPIO pins not listed above may provide inaccurate data without costly and time-consuming reprogramming of the Raspberry Pi operating system. This potential for data inaccuracy was due to the pins being natively aligned to alternate purposes, therefore being prone to occasionally send or receive data unrelated to the experimental process. It should be noted that this potential for inaccuracy may not be ubiquitous among Raspberry Pi units, and should be tested for each unit. To determine if a pin may be used when attempting to program the Pi using MillCollector, a pin that is natively aligned to an alternative purpose will return an error message stating that the pin is already in use. To circumvent this, replace the pin in question with an alternative one until no error message is returned.

The readily accessible wiring instructions available on the Pi circuit board greatly reduce the possibility of user error when connecting the sensors with the Raspberry Pi. The rugged connection of the circuit board to the Raspberry Pi computational module also reduces the possibility of issues resulting from the connection between circuit board and computational module.

The MillCollector program was designed to gather data from magnetic Hall-effect sensors through detection of the voltage increase caused by a decrease in the magnetic field strength around the sensor below its magnetic release point. At that time, the sensor no longer draws voltage from its GPIO pin, and this “rising edge” detection allows the sensor to record a single activation per rotation. In this way, the hardware prevents anomalous data resulting from unchanging low-voltage that may result from the active magnetic arm resting above the Hall-effect sensor. MillCollector is a completely ‘headless’ system, i.e., it requires no keyboard, mouse, monitor, or other traditional computer hardware components with the exception of a single switch and the insertion of an empty flash drive.

Use of the MothMill program provides highly visual representations not only of the movement of the target organism at any given time (Figure 2.7), yet also of the organism’s movements across time and

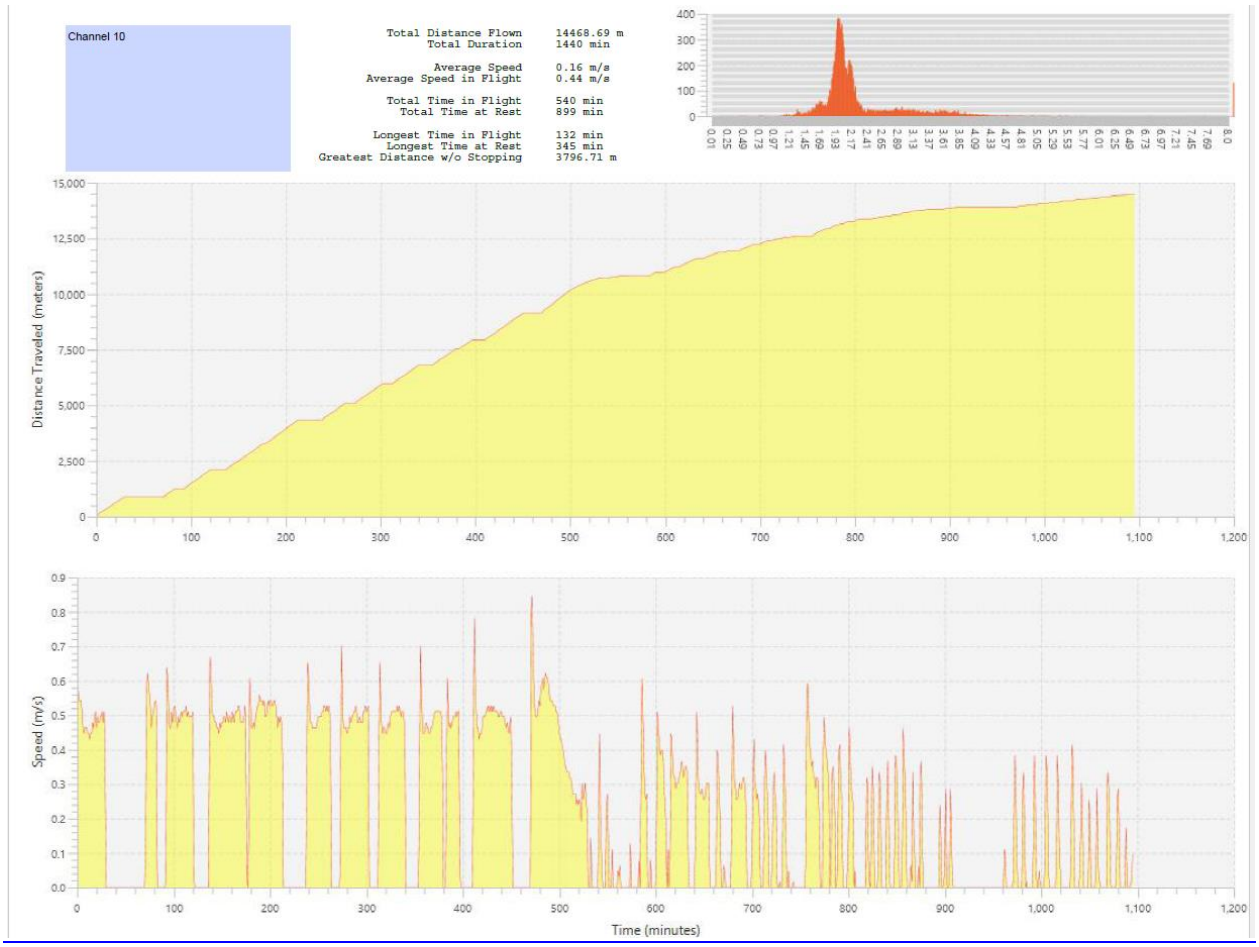


Figure 2.7- MothMill discrete output example. From left to right and top to bottom, this output displays the channel number, a set of mathematical outputs generated by the moth as interpreted by MothMill, and the number of rotations that required a given number of seconds to complete them. The top graph shows the total accumulated distance the insect traveled in meters (y axis) and the total time that the insect had been applied to the flight mill (x axis), with a line graph detailing progression. The bottom graph shows the speed of the insect in meters/second (y axis) at any given time that the insect was on the mill (x axis).

frequency and duration of those movements. The data output also provides a numerical readout of the exact times and distances recoded for test organisms. Issues that were initially detected on the numerical or visual readouts were rapidly corrected, and the output PDFs are a useful tool in comparison between individuals. Similarly, the aggregate PDF allows for a rapid comparison between individuals with data collected through the MillCollector program (Figure 2.8). The resulting RFM and associated software is available as freeware for use in actographic flight mill experiments at GitHub (https://github.com/nchietala/rpi-sensor-controller).

While the system presented is robust and inexpensive to develop and implement, a limitation is the number of insects that may be monitored on RFMs at any given time (20), which is given by the number of available GPIO pins not natively aligned to functions that may interfere with data collection. An increased number of tested insects per replicate could be achieved using a Raspberry Pi Compute Module as the hardware platform, due to the larger number of available pins. This module can then be used with an identical RFM hardware package to increase the number of mills available at a given time, or be advanced to a six-pin system. A six-pin system offers support for superior visualization of individual mill function via a LED for live feedback to the researcher, causing a brief flash during each recorded instance of data collection. In this way, an immediate operational feedback would be available to determine the function of each mill and to verify that data is being recorded accurately over time. A six-pin system would also allow access to multiple voltages for each sensor connector, allowing the operation of a wider range of sensor devices, and the use of a CAT 3 wire to record data. This wire would also allow for the use of RJ11 connectors, rather than jumper leads, which are less prone to user error and more resistant to electrical shorts. While the size of the board for data collection would increase significantly from approximately 100 cm2 to approximately 400 cm2, the result would be an easier and more secure connection of mill to computer hardware. For instance, during experimentation with flight mills it was noted that even minor physical interference with the Pi hardware could cause disconnection between the mill wiring, occasionally leading to the necessity for complete wiring detachment and reattachment.

In the case of either CAT 3 cable or the current wiring paradigm, a new design of customized breadboard is necessary, and a reduction in the size of spacing screws should be made to 2.5 mm to negate the need to enlarge the existing mounting holes on the Pi module. This, in combination with the low cost of Raspberry Pi Compute Module makes it possible to inexpensively design hardware for a large number of simultaneous flight mill projects at a fraction of the cost of a single computer system. With the MothMill

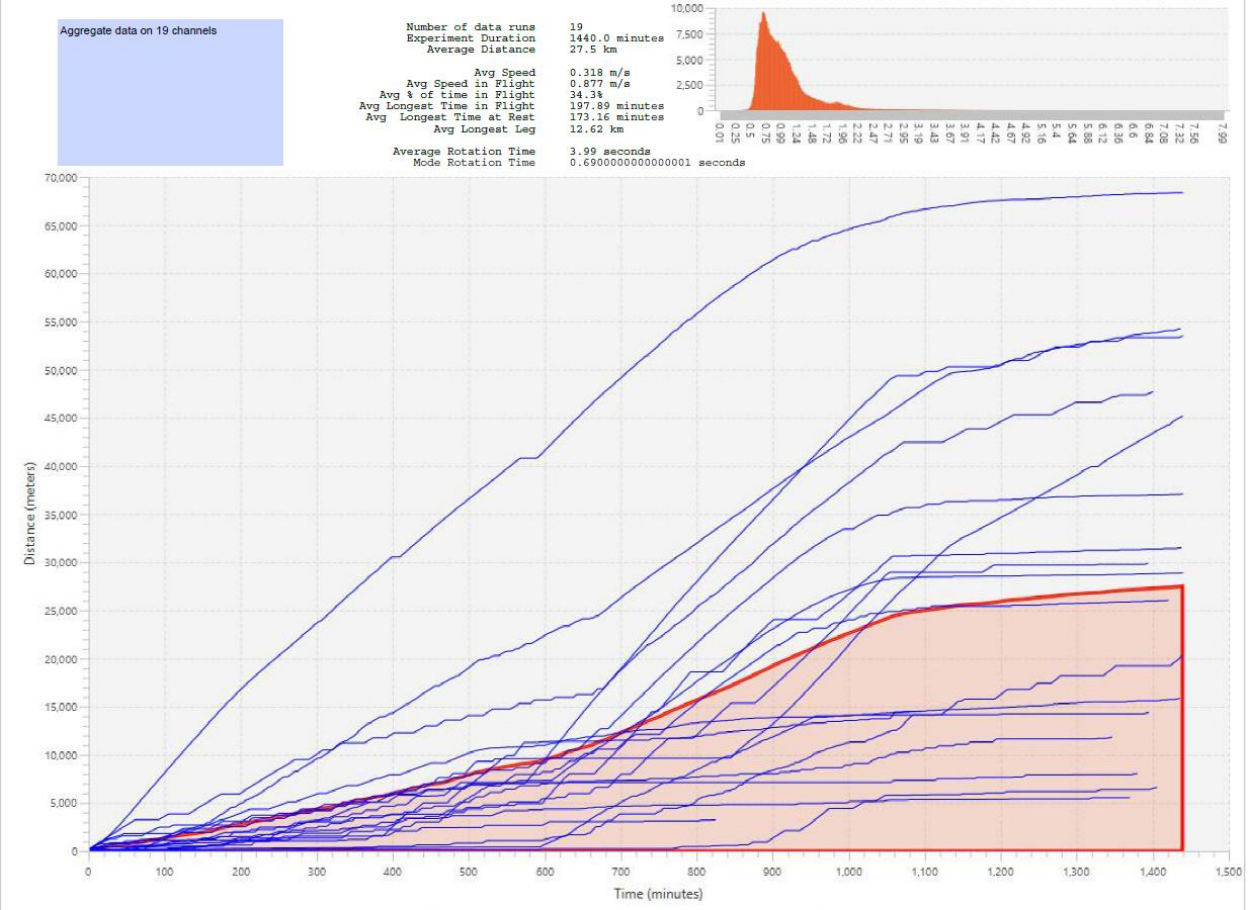


Figure 2.8- MothMill aggregate output example. From left to right and from top to bottom, this output displays the number of channels used for the aggregate data, a set of mathematical outputs generated using the means of all channels used, and the number of rotations that required a given number of seconds to complete them. The large graph details the total distance traveled in meters (y axis) over the duration of the experiment (x axis) for each insect. Each individual is shown as a blue line, while the average insect is shown as a red line with a filled lower area.

analysis software, it is therefore possible to create parallel projects involving large numbers of insects using similar RFMs on a limited budget.

2.3.1 | Troubleshooting

During testing, issues were detected regarding the Raspberry Pi-mill interface and the MothMill program. Below are the most common issues that arose and methods used either to bypass or to determine the simplest method of repairing unresponsive mills (e.g., mills that did not produce a data file when the Hall effect sensor was stimulated):

1. Hall effect sensor is not seated correctly. This may be fixed by switching the Pi to 'pause' and either more firmly seating the sensor wires or removing and replacing the sensor. Ensure that the sensor is aligned correctly.

2. Hall effect sensor is faulty. This may be determined by replacing a Hall effect sensor from a mill that is known to record correctly with the one in question and determining mill recording activity. If it records normally, then the problem lies elsewhere.

3. Wires are not seated correctly. This may be fixed by switching the Pi to 'pause' and either more firmly seating the male ends of the sensor wires into the Pi female connectors, or removing and replacing the wire connectors. Ensure that the wires are aligned correctly.

4. Wire male and/or female ends are not making correct contact with the Pi/sensor. This may occur when the wires are making insufficient contact with the male or female adapters. Due to the negligible cost associated with these connectors, it may be desirable to remove and replace all of the associated pieces rather than attempt to readjust them if this is identified as the issue.

5. Wires are incorrectly labeled. This may occur when all of the portions of the three-part wire are similar in appearance. Ensure that all parts of the wire are correctly labeled and aligned in the Pi connectors and on the sensor.

6. Wires are making incomplete contact with the Pi board. This problem is more detrimental, as it requires the Pi connector to be removed and re-soldered. It is therefore recommended to test the sensor and wire alignment and seating before this is attempted.

Chapter III

Alterations to Flight Behavior of Cry1Fa-Resistant and Susceptible *Spodoptera frugiperda* in Response to Feeding on Cry1Fa Protoxin

This chapter to be submitted to Insect Behavior

Hietala, L., and J.L. Jurat-Fuentes. (2018). Alterations to Flight Behavior of Cry1Fa-Resistant and Susceptible *Spodoptera frugiperda* in Response to Feeding on Cry1Fa Protoxin.

My contributions included: (1) experimental design, (2) data collection and analysis, (3) writing the manuscript and making figures. Juan Luis Jurat-Fuentes assisted with (1) and (3).

Abstract

The use of flight mills is a well-documented method for determining the flight capacity of insects in the laboratory. Data collected from these mill assays may be combined with data from field studies to predict the periodicity and locality of insects in agricultural situations. The fall armyworm, *Spodoptera frugiperda*, is an important pest of various field crops, including corn, soybean, and cotton. Importantly, this insect displays wide yearly migratory range in North America, traveling from southern overwintering sites to Canada. Populations of *S. frugiperda* have developed field-evolved resistance to transgenic corn producing the insecticidal Cry1Fa protein from the bacterium *Bacillus thuringiensis* (Bt). As related insects show increased long-distance flight tendencies when exposed to sublethal Bt toxin doses, we hypothesized that the same phenomenon would be observed for Cry1Fa-resistant *S. frugiperda*. In this study, we used flight mills to test the effect of feeding on Cry1Fa protoxin on the flight behavior of resistant *S. frugiperda* compared to susceptible moths or insects fed on control diets. Results from the flight mill study showed that only pupal weight had significant impact on the flight of adult *S. frugiperda* (p=0.0009), with increased pupal weight resulting in increased flight distance. Pupal weight in turn was influenced by strain, diet, and treatment, but these effects were not found to affect flight distance. These results suggest that Cry1Fa-resistant *S. frugiperda* will not display greater flight behavior than Cry1Fa-susceptible insects under similar conditions in field settings.

3.1 | Introduction

Several species in the *Spodoptera* genus (Lepidoptera; Noctuidae) are known to migrate for long distances, including *Spodoptera exigua*, *S. litura*, *S. frugiperda* and *S. deprevata* [40-43]. According to

previous haplotype mapping and trapping data, *S. frugiperda* displays periodical annual migration in the United States [33, 44-46]. Larvae of this species are a cosmopolitan pest of importance in the United States due to the damage they cause to corn, cotton, and soybean, and pastures among other crops [40, 44, 47, 48]. This wide host range allows them to move between large concentrations of favorable food sources during their migration. In the late fall, winter, and early spring, these insects are primarily found in the southern regions of Florida and Texas, where they overwinter. As the weather warms, they migrate north in two main populations, separated by the Appalachian mountain chain [33, 49]. During an average year, these insects will reach Canada before being killed by cold temperatures and dying back to the overwintering population sites [44, 46].

As an insect of agricultural importance, *S. frugiperda* has traditionally been monitored via pheromone trapping and field sampling [47, 49] and are frequently used in conjunction with predictive models of insects based on average yearly temperature to allow [50] growers to time the applications of insecticides. Currently, control of *S. frugiperda* is often achieved through the use of Bt-transgenic plants (cotton, corn, and soybean) producing Cry toxins derived from the bacterium *Bacillus thuringiensis* [51, 52]. However, the wide use of transgenic crops producing Cry toxins has led to the evolution of resistance in insects that use crop plant species as developmental hosts. Specifically, resistance to the Cry1Fa toxin produced in Bt corn has been reported in populations of *S. frugiperda* at different locations around the world [53-55]. These insects are capable of completing development on these transgenic crops [56, 57], increasing the need for traditional insecticide treatments.

The development of resistant *S. frugiperda* populations becomes more important when considering migratory behavior in light of other insects in the genus.Populations of *S. exigua* that were raised on diet that contained a marginally effective Cry toxin, Cry1Ac, exhibited increased long-distance flight as adults [55, 58, 59]. If this holds true for *S. frugiperda*, the possibility of increased long distance flight triggered by feeding on such marginally effective toxins poses a threat to the spread of resistance and challenge established models for temporal and spatial distribution. Thus, *S. frugiperda* populations resistant to Cry1Fa may display increased migratory tendency after feeding on corn expressing Cry1Fa, creating spatial cohorts of insects that defy model assumptions with migrating populations having higher percentages of resistance alleles contributing to the spread of resistance alleles. Such a cohort may allow for the rapid generation of large numbers of resistant insects, lowering the efficacy of Cry1Fa-producing transgenic crops. Should resistance arise in overwintering populations in Florida and Texas,

the insects may be exposed to these toxins earlier in the year, thus increasing migration and frequency of resistant insects [60].

In order to determine if resistant *S. frugiperda* possess an increased long-distance migratory tendency when exposed to Cry1Fa toxin, we conducted laboratory studies of their flight capabilities. The use of rotational insect flight mills was selected as a rapid method to measure the flight capacity of resistant insects that had been raised on larval diets containing Cry1Fa toxin or Cry1Fa-producing corn versus control diet and non-transgenic corn, respectively. In addition, we compared flight capabilities of susceptible and Cry1Fa-resistant *S. frugiperda* when fed on meridic diet or conventional corn.

3.2 | Materials and Methods

3.2.1 | Flight mill design

The rotational flight mill (RFM) design selected for this project was based on a model currently used to research the movements of the codling moth, *Cydia pomonella* [7, 12, 61]. Design modifications from the design by Jones et al. [12], are listed below. References to Figure 3.1 are listed in parenthesis. The base consisted of three major parts from the lowest to the highest point on the mill (Figure 3.1): The base, the pillar, and the head. The bulk of the base was formed by a triangular polycarbonate resin (Lexan) sheet measuring 16 cm on a side and 6.35 mm in thickness (1). Mill base adjustment was achieved through the use of #8 course-thread wood screws with low temperature hot melt glue on the distant end, placed approximately 1.5 cm from each point of the base (2). No change was made to the acetyl rod (3), but the drill bit size for the dorsal hole (4) was found to be too small to fit the axis (13). This error was corrected by using a no.69 drill bit in a 90° vertical drill jig. Perfect alignment of the axis was corrected through manual bending at the base end. The body ring magnet (5) was attached to the top of the acetyl rod (3) with low-temperature hot melt glue. Placement of switch magnets (6) was oriented to the ventral side of the support pins (7) with opposite poles directed downwards. The top ring magnet (8) was attached to the bottom of the Teflon rod (9) with cyanoacrylate glue to ensure that the magnet and rod remained attached with as little added weight as possible. The diameter of the 22-gauge hypodermic tubing proved too large for the use of a no.70 drill bit, as originally specified in the schematics for the head axis hole (10). A no. 68 mechanic's drill bit was used instead to obtain magnetic levitation while not interfering with the free turning of the head. A slot was cut in the top of the Teflon rod (9) at a depth of 0.5 cm (11) to allow the flight arm (12) to rest without remaining captive and

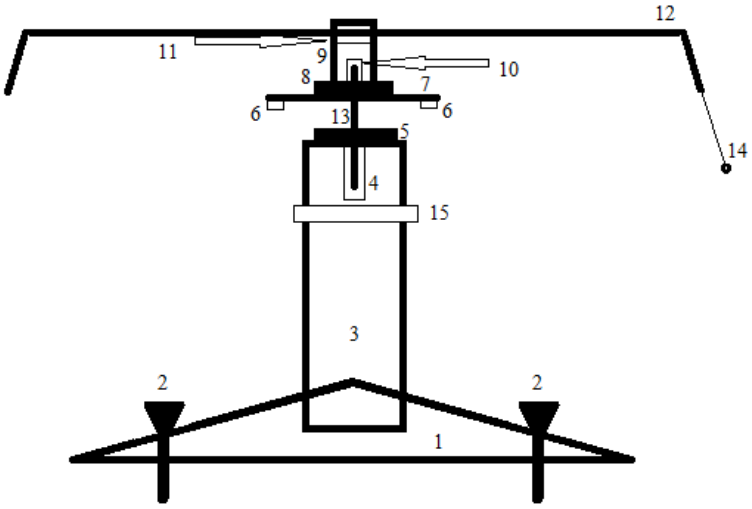


Figure 3.1- Simplified flight mill diagram. This image is derived from the original and modified flight mill design [12]. 1: base, 2: thumb screw (far screw not shown), 3: acetyl rod, 4: rod axis hole, 5: body ring magnet, 6: sensor magnet, 7: sensor magnet support pins, 8: head ring magnet, 9: Teflon head rod, 10: head axis hole, 11: flight arm retention slot, 12: flight arm, 13: axis, 14: insect counterbalance or attachment pin, 15: sensor retention device.

allowing easy removal in case of repairs. Size 2 insect pins stripped of paint on their distal ends (14) were used to attach insects to the flight arm (12). The zip tie (15) was replaced with laboratory tape, providing easy access to the wiring and providing a wider base of connection to the acetyl rod (3) while not interfering with the magnetic or electronic mill parts.

In addition to these parts, each mill arm possessed a pin on the opposite side of where the moth was attached. The original design called only for the pin attaching to the insect, which may be due to the size and weight of the insect for which the design was intended. However, due to the more robust size of *S. frugiperda*, a no. 2 insect pin attached to the cap of a microcentrifuge tube was used as a counterbalance. Each of these cap-pin complexes averaged 140 mg (n25), which was similar to the weight of an adult *S. frugiperda* moth fed on artificial diet (n386, 180.4mg, standard deviation 36.2mg). The hard point (17) used to attach moths to one of the distal ends of the flight arm (12) was a unique addition to the mill design. This hard point consisted of a section of plastic cut into a section 7 mm long and 0.33 cm wide pieced at one end by the insect attachment pin (14). The opposite end of this hard point had the 'hook' portion of an adhesive-backed Velcro brand strip. Each 'hook' portion possessed four rows of 13 hooks for a total of 52 hooks per hard point.

3.2.2 | Insects

Eggs of the Benzon strain of *S. frugiperda* were purchased from Benzon Research Inc. (Carlisle, PA). This strain originated from field-collected moths in Pennsylvania, and are susceptible to the Cry1Fa toxin [62, 63]. The 456 strain of *S. frugiperda* originally collected from Puerto Rico in 2010 [53, 58] was used to generate a near-isogenic colony with the Benzon strain by crossing and selection of larvae at the F2 generation on transgenic corn producing Cry1Fa or artificial diet contaminated with 4.75µg/cm2 of Cry1Fa protoxin as described elsewhere [64]. These two selection methods resulted in the strains 456LS4-C (selected on Cry1Fa corn) and 456LS4-D (selected on diet with Cry1Fa protoxin). Both these resulting strains were highly resistant to the Cry1Fa toxin [56].

All larvae were incubated at 27+-2°C, 60+-5% RH and a 16:8 L:D photoperiod until pupation. Pupae were removed from the rearing container, sexed, weighed, and placed into empty 30 ml (1 oz) translucent plastic cups. Sex was determined based on the morphology of the last three abdominal segments [67], and weights were measured using a Denver Instruments analytical balance to the nearest tenth of a milligram.

3.2.3 | Larval feeding treatments

Neonates larvae were reared on either meridic diet, with or without Cry1Fa protoxin, or on leaf tissue from corn hybrid TC1507 producing Cry1Fa toxin and its non-transgenic isoline (2T777). The beet armyworm meridic diet used was purchased from Frontier Scientific (Logan, UT). Insects reared on this meridic diet were used during the standardization of flight mill operation. Neonates from the Benzon and 456LS4-D strains were reared on meridic diet in individual wells of 128-well bioassay trays (Frontier Scientific). The Cry1Fa toxin treatment for resistant insects consisted of diet that was surface-contaminated with 4.75 µg/cm2 of Cry1Fa protoxin produced and purified as described elsewhere [65]. This protoxin dose was selected based on preliminary bioassays representing the LC99 for Cry1Fa protoxin in the Benzon strain (data not shown).

In treatments using corn (*Zea mays*) leaf material, neonate larvae were fed leaf sections from Cry1Fa-producing (TC1507) or a non-transgenic isoline (2T77). Corn plants were grown following best agronomic procedures in greenhouse planters and leaves were harvested at the V3-5 stage and washed using a mild soap solution to remove potential pathogens. After drying, the corn leaves were cut into 6-9 cm^2 sections and placed into 30 ml (1 oz) plastic cups containing a surface layer of 1% agar to maintain moisture. The corn leaf sections were replaced as necessary in order to maintain a constant supply of food for the insects. Resistant insects reared on TC1507 corn were taken from the 456LS4-C sub-strain, which contains approximately 111ng/mg of Cry1Fa protein in leaf tissue according to the safety report published by Dow Agroscience.

3.2.4 | Adult anesthesia and attachment

Adult *S. frugiperda* were used within 12-18 hours of eclosion, allowing the moth to fully expand its wings and perform excretory activities before being used. Moths were randomly selected, consisting of equal numbers of males and females. Moths were anesthetized for 7-9 seconds using a mixture of 1.5 g of dry ice with water as the source of CO_2, based on significant alteration to insect behavior and physiological effects during longer CO2 anesthesia [66, 67]. After anesthetization, insects were immediately exposed to atmospheric conditions and their dorsal thoracic scales were removed using a natural or soft synthetic hair paint brush with repeated brushing motions from front to back. When increased stiffness of the bristles was required, the brush bristles were wrapped together using laboratory parafilm to reduce the amount of length over which the bristles flexed.

In order to attach an adult *S. frugiperda* cleared of dorsal scales, a section of adhesive backed Velcro 'loops' approximately 1.75-2mm wide and 4-5mm long was applied to a hard point. The protective sheeting was removed prior to insect anesthetization and scale removal in order to expedite the process and allow for maximum reduction in time spent in a CO_2 rich atmosphere. During scale clearing, the Velcro section was kept inside a sealed container in order to reduce the adhesive's exposure to scales that could weaken retention to the insect. The adhesive backing was then applied to the thoracic chitin and gently pressed from each direction to ensure complete coverage, and allowed to rest in a 30 ml (1 oz) plastic cup until used (25-45 minutes).

All flight monitoring experiments were performed in a dedicated room at 24-27°C and 45-50% RH, and a 16:8 L:D photoperiod provided by two fluorescent light ballasts set 1.25 m above the base of all RFMs. Excessive air flow in the room was prevented through the use of a pair of baffles to prevent accidental rotation of the flight arm.

Insects on hard points were randomly assigned to RFMs. These insects were attached via the sharpened end of the no.2 insect pin stripped of black coating affixed to the free end of the flight arm. Each insect was adjusted to an 80-90° angle from the mill arm to optimize force provided by the insect before the head was released from researcher control. After all insects had been attached to hard points, the Raspberry Pi computer module was turned on with an empty flash drive installed. After the indicator light turned on, the power switch was turned from 'pause' to 'on'. The Pi then entered its run/record state and approximately 3-5 seconds later a blue LED light turned on and indicated readiness to record.

3.2.5 | Flight monitoring hardware

All flight mill computer hardware and its associated software interface is described in Chapter 2. Moth flight data was collected using a customized Raspberry Pi 2 B+ (Pi) computer module equipped with custom software (MillCollector). The Pi Computer was equipped with a switch, LED indicator lights, heat sink, shrink-wrap covering, desiccant, and a USB port that supported data logging on a flash drive. The circuit board provided female attachment points for sensor wires, and supported the LED and switch. The entire Pi module, with the exception of an RJ45 port, micro USB port, USB port, and the switch, was coated in a layer of shrink wrap in order to prevent moisture infiltration into the system hardware. Moisture absorption was also provided by approximately 5 g of calcium sulfate (Drierite, WA Hammond Drierite Co Ltd) in the Pi module. A 0.025-gauge aluminum sheet was adhered to aluminum risers, and

allowed to protrude approximately 4.45 cm from the shrink-wrap in order to remove excessive heating that may result from normal operation and environmental conditions.

Data collected through MillCollector was analyzed and used to build reports through the MothMill software (see Chapter 2).

3.2.6 | Data analysis

After an experimental run was complete, the raw data was processed through the MothMill software program to obtain a PDF file in both the discrete and aggregate functions at the 24-hour time point (see Chapter 2). The MothMill software includes functions that display the following variables: total distance flown, total duration of flight used (2,880 minutes for the purposes of this experiment), average speed, average speed in flight, total time in flight, total time at rest, longest continuous flight time, longest continuous rest time, and greatest continuous distance. Time at rest was calculated as a period of time greater than 60 seconds spent without activating the Hall effect sensor. Similarly, time in flight was calculated as a period of time with no two activations of the Hall effect sensor greater than 60 seconds apart.

The data on insect weight by sex, strain, and treatment was run through SAS 9 using weighted values to compensate for the uneven numbers of insects that were available for each treatment and strain (α=0.05). Flight data collected using MothMill was analyzed against treatment factors including diet and treatment, and physiological factors including sex and weight, using SAS 9 and a number of statistical tests as noted below (α=0.05).

3.3 | Results

3.3.1 | Effect of treatment on pupal weight

Pupal weight data was collected for each of the treatments described (Tables 3.1 and 3.2). Table 3.3 displays the effects of multiple variables on pupal weight. For the purposes of these analyses, ranked weights were used to compensate for the uneven number of insects available in each treatment. These ranked weights alter the values of individual weights in order to compensate for fewer samples in given treatments, thereby reducing the potential error of relying on tests based on systems that have fewer

Table 3.1- Pupal weights of *S. frugiperda* from the Ben or 456 strains reared on meridic diet.

Ben- Untreated meridic diet			
	Male	Female	Both
Number	145	101	246
Average Weight (mg) ± SD	179.8 ± 15.5	178.0 ± 15.4	179.0 ± 15.5
Minimum Weight	120.0	134.8	120.0
Maximum Weight	218.4	211.4	218.4
456- Untreated meridic diet			
	Male	Female	Both
Number	69	71	140
Average Weight (mg) ± SD	182.3 ± 30.1	183.1 ± 41.5	182.7 ± 36.2
Minimum Weight	87.7	71.00	71.0
Maximum Weight	244.6	275.0	275.0
456- Cry1Fa-treated meridic diet			
	Male	Female	Both
Number	62	41	103
Average Weight (mg) ± SD	160.3 ± 29.4	156.7 ± 29.0	158.8 ± 29.2
Minimum Weight	60.9	90.0	60.9
Maximum Weight	253.1	221.1	253.1

This table shows the pupal weights of *S. frugiperda* reared on meridic diet, depending on treatment. Shown are means and standard deviation based on two replicates of insects. Potential errors due to unequal numbers of insects in further data analysis by treatment, diet and strain (listed below) were mitigated through the use of weighted statistical modeling.

Table 3.2- Pupal weights of *S. frugiperda* from the Ben or 456 strains reared on corn

Susceptible- Non-transgenic corn			
	Male	Female	Both
Number	45	36	81
Average Weight (mg) ± SD	85.6 ± 14.1	87.9 ± 11.8	86.7 ± 13.1
Minimum Weight	60.0	60.0	60.0
Maximum Weight	127.0	110.0	127.0
Resistant- Non-transgenic corn			
	Male	Female	Both
Number	25	38	63
Average Weight (mg) ± SD	105.6 ± 11.1	102.9 ± 11.5	104.0 ± 11.4
Minimum Weight	88.4	81.4	81.4
Maximum Weight	127.9	126.0	127.9
Resistant- Cry1Fa Corn			
	Male	Female	Both
Number	15	16	31
Average Weight (mg)	86.2 ± 8.8	84.8 ± 17.3	85.4 ± 13.6
Minimum Weight	68.8	52.8	52.8
Maximum Weight	96.2	106.2	106.2

This table shows the pupal weights of *S. frugiperda* reared on corn, depending on treatment. Shown are means and standard deviation based on two replicates of insect. Potential errors due to unequal numbers of insects in further data analysis by treatment type, diet, and strain (listed below) were mitigated through the use of weighted statistical modeling.

Table 3.3- Significance of factors on pupal weight

	Pr>F (*p*-value)	Higher Pupal Weight
Strain	**0.0081**	Cry1Fa- Resistant
Sex	0.3369	-
Diet	**<0.0001**	Meridic Diet
Treatment	**<0.0001**	Untreated
Diet x Treatment	0.0683	-
Strain x Diet	**0.0045**	Cry1Fa- Resistant$_1$

Table showing the significance of factors on pupal weight derived from SAS9.4. Significant detected differences (marked in bold) were found in the strain, diet, treatment, and the interaction between strain and diet. (1) Cry1Fa-resistant insects were found to be significantly larger *(p*=0.0066) than susceptible insects when reared on corn, but were not significantly different *(p*=0.9984) when reared on meridic diet.

samples than competitors. Tables 3.1 and 3.2 show the averages and standard deviations for pupal weights of insects reared on meridic diet and corn larval substrates respectively.

Statistical analysis showed that resistant insects were significantly heavier than susceptible insects when both were fed on meridic diet (p=0.0081), insects reared on artificial diet were heavier than those reared on corn (p<0.0001), and the presence of Cry1Fa toxin in the Cry1Fa-resistant strain had a negative influence on pupal weight (p<0.0001).

Tukey-Kramer multiple analysis tests for probability of significance showed an interaction of the strain and diet and an interaction of the diet and treatment on pupal weight. For the factors of strain and diet, Cry1Fa- resistant insects reared on meridic diet were not significantly different from susceptible insects reared on meridic diet (p=0.9984). When reared on corn, however, Cry1Fa- resistant *S. frugiperda* pupae were significantly larger than susceptible insects (p=0.0066). The pupal weights of resistant insects reared on meridic diet treated with Cry1Fa, resistant insects reared on non-transgenic corn, and resistant insects reared on Cry1Fa expressing corn all significant differed from susceptible insects reared on meridic diet.

3.3.1 | Effect of treatment on flight

For the purposes of determining the effects of treatment variables on the flight capacity of *S. frugiperda*, data collected through the MillCollector and MothMill program were analyzed with SAS 9. For the purposes of this project, weighted values were used in order to compensate for the uneven numbers of insects that were used for each treatment and strain.

The strain (p=0.5611), sex (p=0.0581), diet (p=0.2226), treatment (p=0.2369), and interaction of the diet and treatment (p=0.7182) did not significantly affect the flight capacity of the *S. frugiperda* (Table 3.4). Only pupal weight affected the flight capacity of moths (p=0.0009), and the Spearman correlation coefficient indicates a positive relationship (α=0.05) between flight capacity and weight (Table 3.5).

3.3.2 | Effect of strain, sex, and toxin presence on flight

The statistical models generated by SAS 9 showed that the strain, sex, diet, and presence of Cry1Fa toxin had no effect on the flight of the adult insect. In contrast, it was shown (Table 3.3) that a number of

Table 3.4- Flight of *S. frugiperda* from the Ben or 456 strains reared on meredic diet

Ben- Untreated meridic diet			
	Male	Female	Both
Number	24	17	41
Average Flight Distance in 24 hours (m) ± SD	8463.8 ± 6700.4	13026.3 ± 9614.1	10355.5 ± 8346.2
Maximum Flight Distance	22968.0	34835.9	34835.9
Minimum Flight Distance	743.1	259.5	259.5
456- Untreated meridic diet			
	Male	Female	Both
Number	26	27	53
Average Flight Distance in 24 hours (m) ± SD	7515.5 ± 6780.2	10877.2 ± 7747.6	9228.1 ± 7480.3
Maximum Flight Distance	22794.7	31501.7	31501.7
Minimum Flight Distance	132.1	2.9	2.9
456- Cry1Fa-Treated meridic diet			
	Male	Female	Both
Number	19	4	23
Average Flight Distance in 24 hours (m) ± SD	5525.2 ± 5483.9	2220.81 ± 2209.9	4950.5 ± 5221.2
Maximum Flight Distance	18952.9	4870.1	18952.9
Minimum Flight Distance	12.4	21.1	12.4

Table 3.4 details the flight of *S. frugiperda* adults reared on meredic diet. Susceptible insects were reared without toxin, and resistant insects were reared both with and without toxin.

Table 3.5- Flight of *S. frugiperda* from the 456 strain reared on corn

456- Non-transgenic corn (2T777)			
	Male	Female	Both
Number	9	10	19
Average Flight Distance in 24 hours (m) ± SD	426.1 ± 530.6	978.6 ± 823.6	716.9 ± 757.8
Maximum Flight Distance	1602.0	3103.4	3103.4
Minimum Flight Distance	2.9	85.2	2.9
456- Cr1Fa-producing corn (TC1507)			
	Male	Female	Both
Number	1	3	4
Average Flight Distance in 24 hours (m) ± SD	5.7	242.9± 8.8	183.6 ± 212.2
Maximum Flight Distance	NA	544.9	544.9
Minimum Flight Distance	NA	67.0	5.7

Table 3.5 details the flight of *S. frugiperda* adults reared on corn. Tested insects were reared both on non-transgenic and Cry1Fa-producing (TC1507) corn.

factors influence pupal weights. These factors may influence the flight of *S. frugiperda* through their significant effect on pupal weight, though lack of detection of these factors may also be due to a limited number of insects that were placed on flight mills.

3.4 | Discussion

In this study, the flight Cry1Fa-resistant and susceptible *S. frugiperda* was measured via flight mill in order to detect differences in migratory behavior. The data collected was analyzed in conjunction with the recorded pupal weights of the insects to determine the relationship between factors affecting pupal weight and those that affect flight. There is a positive correlation between pupal weight and flight during the 24 hour period where data was collected, indicating that a larger pupa will result in a moth that has a greater flight tendency compared with a moth developing from a smaller pupa [68]. In this work, exposure to Cry1Fa resulted in smaller pupae in Cry1Fa-resistant *S. frugiperda*, which likely explains the non-significant reduction in flight tendency for these Cry1Fa-exposed insects. However, more behavioral and physiology-linked behavior tests are needed to reveal dietary-to-performance relationships. This observation is in contrast to the increased flight activity of *S. exigua* when exposed to Cry1Ac [59]. It is possible that increased flight behavior following toxin exposure is either unique to the toxin, or may be the consequence of linkage between a marginally effective toxin and a susceptible strain of *S. exigua*. In addition, there may be hidden physiological costs associated with Cry1Fa toxin resistance in *S. frugiperda* that have not been detected previously [56]. Behavioral tests, particularly flight mills in the case of long-distant flying or migratory species, may be needed to accurately determine the physiological cost of resistance to toxins. Furthermore, it is important to note that *S. frugiperda* is normally controlled by Cry1Fa toxin, whereas *S. exigua* is only marginally affected by Cry1Ac [69, 70]. This suggests that the *S. frugiperda* used may be resistant to the lethal effects of the toxin, but have physiological effects that go unnoticed when taking into account only the weight of pupae and the fecundity of adult insects. The effects of marginally effective toxins against *S. frugiperda* have similarly not been determined, and it should be investigated before a sweeping statement is made about how the ingestion of toxins effects the behavior or physiology of resistant insects. Additionally, the results that have been obtained through these experiments are reliant on the use of isogenic strains of *S. frugiperda*, which are based on the Benzon Research insect colony. As these insects have been reared solely on meridic diet for hundreds of generations, the physiology and behavior of these insects may have evolved for laboratory conditions. This may have influenced results recorded for insects

reared on corn, as the 456LS4 strains possess only the resistance of the field strain, and are otherwise near identical to the Benzon Research strain. Additionally, future work needs to be conducted on the lipid profiles of *S. frugiperda* in order to assess baseline amounts of storage lipids as a proxy for flight tendency in adults [71, 72].

Chapter IV

Quantitative Profiling of Phospholipids from Susceptible and Cry1Fa-Resistant *Spodoptera frugiperda* Reared on Diets With and Without Cry1Fa Toxin

This chapter to be submitted to Insect Physiology

Hietala, L. and J.L. Jurat-Fuentes (2018). Lipid extraction and quantification for adult *Spodoptera frugiperda* resistant and susceptible insects reared on diets with and without Cry1Fa toxin.

My contributions included: (1) experimental design, (2) data collection and analysis, (3) writing the manuscript and making figures. Juan Luis Jurat-Fuentes assisted with (1) and (3).

Abstract

Spodoptera frugiperda (Noctuidae) is an important pest of several agricultural crops including cotton, soybean, and corn. In the continental US, these insects migrate annually from two overwintering locations in Florida and Texas as the growing season progresses, eventually reaching the southern border of Canada. Effective control of these insects in corn has been achieved through the use of transgenic plants producing the Cry1Fa protein derived from *Bacillus thuringiensis* (Bt). However, field-evolved resistance to Cry1Fa has arisen at different locations. As lipid metabolism is critical to long-distance flight in Lepidoptera, we were interested in testing a potential physiological linkage between resistance, feeding on Cry1Fa, and lipid metabolism. Levels of phospholipids detecetd by gas chromatography were compared between susceptible and Cry1Fa-resistant *S. frugiperda* after feeding on control diet or on diet containing Cry1Fa. The results support differences in the levels of phospholipids between Cry1Fa-resistant and susceptible fall armyworm, regardless of diet. Diet had an effect on gross phospholipid content for each strain, but exposure to Cry1Fa did not have significant effects when compared with control diet. Taken together, these results indicate relevant physiological differences between susceptible and Cry1Fa-resistant fall armyworm, and that exposure to toxin has no effects on phospholipid content in resistant moths.

4.1 | Introduction

The *Spodoptera* genus in the Noctuidae family contains a number of economically important agricultural pests [41, 47, 52, 73, 74]. Among these, *Spodoptera frugiperda* (J.E. Smith), the fall armyworm, is an important pest of corn, cotton, and soybean plants in the United States [60]. In the continental United

States, these insects overwinter in southern Texas and Florida, and thereafter migrate north as the temperature warms [75]. These two overwintering locations are kept largely separate by the Appalachian mountain chain, but there are major sites at which genetic exchange may occur, such as in Georgia and Alabama in the south, or Louisiana and Pennsylvania in the north [45, 46].

Tracking this pest and behaviors characteristic of its populations' habits is an important aspect in informed integrated pest management decision making. Management of these insects is achieved largely through the use of transgenic crops containing the Cry1Fa protein gene derived from the soil-dwelling bacterium *Bacillus thuringiensis* (Bt), rather than reliance on chemical insecticides. However, field-evolved resistance to corn producing the Cry1Fa protein was first detected for populations of *S. frugiperda* in Puerto Rico in 2006 and described in 2010 [53], only ten years since Puerto Rico had initiated planting of this corn (event TC1507) in 1996. Subsequently, cases of field-evolved resistance to Cry1Fa in populations of *S. frugiperda* have been described in Brazil, the southeastern United States and Argentina {Storer, 2010 #110;Farias, 2014 #114;Li, 2016 #204;Chandrasena, 2018 #221}.

Despite the importance of field-evolved resistance, there is no information available on the physiological and behavioral effects that resistance may have on resistant insects. For example, exposure to sublethal levels of diet containing Cry1Ac in the closely-related Asian armyworm (*Spodoptera exigua*) resulted in adult insects that displayed increased long-distance flight tendency compared to those fed a control diet [59]. Feeding on other sub-optimal food sources has been reported to result in metabolic alterations in Lepidoptera [60, 68, 77, 78]. Consequently, we hypothesized that there would be metabolic differences in Cry1Fa-resistant *S. frugiperda* exposed to diet with Cry1Fa compared to those fed a control diet.

Resistance to Cry1Fa in *S. frugiperda* from Puerto Rico is genetically linked to a mutation in an ATP binding cassette subfamily 2 (*SfABCC2*) gene, which is expected to have a role in metabolism and detoxification [64, 79]. The resistant insects do not express a functional SfABCC2 protein, which could lead to metabolic differences when compared to susceptible insects due to the functions that this protein performs in cellular metabolism and the transport of anionic molecules [64].

The purpose of this study was to test for alterations in phospholipid profiles as a proxy for potential metabolic differences between susceptible and Cry1Fa-resistant fall armyworms and to determine whether exposure to Cry1Fa has a relevant effect on the metabolism of fats in resistant insects. We describe a thorough lipidomic (lipid quantifying) analysis of the phospholipid profiles in the abdominal

contents of susceptible and Cry1Fa-resistant *S. frugiperda* moths reared on control or Cry1Fa-contaminated diet.

4.2 | Materials and Methods

4.2.1 | Insects

Eggs from a susceptible (Ben) strain of *S. frugiperda* were obtained from Benzon Research Inc. (Carlisle, PA). Resistant (456LS3) *S. frugiperda* derived from a field population collected from Puerto Rico have been maintained in the laboratory for more than 100 generations with constant selection on Cry1Fa-producing corn (event TC1507) leaves, which are noted to contain approximately 111 ng of Cry1Fa toxin/ mg of tissue (Dow Agrosciences). Before this project, resistant and susceptible strains were crossed and selected in the F2 generation three times in order to create isogenic lines [56]. Insects were reared for experimentation in 128 well bioassay trays (Frontier Agricultural Sciences, Newark, DE) with artificial diet (Beet Armyworm Diet, Frontier Agricultural Sciences product #F9220B) under a 16:8 light: dark photoperiod at 27ºC (±1ºC) and 60% ± 10% relative humidity.

To expose larvae to the toxin, a solution of Cry1Fa protoxin (5 µg/cm^2) was deposited on top of dry artificial diet plugs in bioassay trays, and the larvae were introduced once the diet was dried. The Cry1Fa dose used was the LC99 dose for Ben larvae (data not shown), but a sublethal dose for 456LS3 (10-18% observed mortality). Larvae were incubated until pupation and survivors were collected and weighed. The pupae were allowed to eclose into adult moths in individual 30 ml (1 oz) plastic containers. Moths were allowed to rest, dry, and excrete without food for 12-18 hours before freezing them at -20ºC.

4.2.2 | Insect Dissection and Sample Preparation

Moths were thawed from -20^{o}C and the abdominal contents (including fat body, reproductive organs, and gut) of three moths were dissected and pooled in borosilicate glass vials. These samples were later pooled until approximating 50 mg of abdominal contents, which constituted a biological replicate. Samples were suspended in 300 µl of MeOH: CHCl3 9:1 before being stored for 18 hours at 4^{o}C before sending to the University of Tennessee Biological and Small Molecule Mass Spectrometry Core (BSMMSC) for analysis by electrospray-ionization mass spectrometry (ESI-MS) [80]. Samples analyzed included male (456MAD) and female (456FAD) Cry1Fa-resistant moths exposed to control artificial diet

(456MAD, 5 biological replicates totaling 33 individuals, 456FAD, 3 biological replicates totaling 26), male (456MTx) and female (456FTx) Cry1Fa-resistant moths exposed to artificial diet containing Cry1Fa protoxin (456MTx 3 biological replicates totaling 21 individuals, 456FTx 2 biological replicates totaling 13 individuals), female Cry1Fa-resistant moths exposed to corn event TC1507 (456FBt, 1 biological replicate totaling 5 individuals) or the non-transgenic isoline 2T777 (456FCorn , 1 biological replicate totaling 9 individuals), and male (BenMAD) and female (BenFAD) moths from the Cry1Fa-susceptible line exposed to control artificial diet (BenMAD, 15 biological replicates totaling 102 individuals, BenFAD 14 biological replicates totaling 71 individuals).

4.2.3 | Sample Homogenization and Extraction

After collection andlabeling of replicates, each sample was homogenized using a micro pestle in a 2 mL centrifuge tube containing 800µL of 1:1 0.1M HCl: Methanol and 400µL of chloroform. Samples were then vortexed 30 seconds to ensure complete lipid extraction, then centrifuged for 5 minutes at 16,600 x g. The lower phase was isolated and transferred to a clean tube and dried under nitrogen. The pellet was resuspended in 300 µL of 9:1 methanol: chloroform and then transferred to an autosampler vial.

All samples were kept in an autosampler controlled at 4°C prior to analysis via Ultra Performance Liquid Chromatography-High Resolution Mass Spectrometry (UPLC-HRMS). Extracts were separated using an Ultimate 3000 ultra-performance liquid chromatography system (Dionex, Waltham, MA). Sample (10 µl) was injected onto a Kinetex HILIC column (150mm x 2.1mm, 2.6µm, Phenomenex, Torrance, CA), kept at 25°C, using a binary solvent system. Mobile phase A consisted of 100% water with 10 mM ammonium formate adjusted to pH 3.0, and B consisted of 93:7 (v/v) acetonitrile: water with 10 mM ammonium formate adjusted of pH 3.0. A constant flow rate of 0.2 mL/min was used over the complete 30 min elution gradient. Initial conditions were 100% B for 1 minute; a decrease to 81% B from 1 to 15 minutes, a sharp decrease to 48% B from 15 to 15.1 minutes, and then the conditions were held constant at 48% B from 15.1 to 25 min to clean the column. The column was re-equilibrated to the initial conditions of 100% B from 25 to 30 minutes.

Eluent was coupled to an Exactive Plus Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA) via an electrospray ionization (ESI) source. Sheath gas was set to 25 (arbitrary units), auxiliary gas to 10 (arb. Units), heated capillary was set at 350°C and the spray voltage was 4kV. The parameters were identical for both positive and negative ionization modes. The mass spectrometer was operated in full scan/all

ion fragmentation (30eV) mode across a range of 100-1500 m/z at a resolution of 140,000. Negative mode was used to detect 5 classes of phospholipids: phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidylserine (PS), and phosphatidylethanolamine (PE), while positive mode was used to detect phosphatidylcholine (PC). Unique phospholipids were identified based on their exact mass (+/- 5ppm) and retention time, which was verified by a standard from each class of phospholipid. Compounds were annotated in short hand notation by a two-letter abbreviation for the phospholipid head group, the sum of total numbers of carbons in the tails, and the total number of degrees of unsaturation. For example, PG subgroup (36:1), means the mass/charge that was detected corresponds to a phosphatidylglycerol with 36 total carbons in the tails, and 1 degree of unsaturation.

Raw data files generated by the mass spectrometer were converted to mzML using MSConvert [81] and uploaded to MAVEN [82, 83], where all the processing occurred. The software performed a retention time correction to align peaks and account for any peak drift. Areas under the curve were integrated for all samples and reported as intensity units and data were normalized by sample mass prior to any statistical analysis. Analysis was carried out based on the lipid content of Moth Equivalent Units (MEUs), which are the result of the weight-normalized lipid data divided by the number of individual moths that made up the sample in question. For graphical display, MEU abundance for each lipid head group (ex. Phosphaditic acid or Phosphaditylserine) for each sample was calculated by adding the detected levels for all subgroups (ex. PA 38:2 or PA 38:3) within that head group and averaging by the number of biological replicates

4.2.4 | Statistical Analyses

Averaged data for each headgroup category was applied to SAS9.4 using a Tukey-Kramer multiple analysis statistical test against a significance level of $p<0.05$.

4.3 | Results

Based on the limited sample size for some of the treatments, we limited statistical analyses and discussion of results to the samples that included a minimum of three biological replicates. Statistical comparisons (Table 4.1) showed no significant effects ($p<0.05$) of strain, sex, treatment, strain x lipid interaction, or diet x treatment interaction on phospholipid levels. Diet and lipid type were not relevant

Table 4.1- Statistical analysis for phospholipids in analyzed samples

Source	Pr>F (*p*-value)	Higher Phospholipid Content
Strain	0.1089	-
Sex	0.7612	-
Diet	**0.0002**	Corn
Treatment	0.3738	-
Lipid	**<.0001**	NA_1
Strain*Lipid	0.3802	-
Treatment*Lipid	0.0754	-

Table 4.1 shows the results of SAS9.4 analysis based on MEUs with significant factors listed in bold: Lipids are listed as NA due to the natural variation in the amounts of each head group of phospholipid, and are therefore expected to be different in their concentrations. The exceptions to this are the pairs of PA and PE (p=0.6754) and PI and PS (p=0.8210), which did not differ significantly.

considering that only 2 biological replicates were available for corn and the expected differences in lipid type percentages between animal cells [84-86]. Each phospholipid headgroup is significantly different from each other in terms of total average abundance, as shown in Table 4.2, which is expected from previous reports [84]. In the comparison between strains, analyses indicated a non-significant (p=0.1089) tendency for higher total levels of phospholipids for the resistant (456LS3) compared to the susceptible strain.

4.3.1 | Phosphatidic Acid (PA)

Phosphatidic acid (PA) is a precursor molecule known to influence the curvature of cellular membranes and for acting as a signaling lipid [87]. These anionic lipids are composed of a mix of mono- and di-anionic species which support several biosynthetic pathways and signaling functions in cells, including interactions with protein kinases, sphingosine kinases, and vesicular trafficking proteins [87, 88]. However, these lipids are often short lived inside the cell and will often undergo degradation and conversion to triacylglycerol (TAG) [89]. In eukaryotic animal cells, PA generally represents approximately 1-2% of the total phospholipid content [89, 90]. In the *S. frugiperda* samples analyzed, the levels of PA made up to 0.7-3.3% of total detected phospholipids in all cases (Table 4.2).

The PA head groups detected in our lipidomic analysis included 13 different subgroups (38:4, 36:5, 36:8, 34:1, 34:3, 34:4, 34:5, 34:6, 32:3, 30:1, 28:1, 28:2, 28:3, and 26:1). When the amounts of all these PA classes were combined and the average amounts compared among treatments (Fig. 4.1), no significant differences were found between strains and treatments.

4.3.2 | Phosphatidylethanolamine (PE)

In general, PE is a phospholipid particularly abundant in the membranes of mitochondria, but is also found in the interior and exterior surface of the cell membrane [91]. This anionic phospholipid is an important signaling factor in macroautophagy and a number of other cellular functions, and high levels may create a more viscous cell membrane or increase the chances of oxidative stress [92]. This lipid type

Table 4.2- Lipid abundance for detected head groups in MEUs

	PA	PE	PI	PG	PS	PC
456MAD	17929.4	9162.9	25783.7	3134.5	19021.3	917310.4
456FAD	6264.7	2203.3	8225.7	1187.4	5487.5	163958.3
456MTx	2401.6	4503.1	14229.1	687.0	10216.1	227061.1
456FTx	2730.9	5161.8	16356.5	1192.1	11918.8	352467.0
456FCrn	1772.3	23199.7	69782.2	1480.9	51673.6	71813.6
456FBt	24009.2	56113.8	119070.2	4439.6	96855.3	687698.3
BenMAD	4697.6	5269.8	13889.9	567.3	10488.7	200184.6
BenFAD	7270.8	8573.5	20933.8	839.4	16323.5	197223.3

Table 4.2 displays the levels of each detected lipid headgroup in terms of average intensity unit abundance by strain, treatment, sex, and diet. These levels are in intensity units, and are based on the MEU model, with each listed number representing a single average moth worth of the listed head group.

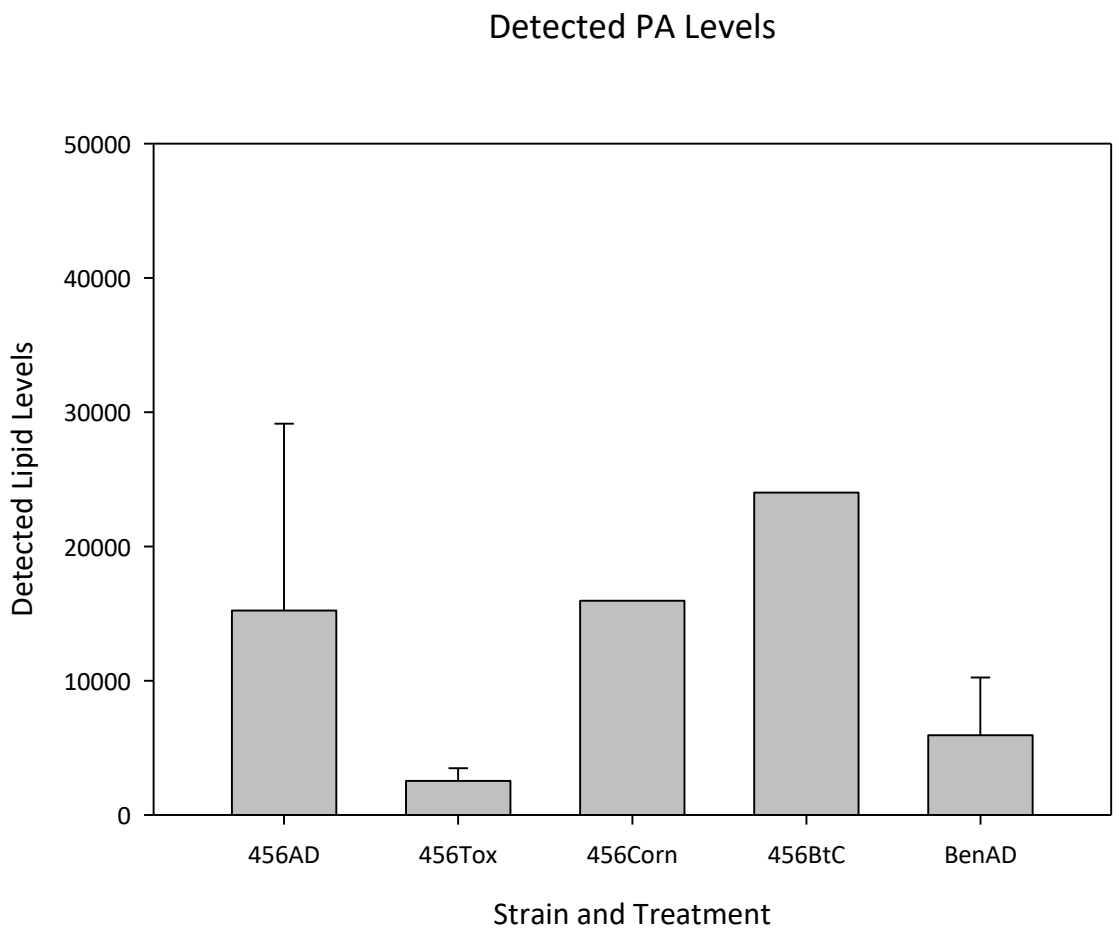


Figure 4.1- Average detected PA levels in samples of *S. frugiperda* adult abdominal tissues. 456, resistant strain; Ben, susceptible strain; AD, artificial diet; Tox, artificial diet treated with Cry1Fa toxin; Corn, 1% agar solution with a section of non-transgenic corn leaf (2T777 strain); Bt, 1% agar solution with a section of transgenic corn leaf (TC1507). Detected levels are listed as average MEU.

makes up approximately 15-25% of total phospholipid content in mammalian cells [89, 90]. In the *S. frugiperda* samples analyzed (Fig. 4.2), PE made up approximately 1-3.5% of the total phospholipids, with the exception of Cry1Fa-resistant females reared on non-transgenic and transgenic corn, which were reported as 10.6% and 5.7% respectively. Detected PE levels were found to be non-significantly different (p<0.05) among all samples derived from artificial diet, but were detected to be approximately 6 fold and 9 fold higher for female resistant insects reared on non-transgenic and transgenic corn, respectively, compared to resistant females reared on Cry1Fa-treated artificial diet (Fig. 4.2). The detected head groups for PE included 4 different subgroups (26:5, 26:3, 30:4, and 30:3). No significant differences were detected for PE average abundance based on strain (p=1.0) or treatment (p=1.0)

4.3.3 | Phosphatidylglycerol (PG)

In mammalian cells, PG is a cellular membrane lipid recognized as pulmonary surfactant involved in the movement of oxygen into lung alveoli [93]. This lipid class has numerous functions, including membrane stabilization for certain classes of PG [94]. PG comprises approximately 1-2% of total cellular phospholipids for eukaryotic organisms [90]. The levels of PG detected in our study all consisted of less than 1% of the total phospholipids, ranging from 0.2-0.7%. In samples collected, detected PG was found to be higher by approximately 2 fold in resistant insects over susceptible insects with the exception of male resistant insects reared on toxin-treated artificial diet.

The detected head groups for PG in *S. frugiperda* moths included 11 different subgroups (36:4, 36:3, 36:2, 36:5, 34:1, 34:2, 34:3, 34:6, 28:2, 28:3, 26:0, and 26:1). The total levels of PG were generally higher in insects fed Cry1Fa or corn leaf material (Fig. 4.3). See Appendix Table 4.3 for the complete table of detected PG data. Table 4.1 shows that there is no significant difference between PG levels for interactions between the phospholipid level and strain (p=0.3802).

4.3.4 | Phosphatidylserine (PS)

Phosphatidylserine is an anionic phospholipid found mostly in the endoplasmic reticulum, where it is synthesized, and in the cellular membrane [90]. This phospholipid acts as a signaling molecule and is particularly important for its role in signaling for apoptotic phagocytosis. Additionally, PS interacts with a large number of proteins, though its role on the interior of the cell has not yet been thoroughly

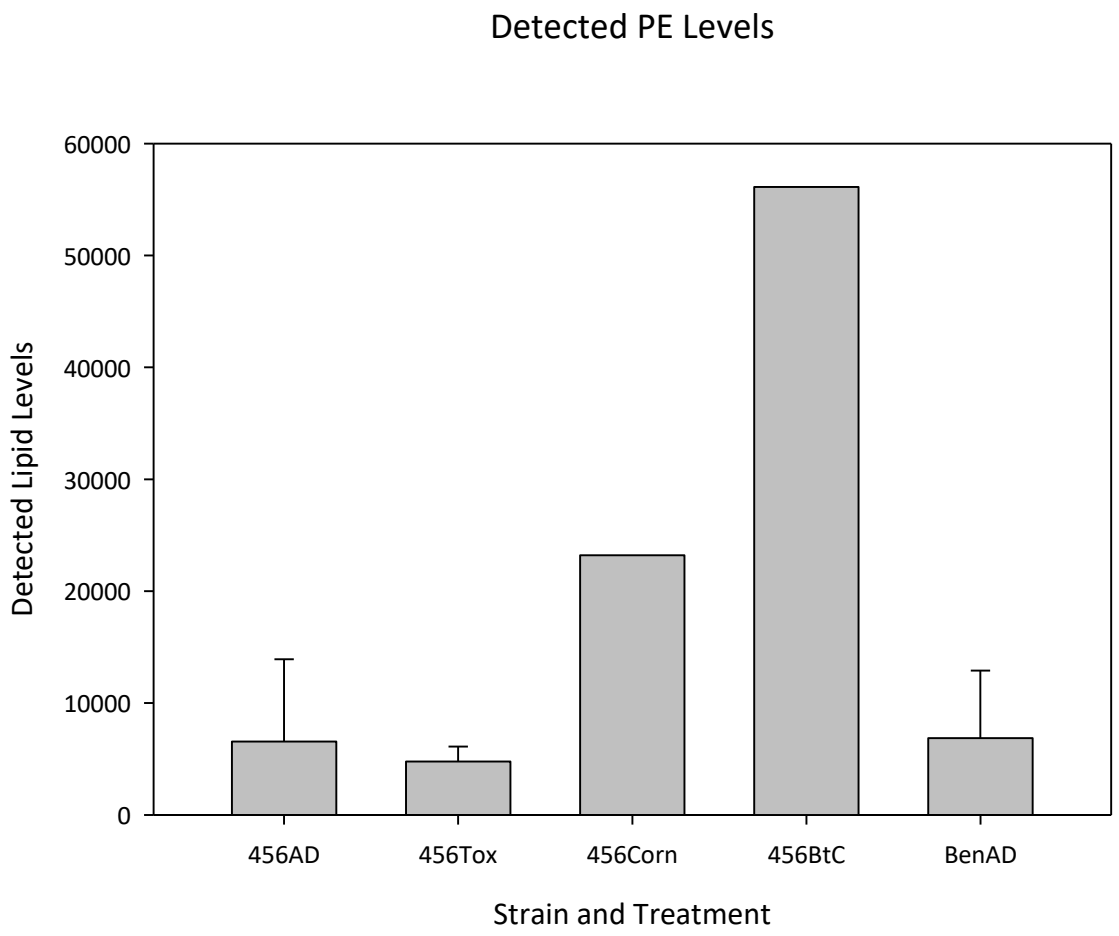


Figure 4.2- Average detected PE levels in samples of *S. frugiperda* adult abdominal tissues. 456, resistant strain; Ben, susceptible strain; AD, artificial diet; Tox, artificial diet treated with Cry1Fa toxin; Corn, 1% agar solution with a section of non-transgenic corn leaf (2T777 strain); Bt, 1% agar solution with a section of transgenic corn leaf (TC1507). Detected levels are listed as average MEU.

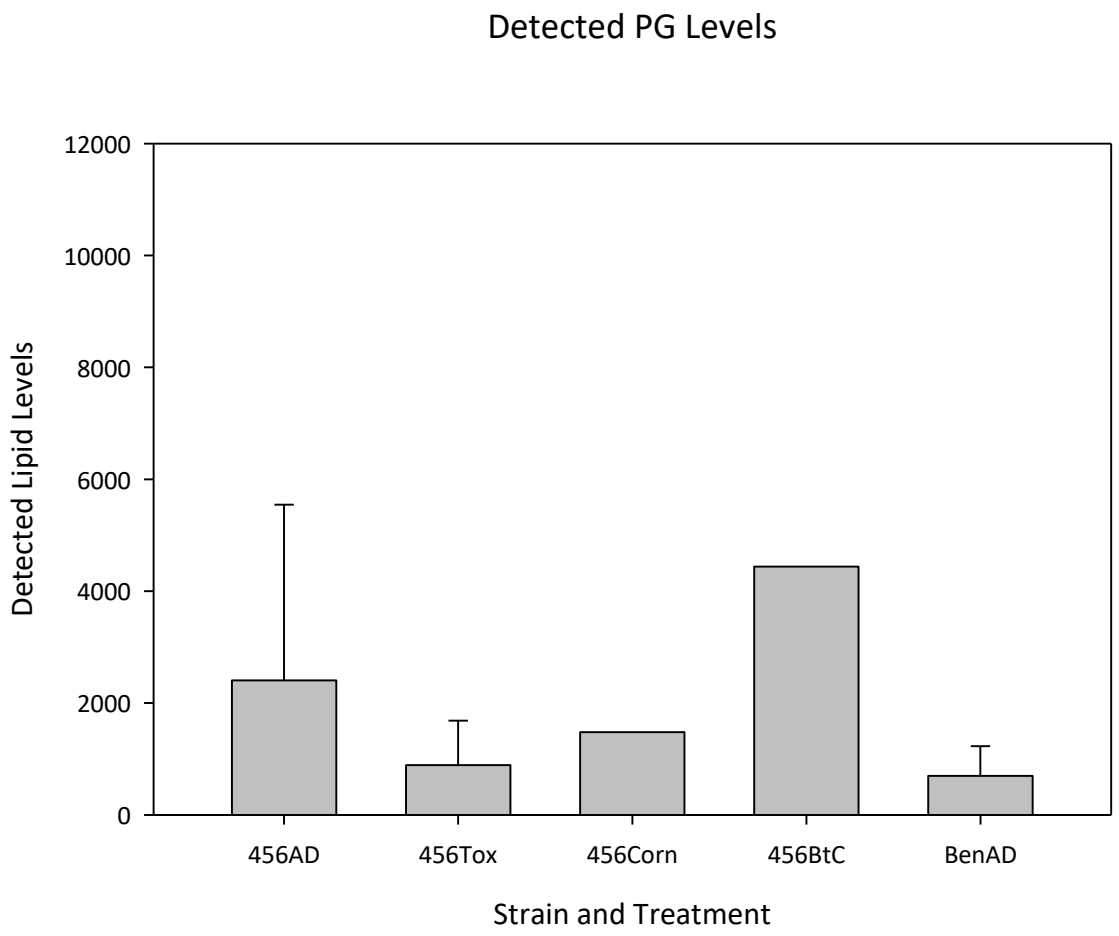


Figure 4.3- Average detected PG in samples of *S. frugiperda* adult abdominal tissues. 456, resistant strain; Ben, susceptible strain; AD, artificial diet; Tox, artificial diet treated with Cry1Fa toxin; Corn, 1% agar solution with a section of non-transgenic corn leaf (2T777 strain); Bt, 1% agar solution with a section of transgenic corn leaf (TC1507). Detected levels are listed as average MEU.

investigated and its action is therefore not wholly understood. In animal cells, PS comprises approximately 2-10% of total cellular phospholipids [90, 92]. The samples of *S. frugiperda* abdominal contents generally held to the expected range of 2-10%, with the exception of resistant females feeding on non-transgenic corn (23.5%). The detected levels of PS in samples collected were notably higher on average (>20-fold) in insects reared on corn-based diets than those reared on artificial diets, as shown in Figure 4.4. No significant differences (p<0.05) were detected when comparing PS levels and considering *S. frugiperda* strain or treatment, as shown on Table 4.1. The detected head groups for PS included 5 different subgroups (46:7, 46:8, 46:9, 42:7, 30:2, and 26:6).

4.3.5 | Phosphatidylinositol (PI)

In animal cells, PI is an anionic phospholipid generally found on the inner edge of the cellular plasma membrane, where it functions in protein targeting and signal transduction [95]. This phospholipid is notably important for its ability to become phosphorylated to form PI-4-P, PIP2, PIP3, and a large number of other compounds involved in lipid and cellular signaling, as well as membrane trafficking [96, 97]. In animal cells, PI generally makes up about 2-10% of the total cellular phospholipid content [98]. This trend was also observed in the samples analyzed here, which ranged from an average of 3-8% in most samples. In resistant insects fed on non-transgenic and transgenic corn, however, the percentages were found to be 31.8 and 12% respectively, which represents approximately a 5-fold increase over those reared on toxin-treated artificial diet, and approximately 9-fold higher than those reared on untreated artificial diet, shown in Figure 4.5. We did not detect significant differences (p<0.05) for comparisons of strain and treatment. The detected head groups for PI included 4 different subgroups (40:5, 40:4, 40:3, 40:2, and 44:6).

4.3.6 | Phosphatidylcholine (PC)

In general, PC is considered to be the most abundant phospholipid in animal cells, with a few exceptions where it is replaced with PE [92]. Phosphatidylcholine contains an additional quaternary amine group and fulfils a largely structural role in the cell although it also functions in signal transduction [99]. For instance, PC is the precursor phospholipid for a number of different signaling molecules that are necessary for normal cellular function [100]. Generally, PC makes up about 45-55% of the total cellular phospholipid content in animal cells [101]. In our samples, samples from insects fed on corn material

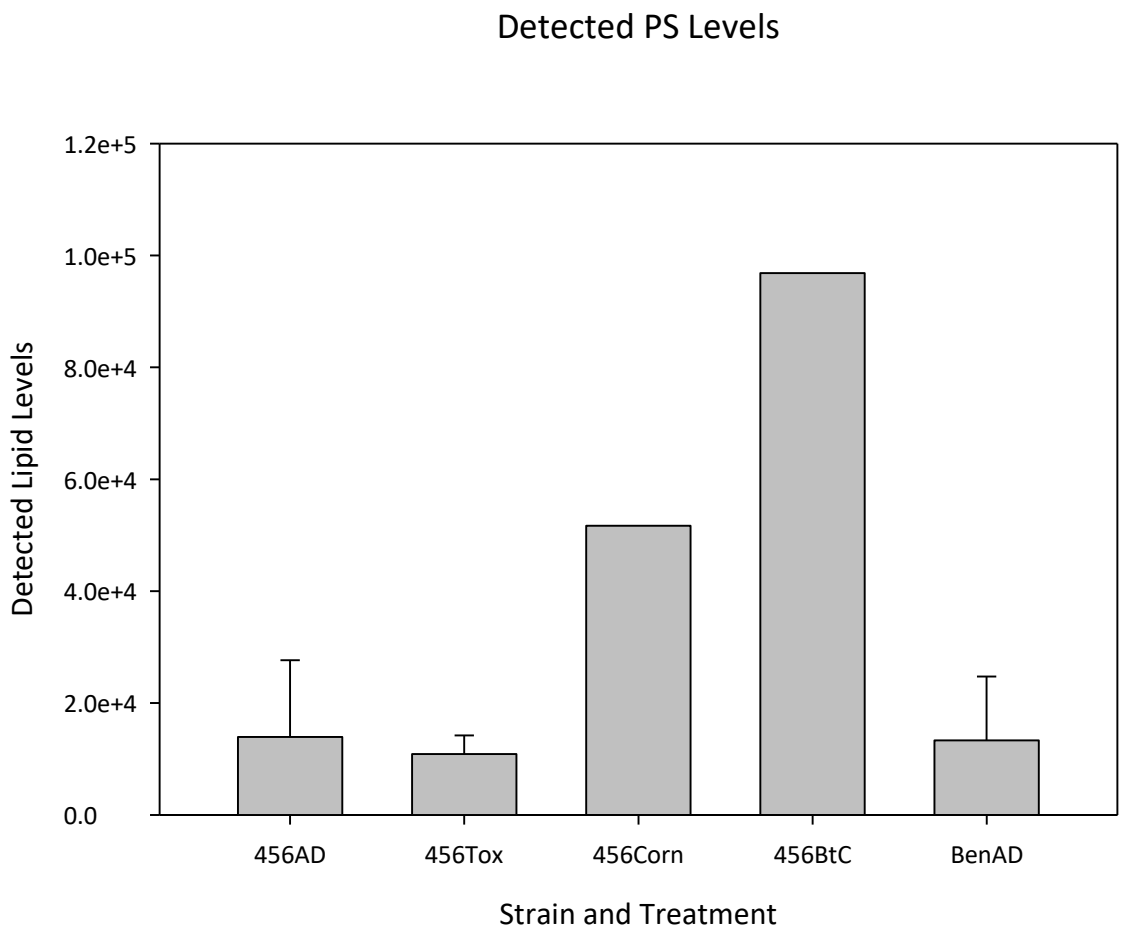


Figure 4.4- Average detected PS in samples of *S. frugiperda* adult abdominal tissues. 456, resistant strain; Ben, susceptible strain; AD, artificial diet; Tox, artificial diet treated with Cry1Fa toxin; Corn, 1% agar solution with a section of non-transgenic corn leaf (2T777 strain); Bt, 1% agar solution with a section of transgenic corn leaf (TC1507). Detected levels are listed as average MEU.

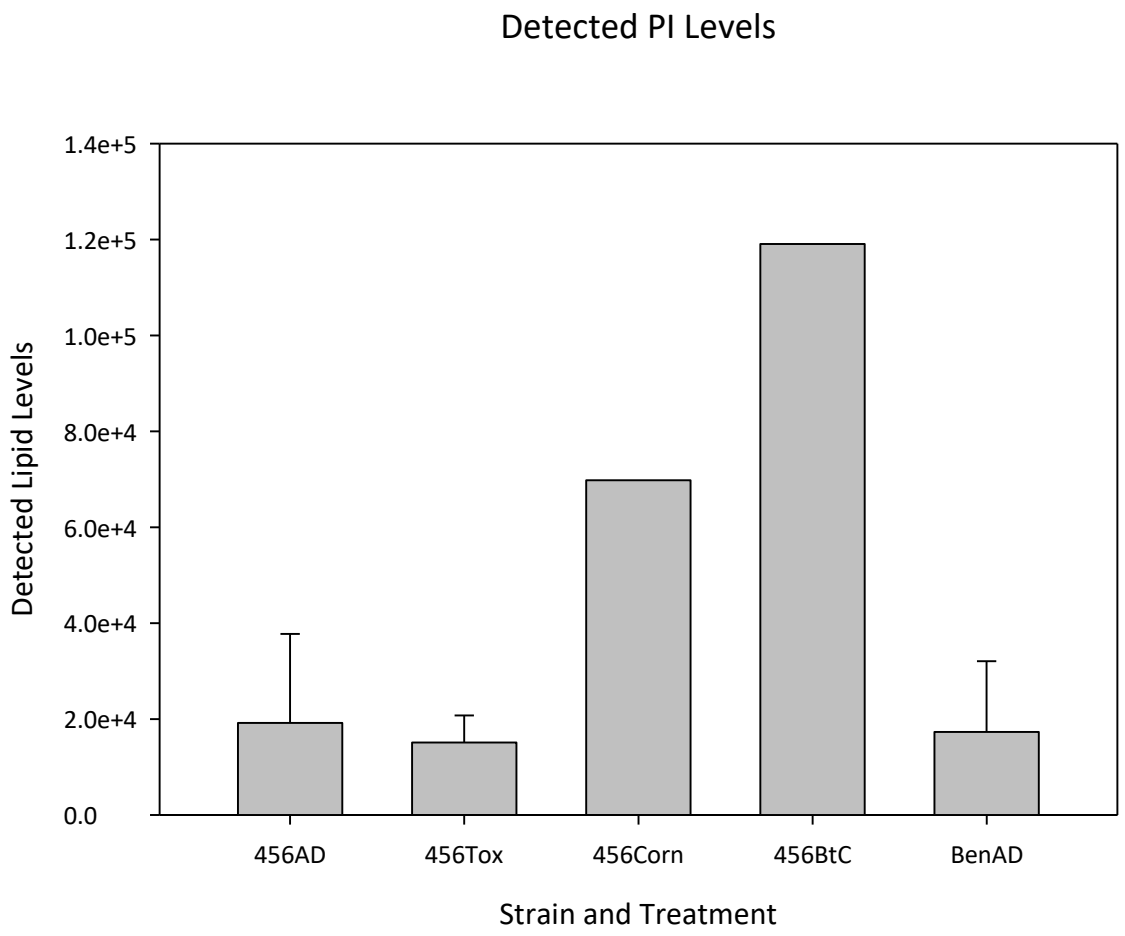


Figure 4.5- Average detected PI in samples of *S. frugiperda* adult abdominal tissues. 456, resistant strain; Ben, susceptible strain; AD, artificial diet; Tox, artificial diet treated with Cry1Fa toxin; Corn, 1% agar solution with a section of non-transgenic corn leaf (2T777 strain); Bt, 1% agar solution with a section of transgenic corn leaf (TC1507). Detected levels are listed as average MEU.

contained 32.7% (456FCorn) and 69.6% (456FBt), while PC relative levels ranged from approximately 78-92% of total detected phospholipids in the rest of samples. The levels of PC were not found to be significantly different between strains *(p*=1.00), or the presence or absence of Cry1Fa toxin (*p*=1.00) as shown on Table 4.1. The detected head groups for PC 25 different classes (46:6, 46:7, 46:8, 46:9, 44:5, 44:4, 44:6, 44:7, 42:3, 42:4, 42:5, 42:10, 40:2, 40:3, 40:4, 38:1, 38:2, 38:3, 32:6, 30:2, 28:0, 28:1, 28:2, 28:3, 26:1, and 26:2). The following image (Fig.4.6) is a graph of detected PC in analyzed samples.

4.4 | Discussion

In this work we performed a lipidomic analysis of phospholipids in newly emerged *S. frugiperda* adults that had been reared on artificial diet or corn tissue during the larval stage. We also performed comparisons between susceptible and Cry1Fa-resistant strains of *S. frugiperda* reared on control versus diet containing Cry1Fa protoxin (See Appendix B, Table 4.3 and 4.4). Our hypothesis was that treatment with Cry1Fa in the Cry1Fa-resistant strain would increase the levels of phospholipid classes detected in comparison to unexposed Cry1Fa-resistant insects. The results showed no significant alterations in phospholipid levels between susceptible and Cry1Fa-resistant *S. frugiperda*.

The total detected abundance of phospholipids for *S. frugiperda* was higher, albeit not significantly, in the case of Cry1Fa-resistant insects compared to susceptible insects reared on artificial diets. In the case of Cry1Fa-resistant insects, we detected no significant differences associated with the absence or presence of Cry1Fa toxin in the diet. These results suggest that Cry1Fa-resistant insects do not display an alteration to their phospholipid profile when exposed to Cry1Fa. Although the number of replicates was much lower, we did detect a non-significant tendency for higher levels of PE, PS, and PI in resistant insects reared on corn compared to resistant or susceptible insects reared on artificial diet (See Appendix B, Table 4.4).

Dawaliby et al. (2016) compared the levels of phospholipid classes in mammalian and insect cells, using the ovarian-derived *S. frugiperda Sf*9 cell line as a model [102]. In that work, authors reported different concentrations for each of the phospholipid categories compared to those reported in this study. This discrepancy may be explained by the use of an ovarian cell line in comparison to the samples taken from adult abdomens used in this study. However, the expected rates of PI (7-13%) and PG (3%) are similar to those reported by Dawaliby et al. (2016). The reported levels of PC (16%), PS (18-24%) and PE (38%) by Dawaliby et al. differed from the levels found in this study, which again may be explained by the

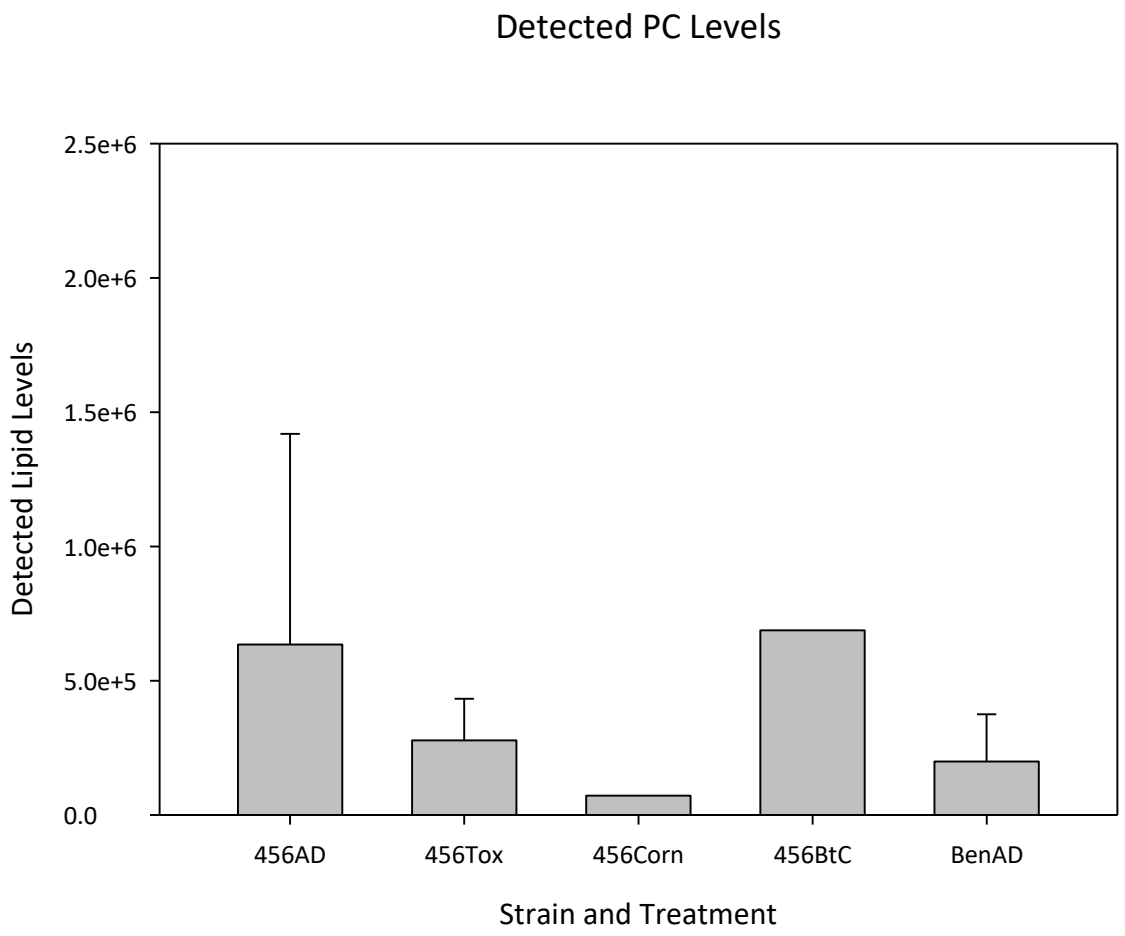


Figure 4.6- Average detected PC in samples of *S. frugiperda* adult abdominal tissues. 456, resistant strain; Ben, susceptible strain; AD, artificial diet; Tx, artificial diet treated with Cry1Fa toxin; Corn, 1% agar solution with a section of non-transgenic corn leaf (2T777 strain); Bt, 1% agar solution with a section of transgenic corn leaf (TC1507). Detected levels are listed as average MEU.

different cell model used (*Sf*9 ovarian cells versus abdominal tissues. Stem and ovarian cell culture lines show different phospholipid profiles from other tissues as a result of lipid and protein sequestration that occurs *in vivo* during egg production [103]. Research performed on the phospholipid content of *Pieris brassicae* larvae by Turunen et al (1989) showed a lower level of PC (33%) than observed here for *S. frugiperda* reared on artificial diet [77]. In contrast, PS (6%) andPI (6%)levels were closer to the observed levels in *S. frugiperda* (6.3% and 8.2%, respectively). Additionally, the Turunen group performed similar work with *S. frugiperda* larvae reared on artificial diet and found phospholipid levels similar to the levels that were detected in this study, except for reduced amounts of PS compared to PI [77], which is not observed in the data collected in this study .

The lipids that this study focused on are primarily involved in cellular signaling, protein interactions, and cellular membrane integrity [100, 102, 104]. Through the alteration of levels of these phospholipids, PE in particular, alterations may be made to the behavior of the cellular membrane [102]. Interactions with the cellular membrane are particularly important when considering the mode of action of Cry toxins. In lepidopteran larvae, these toxins are activated in the midgut and then bind to a series of proteins before inserting into the cellular membrane [105]. The interaction of the toxin with the cell membrane is often overshadowed by the interaction of the toxin protein with different receptor molecules, though pore formation is considered important. It should be noted that in the absence of the brush-border membrane, Cry toxins were found to be up to three orders of magnitude less effective in producing mortality than when the brush border membrane of the midgut was present [106]. With this in mind, and considering the importance of the detected phospholipids for cell membrane structure, the presence of different levels of phospholipids would probably have an effect on the conformation of the cellular membrane and possibly an insect's susceptibility to Cry toxins. Additionally, these lipids are required for cellular homeostasis and the correction of homeostatic imbalances, such as the role of PI and PE in regulation of macroautophagy and apoptotic cell death [91, 107].

When exposing larval *S. frugiperda* of the Cry1Fa-resistant strain to Cry1Fa protoxin, there was little effect compared with larvae fed a control diet or susceptible insects. However, in the case of resistant female insects, there was a large difference noticed in the percentages of phospholipid classes for those insects reared on corn (supplementary Table 4.4). This result may indicate a necessary physiological alteration in reaction to exposure to plant material or the artificial diet presented. Most notably, the abundance of PI and PS in the samples derived from corn-fed insects would indicate either a

physiological allocation of resources to these phospholipids in particular, or a large amount of these phospholipids that are presented within the corn-based diet. In either case, future work should be conducted to determine whether a larval diet intentionally enriched with PS and PI results in an increased level of PS and PI in adult insect abdominal contents [108, 109].

With the collected data, it is possible to create a basic understanding of the levels of phospholipids and general trends related to the exposure of Cry1Fa-resistant insects to Cry1Fa toxins compared to no exposure. These trends may prove to hold true for multiple sources of larval diet, and exposure to varied toxins. A previous report supports that lepidopteran species possess an altered phospholipid profile under different rearing conditions, particularly in those that possess overwintering or cold climate behavior [110]. Further research conducted on *S. frugiperda* phospholipid content could better define the composition of membrane lipids in these insects and also elucidate how different physiological states affect phospholipid concentrations.

Chapter V

Conclusions and Future Work

5. 1 | Conclusions

The movement capabilities and behaviors of *Spodoptera frugiperda* are extremely important to agricultural systems in the United States. This polyphagous pest is capable of long distance flight, and larvae are voracious feeders of both leaf material and fruits [44, 60]. While control of these insects is possible through chemical insecticides, it is more common that growers mitigate damage through the use of transgenic crops. Because of high adoption or possibly because of improper utilization of refuge crops, *S. frugiperda* populations has developed resistance to transgenic crops producing Cry toxins, particularly Cry1Fa [51, 52, 56]. Resistance monitoring and management therefore becomes extremely important in these insects, as they have already proved their capability to rapidly evolve resistance to control measures.

Another member of the *Spodoptera* genus, *S. exigua*, possesses an increased flight tendency when exposed to a marginally effective toxin, Cry1Ac [59]. Thus, we hypothesized that *S. frugiperda* may respond similarly to marginally effective toxins such as Cry1Ac. More importantly, *S. frugiperda* may possess a similar reaction when resistant populations are exposed to previously effective Cry toxins, such as Cry1Fa. In this project, a combination of flight actograph tests and lipid quantification techniques were used to determine the relationship between resistant *S. frugiperda* exposed to Cry1Fa and their increased ability to achieve longer distance flights. Flight actograph tests were used to directly correlate the ability for long distance flight with exposure to Cry1Fa, while lipid quantification techniques were applied to the insect abdominal contents [71, 111].

Through the use of the rotational flight mills and Raspberry Pi computer programs MillCollector and MothMill, data was collected on the flight activity of adult *S. frugiperda*. For the purposes of this experiment, both susceptible and resistant insects were used. Testing with flight mills was performed with susceptible insects obtained from a commercial rearing company. Resistant insects were maintained in the lab from field-collected resistant insects from Puerto Rico in 2010. These field-collected insects were subsequently bred with the commercially available susceptible insects and selected on transgenic (TC1507) corn in the F2 generation, generating an isoline to the susceptible insects.

Over the course of the project, pupal weights were taken for insects used in the experiments. For the purposes of accuracy, pupal weights were used instead of 5th instar larvae or adults due to the

variability in times when each of those stages could be reached, outside of researcher control, while the pupal weight offers a relatively static point at which the statistic may be recorded.

Resistant insects possessed higher average pupal weights than the susceptible insects in all cases. Larvae exposed to Cry1Fa had lower pupal weights, which was expected and similar to *S. exigua* on which this study was based, and it also holds true in other tests involving *S. frugiperda* reared on Cry toxins [48, 51, 59, 60]. Also not unexpected and previously reported [56], resistant insects displayed no significant physiological costs in regards to their weight or fitness as a result of their resistance.

Results taken from the rotational flight mills provided an idea of how resistant and susceptible *S. frugiperda* may react to different diet sources. Information was initially analyzed through the use of the MothMill program to provide raw data, which was further analyzed through SAS software to determine the effects of multiple factors on flight behavior. Results indicated that only pupal weight had a positive (p<0.0001) significant effect on adult flight in the first 24 hours (p=0.0009). Strain (p=0.561), sex (p=0.0581), diet (p=0.2226), and treatment (p=0.2369) were not found to significantly influence the flight. This effect of pupal weight on flight is somewhat more difficult to explain when considering all the different factors significantly affecting pupal weight. The main factor in the long distance flight, according the data obtained, was pupal weight. Pupal weight was shown to be affected by strain, diet, and treatment, though these factors did not affect significantly the flight behavior of these insects. This may be due to several different factors. The insects used were allowed to fly for 24 hours, after which the experimental run was ended and data collected. It is possible that longer flight experiments beyond this 24-hour period could have shown significant differences. For example, other experiments have used 24 or 72 hours, or until the death of the insect [59, 61, 71]. However, previous research conducted on the flight behavior of *S. exigua* that detected differences based on treatment with a Cry1 toxin allowed flight for 12 hours {Jiang, 2013 #33}. The diet or treatment may also have had an effect on the insect propensity to undergo flight due to nutritional deficits or surpluses. Finally, there may be differences in the flight behavior of the insects due to strain, diet, or treatment as found in the pupal weight data, but the number of insects available for testing may have been too low to detect them.

The strain and diet of *S. frugiperda* had an effect on total phospholipids detected in the insects, but only one head group category (phosphatidic acid, PA) was significantly influenced by the interaction of the strain and diet or strain and treatment. PA is a relatively small portion of the insect phospholipid contents, and many traditional methods of detecting phospholipid classes such as chromatographic tests do not differentiate this headgroup as well as costlier mass spectrometric methods. The availability

of such mass spectrographic methods for determining phospholipid contents is also limited by the availability of specialized machinery and trained personnel, as it becomes much more difficult to use PA levels as a marker for physiological effects in field specimens. The changes in PA levels and total phospholipids based on the diet and strain, however, have potential use as a point of future research into the compared cellular structures of resistant and susceptible insects, and the physical binding of Cry1Fa toxins to cell membranes possessing varied levels of such phospholipid groups.

The results of this research are of use in current research on flight behavior. While flight behavior was not affected by the diet or exposure to Cry1Fa, flight capabilities were positively related to pupal weight. This may be important information when considering the fitness of *S. frugiperda* resistant insects reared on Cry1Fa corn compared with insects with partial resistance (or susceptible insects that have been affected by Cry1Fa corn in the field and subsequently remain smaller in size) [48, 58, 112]. There is a strong linkage between the increase in weight and increase in flight capacity, meaning that highly resistant insects that feed on Cry1Fa producing crops with a higher pupal weight than more susceptible insects will likely have a higher propensity for long-distance movement.

Phospholipid data collected in this project is useful in future work determining the metabolism of and physiological costs of Cry toxin resistance. The cost of resistance, generally determined by studies regarding fecundity, weight, and insect longevity may be expanded to include the fatty acid composition of the insect, which was shown in the project above to be higher in insects that possess Cry1Fa resistance. It should be noted that with these insects, the presence or absence of Cry1Fa toxin was not a significant factor at a rate of 5µg/ cm^2, meaning that this increase in phospholipids may only be a cost of the resistance itself and not an additional cost incurred by the presence of the inactive toxin.

Future work on this subject may include the exposure of the resistant 456 strain of insects to toxins that share a binding site with Cry1Fa, as well as those toxins that are only marginally effective or ineffective at controlling *S. frugiperda* susceptible strains. In doing so, the alteration or lack of alteration to the metabolic production and use of phospholipid categories could be determined as being linked with or not linked with the presence of Cry toxins, or those that specifically would incur mortality in susceptible members of the species. This information could then be used to further research into the molecular physiology of these insects, particularly in the cellular membrane of the insect gut. If altered levels of individual phospholipid headgroups can create conformational changes or physical changes that assist in the reduction of Cry toxin binding, or in the repair or elasticity of the gut membrane [113], it may be the that the resistance mechanism in these insects is further-reaching that previously suspected.

Data collected on any form of actograph, including rotational flight mills, is influenced by a controlled environmental setting and may not reflect true natural behavior. The insects are tethered to a device which they must turn, in addition to their own mass, overcoming the inertia of the flight arm and head. The insects are also maintained in a relatively small area under artificial lighting that possesses no 'evening' or 'morning' phase. Males and females are kept together, mixed in equal number and in close proximity to one another for the duration of the experiment. Any of these factors may pose significant influences on the flight of these insects. Each mill remains slightly different, and despite researcher compensation and adjustment, errors in machining may cause the variability among some recorded flight activity data. Finally, the insects used in this study are not entirely applicable to field populations. In creating an isoline with a known susceptible strain, the original field collected resistant insects became almost genetically identical to the susceptible strain, with the exception of the resistance gene that they carry. However, the susceptible strain has been maintained for more than 20 years on meridic diet, which likely alters their genetic profile in regards to flight behavior or their response to difference food sources. Given these caveats, and with the results of this project in hand, there are several different directions to continue this project.

A great deal is known about the feeding habits and population movement of *S. frugiperda* over time in the United States. However, studies on the flight capacity and behavior of *S. frugiperda* are the next in a series of steps in understanding and preventing damage that this pest can cause. The results presented in the thesis are only an important starting point, and there are several areas of future study that should be undertaken.

First and foremost, susceptible and resistant strains of *S. frugiperda* obtained from wild populations need to be tested for behavioral and physiological changes in a laboratory setting. These insects ideally would be exposed to the most natural setting possible in order to retain their field-derived behavior. In this way, the accuracy and application of all such experiments would be increased in the short and long term. Comparisons may also be made with these field-derived strains and the established laboratory strains that are currently in use.

Further research should be completed on the effects of diets and other treatment factors on the flight behavior, pupal weights, and lipid profiles of *S. frugiperda*. Modern transgenic corn hybrids often have pyramids of several Cry toxin genes, including two or more toxins with different modes of action. For example, resistant *S. frugiperda* may react differently when exposed to plants possessing a formerly effective Cry toxin, such as Cry1Fa (TC1507) and a toxin that was only ever marginally effective, such as

Cry1Ac (corn transgenic event DKB-89614-9). Further research could help predict and model changes in flight behavior as new varieties of insecticidal transgenic crops are introduced.

Likewise, tests with *S. frugiperda* should be tested on different crops. Corn is a common host plant resource for these insects, but *S. frugiperda* is also capable of reproductive development cotton, soybean, and several other agricultural crops{Silva, 2017 #222}. The polyphagous nature of these insects makes them a particularly noxious pest, and physiological and behavioral difference when feeding on alternate hosts could have important implications for their management. For example, insects that developed on corn may have different flight behaviors and capabilities than those from soybean. This may lead to more accurate modeling of *S. frugiperda* migration over time based on the regional availability of various food sources.

The relative number of individuals from each sex should be determined during flight mill studies to test for any effect of sexual prevalence on flight in laboratory studies. Likewise, the mated status of insects used should be considered. Mating behaviors, particularly how quickly virgin females are mated, and the flight capabilities of moths before and after mating should be considered in order to determine if the flight tendencies of the insects are incorrectly represented in similar studies undertaken with virgin moths [11].

Finally, there are many designs of flight mill and almost as many varieties of program made for interpreting data taken from them. In order to better compare results among researchers, a single variety of flight mill using modular parts should be created using commonly available materials. Ideally, this should be easy to manufacture and calibrate, and should be sized for the most common species of alate agricultural pests.

The experiments performed during the course of this project have resulted in the development of improved technology for measuring the activity of an important agricultural pest and many other insects due to the flexibility of the actograph platform. A greater understanding of the movement of these insects as they are exposed to Bt toxins has been garnered as well, showing that the flight behavior of Cry1Fa-resistant insects was not affected by exposure to Cry1Fa. These results should be useful baseline for in future research on the behavior of *S. frugiperda* as compared to its close relative, *S. exigua*; especially when considering that the changes in flight behavior exhibited by *S. exigua* when feeding on Bt toxins were not observed in *S. frugiperda*.

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Appendices

Appendix A:

Kudzu Bug RNAi Project

Project components included design of experiment (1), design and preparation of dsRNA (2), design and implementation of bioassays (3) writing and conclusions (4). Lucas Hietala performed (2) (3), and (4). Dr. Jerreme Jackson assisted with (1) and (2). Dr. Juan Luis Jurat-Fuentes assisted with (1) and (2).

A1.1 - Introduction

The kudzu bug (*Megacopta cribraria*; Plataspidae) is an invasive hemipteran insect that appeared in the southern United States in 2009 [114]. These Hemipteran insects originated in eastern Asia, most likely from Japan, where they are noted for feeding on the kudzu vine (*Pueraria* sp.) as well as other plants in the family Fabaceae. In their native range, these insects are considered secondary pests due to the presence of natural predation and environmental factors that reduce the effectiveness of the insect as a pest [115]. However, in the United States, the parasitoids and predators that may reduce the populations of these insects are not natively present, and populations may reach levels that potentially threaten the production of agriculturally important crops.

Most concerning of the agriculturally important crops that these insects may affect in the southeastern United States is the soybean (*Glycine max).* These insects may cause significant yield loss if uncontrolled due to a combination of reduced plant health via sucking feeding behavior and damage to bean pods caused by mouthpart insertion [116]. Furthermore, while it is unreported that these insects are vectors for agriculturally important diseases in the United States at the moment, concerns exist involving the development of these insects as future plant disease vectors.

In addition to the agricultural threat that these insects pose, they are considered to be a nuisance pest. The insects when disturbed produce a yellow-orange substance that is anecdotally capable of staining wood and permanently discoloring fabrics. This exudate additionally possesses a noxious odor, and reports exist of allergic reactions to the exudate causing prolonged itching, swelling, and discomfort in the reporting individuals [117].

Control of these insects is currently achieved through the use of commercial insecticides containing substances including pyrethroids and neonicotinoids, both of which are potentially threatening to non-target species[118]. In order to both control these insects with greater specificity and with fewer

continual or repeated expenses, the development of a transgenic soybean plant line containing specific insecticidal substances was proposed.

The use of insecticidal RNAi (RNA interference) has been shown as an effective means of control for several species of Coleoptera [119]. However, limited success has been found during feeding assays with RNAi in lepidopteran species [119]. RNAi is the process by which a cell takes up foreign double-stranded RNA (dsRNA), and through a series of processes uses this RNA in conjunction with a protein complex called Argonaut to cleave matching messenger RNA in the cell, thus reducing the amount of that specific protein being produced [119]. In order to determine the effectiveness of RNAi in *M. cribraria*, it was necessary to determine and design potential double stranded RNA (dsRNA) segments that could later be expressed in a transgenic soybean line for crop protection.

A1. 2 - Materials and Methods

A1.2.1 | Insects

Adult *M. cribraria* were collected from a field population (Knoxville, TN, 35°57'43.2"N 83°54'37.5"W). Insects that were to be used for bioassay immediately were allowed to rest for a minimum of 1 hour in laboratory conditions to confirm survival on removal from the field before being placed into assay conditions. Insects that were to remain in the laboratory for a protracted period of time were placed in BugDorm cages (BugDorm Product no. DP1000). Nutrition was provided with collected cuttings of local kudzu plants (*Pueraria* sp., 35°56'56.6"N 83°56'24.0"W) placed in filtered water. These cages were placed in incubators set for a 16:8 hour light: dark photoperiod, 27±1°C, and 55±3% RH. Insect populations were found to be most stable at concentrations of approximately 150 insects/ cage, and the volume of insects needed necessitated the use of larger dorms after the commencement of bioassays. Populations maintained in the laboratory for bioassays and attempted population breeding were placed in large mesh enclosure and provided nutrition was increased to fit the enclosure volume.

A1.2.2 | Preliminary bioassay tests

An efficacy test was designed and performed in order to ascertain the feeding behavior of *M. cribraria* in the assay tube. Three individuals taken from the same field population as the dsRNA constructs were derived from were placed in assay vials containing 0.15 µg/7µL of Congo Red solution, and three were placed on a sucrose only control. These insects were allowed to feed under bioassay conditions for 72 hours, and then their guts dissected.

A1.2.3 | Gut dissection

A dissection technique was developed by this researcher for the purposes of collecting *M. cribraria* gut, using minimal effort and with little contamination. The researcher firmly grasps the specimen in the off-hand, facing with the head dorsally protruding between the index finger and thumb. Fine-pointed forceps are then applied to one corner of the scutum. A twisting motion of approximately 85° forward cracks the scutum away from the insect, detaching the head and scutum from the body and leaving the thorax and abdomen in the gripping hand. The scutum-head complex may then be slowly drawn away from the insect directly, pulling with it the gut. A well-removed gut will be approximately 1.5cm long when stretched and possess a rounded bulb on the posterior end. Elasticity of gut tissues often results in the need to remove the gut from the inside of the scutum, which may be done with a second pair of forceps before being placed into a receptacle for preservation or use. *M. cribraria* dissection will result in alarm chemical release onto the researcher's glove, which will stain through up to 3 layers of standard latex exam gloves for 50-60 insects dissected and leave a brown and orange stain that will remain for approximately 7-10 days but does not appear to provide longer lasting discoloration or discomfort. A respirator or filter mask is recommended for dissections of more than 30 insects due to the alarm chemical.

A1.2.4 | Bioassays

A number of dsRNA constructs were developed by Dr. Jerreme Jackson using a custom transcriptome and genetic material taken from wild-collected *M. cribaria.* The target genes were selected by Dr.

Jackson and Dr. Jurat-Fuentes as homologs to genes previously reported to be effective in insecticidal RNAi. The dsRNA for use in bioassays was made from stock solutions prepared using the T7 High Yield Transcriptase kits (Fisher Science product no.K0441) with amplicons of ~200 bp fragments matching target genes and containing T7 promoter regions in both orientations, as provided by Dr. Jerreme Jackson using . Yields were quantified through the use of a Qubit fluorometer, with dsRNA quality ascertained through detected 260/280 ratios. Aliquots of dsRNA obtained from the T7 kit were made for assays of 10 insects and placed in -20°C storage until used.

Bioassays were performed with the dsRNA constructs in individual assay vials. These vials were prepared using 1.5mL tubes pierced with a section of 18-gauge wire. Assay solution (10% sucrose base) was placed in the cap of the vial and covered with a single layer of pre-stretched parafilm. Insects were placed individually in each vial, which was placed inverted (cap down) into a 128 well bioassay tray (Frontier Scientific). Bioassay conditions were identical to rearing conditions.

All dsRNA solutions used for testing (Table A.1) were used at a rate of 7µg/75µL for all 10 contigs (Figure A.1). Insects were placed at a rate of 10 insects/ dsRNA contig in each replicate, and were allowed to feed for 10 days. Mortality was recorded every 24 hours, and survivorship calculated each day (Table A.1).

A1.3 | Results

The results from preliminary bioassays (Figure A.1) show that feeding took place in the bioassay setting, as all three cases of Congo Red test subjects displayed red guts. The average results of the bioassays over three replicates are displayed in Table A.1 below. These results indicate that at 9 days there is a high degree of mortality in both GFP (Green Fluorescent Protein dsRNA) and sucrose only controls (93.33% and 76.64%, respectively). As a result, 3 and 4 day bioassays were considered to identify potential target genes. From these, dsRNA targeting contig numbers 1209, 173, and 860 were selected as possessing the greatest potential, and were used as the focus of future experiments and tests.

A1.4 | Conclusions and Discussion

The results of this project indicated that *M. cribraria* is affected by RNAi using feeding of dsRNA in a laboratory setting. A bioassay technique was developed and shown to be effective in a comparative test

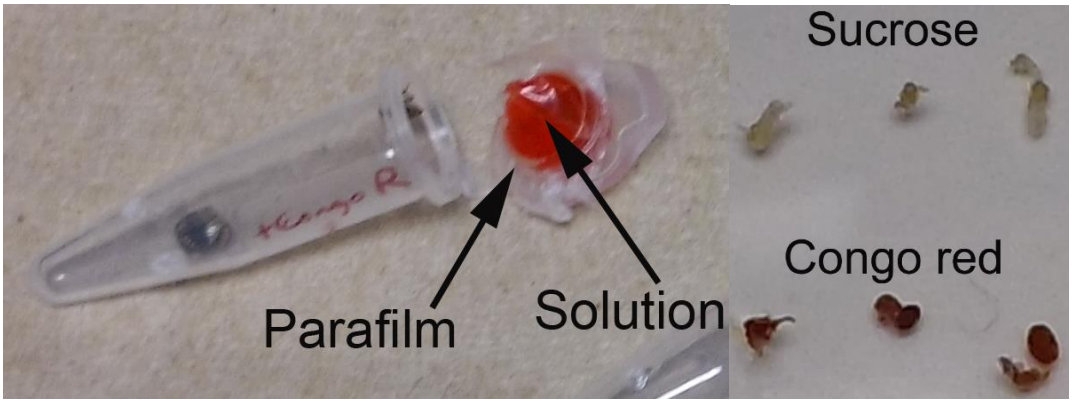


Figure A.1- Congo red feeding assay vial and results. Figure displays the results (right) after 72 hours of feeding, showing the difference in gut coloration between presence and absence of Congo Red in 10% sucrose solution. Displays that the feeding assay vial (left) functions and the insects take up sucrose solution.

Table A.1- Survivorship of M. cribraria on varied dsRNA contigs

		Survival %									
Treatment	Contig	Day0	Day1	Day2	Day3	Day4	Day5	Day6	Day7	Day8	Day9
1	1217	100	96.67	86.67	80	63.33	60	46.67	30	20	16.67
2	9114	100	100	90	75	70	65	45	45	40	40
3	1535	100	93.33	86.67	73.33	63.33	53.33	40	36.67	30	23.33
4	738	100	96.67	86.67	80	63.33	43.33	30	30	23.33	20
5	6176	100	86.67	90	66.67	46.67	30	23.33	10	6.67	3.33
6	1209	100	93.33	80	60	43.33	30	20	13.33	13.33	13.33
7	173	100	95	85	60	50	25	10	10	5	0
8	1167	100	76.67	60	56.67	50	20	10	10	10	6.67
9	5929	100	96.67	83.33	73.33	70	40	33.33	23.33	20	20
10	860	100	93.33	76.67	66.67	36.67	23.33	20	13.33	13.33	10
11	GFP	100	96.67	83.33	83.33	60	30	23.33	16.67	6.67	6.67
12	Sucrose	100	96.67	93.33	86.67	73.33	50	36.67	26.67	23.33	23.33

The above table shows the survivorship of *M. cribraria* treated with 7µg/75µL dsRNA after the listed number of days.

using visual diagnostics, and then used to test the efficacy of a number of dsRNA fragments. This project creates a foundation upon which future research may be conducted on the insecticidal effects of RNAi on *M. cribraria* and related species, which are known pests of soybean, and may potentially become more agriculturally important in the future.

The most damaging life stages of the kudzu bug are late instar nymphs [116, 120]. Therefore, future work on the efficacy of RNAi in kudzu bug should include assays using the late instar nymphs as experimental subjects. Furthermore, future work with RNAi in kudzu bug should be conducted with captive laboratory strains, which will both serve to reduce the potential effects of fungal, bacterial, or viral infections that may be present in wild populations and affect the observed mortality in a bioassay. Confirmation of efficacy for these bioassays may be gained through comparative analysis of protein expression in insects to determine knockdown, or through comparison between levels of present mRNA for the gene of interest.

Additional research into the roles of Juvenile Hormone (JH) and 20-Hydroxyecdysone (20-HE) should also be conducted on kudzu bug nymphs. In other insects, RNAi of JH or 20-HE is capable of disrupting the insects' life cycle, thus preventing future adults [121, 122]. Should such an alteration to the JH or 20-HE gene in *M. cribiraria* prove effective, it may be possible to remove future adults from field populations through application of RNAi. Future research in this area and in the area of RNAi in *M. cribraria* includes the targeting of more genes involved in insect survival, the modification and testing of transgenic plants coding for dsRNAs targeting *M. cribraria* specific genes, and testing for effects on closely related species, such as *Nezara viridula*.

The kudzu bug remains an invasive species in North America, and while it poses a potential threat to the soybean industry, it is currently being controlled through chemical insecticide and a variety of the entomopathogenic fungus *Beauveria bassiana* [123]. The kudzu bug, due to the fact that it is one of a relatively small family (Plataspidae) and none of the known species are native to North America, makes an excellent organism for research into RNAi. The specificity of RNAi creates a unique opportunity to test the efficacy of this mechanism in wide field conditions with relatively little risk, both due to the RNAi mode of action and the invasive and unique nature of the insect that the trials may target.

Appendix B:

Supplementary Data

Table 4.3- Heat map of detected phospholipids

	456MAD	456FAD	456MTx	456FTx	456FCrn	456FBt	BenMAD	BenFAD
PA(38:4)	239.661	152.796	80.412	182.888	177.074	1545.955	83.322	234.622
PA(36:5)	19.593	7.592	13.327	19.554	0.000	124.279	25.389	24.011
PA(36:8)	2606.929	479.710	533.281	328.671	431.599	7554.980	1291.458	1656.081
PA(34:1)	298.394	122.241	24.152	41.897	0.000	0.000	11.834	67.358
PA(34:3)	282.575	47.480	56.794	9.706	0.000	178.089	108.551	221.639
PA(34:4)	2789.727	508.896	291.500	177.217	254.169	3927.192	736.091	1350.317
PA(34:5)	3763.765	709.519	441.898	395.190	479.565	6222.341	1061.319	1772.010
PA(34:6)	745.589	194.217	142.301	59.541	116.080	1826.541	325.102	470.525
PA(32:3)	327.759	93.857	43.419	9.384	0.000	209.934	95.593	164.785
PA(30:1)	387.126	184.379	55.292	152.293	93.526	2131.462	50.180	134.012
PA(28:1)	1658.618	918.970	256.167	479.371	112.630	0.000	178.551	403.704
PA(28:2)	1487.857	1038.281	186.124	357.124	72.889	247.209	375.559	345.346
PA(28:3)	513.956	446.098	119.870	222.501	12.543	0.000	153.575	155.073
PA(26:1)	2807.856	1360.644	157.013	295.565	22.192	41.192	201.061	271.366
PA TOTAL	17929.406	6264.680	2401.551	2730.903	1772.266	24009.173	4697.585	7270.849
PE(26:5)	1331.824	352.162	794.818	805.977	2124.399	8703.725	727.128	1147.563
PE(26:3)	386.868	52.542	129.439	166.926	1004.802	2443.707	228.552	343.726
PE(30:4)	1174.300	469.565	529.293	920.844	637.101	2314.641	217.274	421.573
PE(30:3)	6269.954	1329.058	3049.585	3268.015	19433.387	42651.765	4096.868	6660.642
PE TOTAL	9162.946	2203.328	4503.135	5161.762	23199.689	56113.838	5269.823	8573.505
PI(40:5)	1338.940	530.740	288.393	472.388	33.766	144.033	24.941	45.285
PI(40:4)	10209.951	3969.548	4977.169	7739.976	7707.980	19974.102	2357.824	3935.591
PI(40:3)	11863.359	3144.982	7503.861	6830.719	51001.889	83579.843	9539.403	14081.752
PI(40:2)	2287.733	561.615	1393.322	1270.209	10607.922	14689.329	1894.711	2762.261
PI(44:6)	83.749	18.854	66.371	43.173	430.678	682.860	73.026	108.945

Table 4.3 Continued

	456MAD	456FAD	456MTx	456FTx	456FCrn	456FBt	BenMAD	BenFAD
PI TOTAL	25783.732	8225.740	14229.115	16356.465	69782.236	119070.168	13889.905	20933.834
PG(36:4)	404.267	118.603	131.760	135.181	127.306	0.000	54.740	53.792
PG(36:3)	223.013	65.848	64.790	59.808	130.846	0.000	32.360	29.673
PG(36:2)	184.353	52.847	11.323	18.289	46.781	33.154	11.981	14.614
PG(36:5)	261.586	65.514	55.150	81.594	52.330	0.000	30.267	23.228
PG(34:1)	334.855	136.234	92.881	95.727	125.080	0.000	34.492	25.549
PG(34:2)	387.464	134.633	71.594	97.691	47.823	0.000	34.399	21.411
PG(34:3)	414.352	125.053	55.917	55.457	17.438	0.000	26.528	18.284
PG(34:6)	105.563	57.702	2.373	21.492	13.625	0.000	47.892	61.095
PG(28:2)	306.433	177.393	91.659	292.258	407.509	2098.981	126.865	304.480
PG(28:3)	167.904	113.469	55.622	190.530	250.242	956.197	43.981	84.656
PG(26:0)	177.930	45.913	39.277	61.011	183.030	631.698	46.965	80.442
PG(26:1)	166.748	94.243	14.645	83.074	78.900	719.593	76.807	122.127
PG TOTAL	3134.470	1187.452	686.991	1192.111	1480.911	4439.624	567.277	839.352
PS(46:7)	6930.202	1822.475	4550.357	4199.377	32222.254	48711.216	5819.868	8518.927
PS(46:8)	6490.824	2384.898	3101.757	4845.164	4383.027	11639.475	1497.674	2435.676
PS(46:9)	813.475	315.537	162.075	298.365	14.005	0.000	12.761	27.342
PS(42:7)	763.750	169.684	439.207	391.295	2933.801	6049.165	528.407	820.090
PS(30:2)	3907.878	764.695	1897.411	2118.355	12004.184	29358.278	2558.601	4313.516
PS(26:6)	115.186	30.171	65.281	66.233	116.303	1097.163	71.406	207.905
PS TOTAL	19021.315	5487.459	10216.088	11918.790	51673.572	96855.296	10488.717	16323.456
PC(46:6)	108404.996	16120.421	13671.180	23868.044	5751.772	37736.808	20871.453	20587.668
PC(46:7)	22290.706	3180.322	1655.535	2836.471	2130.682	2112.735	4288.025	4173.567
PC(46:8)	1365.616	277.655	57.588	230.629	245.269	0.000	1125.153	758.493
PC(46:9)	18.120	29.422	0.000	0.000	0.000	0.000	1388.597	628.949

Table 4.3 Continued

	456MAD	456FAD	456MTx	456FTx	456FCrn	456FBt	BenMAD	BenFAD
PC(44:5)	128018.732	22314.958	21402.084	38272.689	9157.229	62560.353	26969.978	26148.788
PC(44:4)	364418.974	61909.953	77443.609	114625.598	22714.215	240546.824	64640.790	66411.934
PC(44:6)	20332.202	3557.040	2406.626	4242.861	2076.161	3527.129	6914.249	5600.090
PC(44:7)	1206.356	271.971	68.565	232.951	66.629	0.000	284.365	231.708
PC(42:3)	95189.700	19853.556	45502.322	66863.619	12391.732	148826.941	26420.146	25206.526
PC(42:4)	59540.659	11332.792	12905.224	20905.165	5731.066	33160.816	12415.705	12418.152
PC(42:5)	8718.085	1840.124	1804.444	3467.854	710.539	2262.274	1591.138	1774.391
PC(42:10)	714.795	141.940	245.420	227.276	167.314	714.874	283.175	228.528
PC(40:2)	39476.384	9489.544	26337.329	40581.311	4863.149	95799.216	13136.723	12994.383
PC(40:3)	19729.701	4035.193	6142.620	10882.694	1391.346	14759.702	5382.038	4801.547
PC(40:4)	10439.011	1912.250	2054.017	3595.584	140.351	3096.127	2735.599	2576.663
PC(38:1)	9288.989	2540.482	6104.340	8378.737	1114.533	10282.361	3234.121	3359.451
PC(38:2)	11331.869	2390.415	5389.819	8185.197	230.030	10746.867	3625.915	3465.942
PC(38:3)	2733.021	319.864	401.289	448.380	48.998	985.588	536.185	656.062
PC(32:6)	1581.996	496.677	735.003	1104.259	746.453	10552.416	788.340	1323.771
PC(30:2)	708.398	128.020	148.974	353.811	606.064	6700.435	382.790	686.006
PC(28:0)	987.334	51.182	345.686	690.768	385.238	1088.716	548.719	471.283
PC(28:1)	3386.028	528.821	137.362	138.044	24.972	0.000	245.537	484.918
PC(28:2)	2786.333	576.103	699.361	879.624	347.674	1156.813	963.638	864.661

Table 4.3 Continued

	456MAD	456FAD	456MTx	456FTx	456FCrn	456FBt	BenMAD	BenFAD
PC(28:3)	2721.518	401.243	974.395	1069.211	443.458	258.769	871.806	646.885
PC(26:1)	1078.846	105.471	208.158	60.993	229.419	432.759	267.830	423.397
PC(26:2)	842.032	152.924	220.187	325.184	99.347	389.800	272.599	299.582
PC TOTAL	917310.403	163958.341	227061.136	352466.954	71813.640	687698.321	200184.613	197223.345

The above table is a heat map of detected lipid subclasses for each headgroup, for each strain and diet combination tested. The heat map is arranged vertically, displaying a greater abundance of a given subclass (ex PC44:4) in green, versus lower abundance of a given subclass (ex PC46:9) in red. These abundances are given for a single EMU.

Table 4.4- Headgroup subclass average abundance percentages.

	456MAD	456FAD	456MTx	456FTx	456FCrn	456FBt	BenMAD	BenFAD
PA(38:4)	1.3%	2.4%	3.3%	6.7%	10.0%	6.4%	1.8%	3.2%
PA(36:5)	0.1%	0.1%	0.6%	0.7%	0.0%	0.5%	0.5%	0.3%
PA(36:8)	14.5%	7.7%	22.2%	12.0%	24.4%	31.5%	27.5%	22.8%
PA(34:1)	1.7%	2.0%	1.0%	1.5%	0.0%	0.0%	0.3%	0.9%
PA(34:3)	1.6%	0.8%	2.4%	0.4%	0.0%	0.7%	2.3%	3.0%
PA(34:4)	15.6%	8.1%	12.1%	6.5%	14.3%	16.4%	15.7%	18.6%
PA(34:5)	21.0%	11.3%	18.4%	14.5%	27.1%	25.9%	22.6%	24.4%
PA(34:6)	4.2%	3.1%	5.9%	2.2%	6.5%	7.6%	6.9%	6.5%
PA(32:3)	1.8%	1.5%	1.8%	0.3%	0.0%	0.9%	2.0%	2.3%
PA(30:1)	2.2%	2.9%	2.3%	5.6%	5.3%	8.9%	1.1%	1.8%
PA(28:1)	9.3%	14.7%	10.7%	17.6%	6.4%	0.0%	3.8%	5.6%
PA(28:2)	8.3%	16.6%	7.8%	13.1%	4.1%	1.0%	8.0%	4.7%
PA(28:3)	2.9%	7.1%	5.0%	8.1%	0.7%	0.0%	3.3%	2.1%
PA(26:1)	15.7%	21.7%	6.5%	10.8%	1.3%	0.2%	4.3%	3.7%
PA TOTAL	17929.41	6264.68	2401.551	2730.903	1772.266	24009.17	4697.585	7270.849
PE(26:5)	14.5%	16.0%	17.7%	15.6%	9.2%	15.5%	13.8%	13.4%
PE(26:3)	4.2%	2.4%	2.9%	3.2%	4.3%	4.4%	4.3%	4.0%
PE(30:4)	12.8%	21.3%	11.8%	17.8%	2.7%	4.1%	4.1%	4.9%
PE(30:3)	68.4%	60.3%	67.7%	63.3%	83.8%	76.0%	77.7%	77.7%
PE TOTAL	9162.946	2203.328	4503.135	5161.762	23199.69	56113.84	5269.823	8573.505
PI(40:5)	5.2%	6.5%	2.0%	2.9%	0.0%	0.1%	0.2%	0.2%
PI(40:4)	39.6%	48.3%	35.0%	47.3%	11.0%	16.8%	17.0%	18.8%
PI(40:3)	46.0%	38.2%	52.7%	41.8%	73.1%	70.2%	68.7%	67.3%
PI(40:2)	8.9%	6.8%	9.8%	7.8%	15.2%	12.3%	13.6%	13.2%
PI(44:6)	0.3%	0.2%	0.5%	0.3%	0.6%	0.6%	0.5%	0.5%
PI TOTAL	25783.73	8225.74	14229.12	16356.46	69782.24	119070.2	13889.91	20933.83
PG(36:4)	12.9%	10.0%	19.2%	11.3%	8.6%	0.0%	9.6%	6.4%
PG(36:3)	7.1%	5.5%	9.4%	5.0%	8.8%	0.0%	5.7%	3.5%
PG(36:2)	5.9%	4.5%	1.6%	1.5%	3.2%	0.7%	2.1%	1.7%
PG(36:5)	8.3%	5.5%	8.0%	6.8%	3.5%	0.0%	5.3%	2.8%
PG(34:1)	10.7%	11.5%	13.5%	8.0%	8.4%	0.0%	6.1%	3.0%
PG(34:2)	12.4%	11.3%	10.4%	8.2%	3.2%	0.0%	6.1%	2.6%

Table 4.4 Continued

	456MAD	456FAD	456MTx	456FTx	456FCrn	456FBt	BenMAD	BenFAD
PG(34:3)	13.2%	10.5%	8.1%	4.7%	1.2%	0.0%	4.7%	2.2%
PG(34:6)	3.4%	4.9%	0.3%	1.8%	0.9%	0.0%	8.4%	7.3%
PG(28:2)	9.8%	14.9%	13.3%	24.5%	27.5%	47.3%	22.4%	36.3%
PG(28:3)	5.4%	9.6%	8.1%	16.0%	16.9%	21.5%	7.8%	10.1%
PG(26:0)	5.7%	3.9%	5.7%	5.1%	12.4%	14.2%	8.3%	9.6%
PG(26:1)	5.3%	7.9%	2.1%	7.0%	5.3%	16.2%	13.5%	14.6%
PG TOTAL	3134.47	1187.452	686.9912	1192.111	1480.911	4439.624	567.277	839.3517
PS(46:7)	36.4%	33.2%	44.5%	35.2%	62.4%	50.3%	55.5%	52.2%
PS(46:8)	34.1%	43.5%	30.4%	40.7%	8.5%	12.0%	14.3%	14.9%
PS(46:9)	4.3%	5.8%	1.6%	2.5%	0.0%	0.0%	0.1%	0.2%
PS(42:7)	4.0%	3.1%	4.3%	3.3%	5.7%	6.2%	5.0%	5.0%
PS(30:2)	20.5%	13.9%	18.6%	17.8%	23.2%	30.3%	24.4%	26.4%
PS(26:6)	0.6%	0.5%	0.6%	0.6%	0.2%	1.1%	0.7%	1.3%
PS TOTAL	19021.32	5487.459	10216.09	11918.79	51673.57	96855.3	10488.72	16323.46
PC(46:6)	11.8%	9.8%	6.0%	6.8%	8.0%	5.5%	10.4%	10.4%
PC(46:7)	2.4%	1.9%	0.7%	0.8%	3.0%	0.3%	2.1%	2.1%
PC(46:8)	0.1%	0.2%	0.0%	0.1%	0.3%	0.0%	0.6%	0.4%
PC(46:9)	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.7%	0.3%
PC(44:5)	14.0%	13.6%	9.4%	10.9%	12.8%	9.1%	13.5%	13.3%
PC(44:4)	39.7%	37.8%	34.1%	32.5%	31.6%	35.0%	32.3%	33.7%
PC(44:6)	2.2%	2.2%	1.1%	1.2%	2.9%	0.5%	3.5%	2.8%
PC(44:7)	0.1%	0.2%	0.0%	0.1%	0.1%	0.0%	0.1%	0.1%
PC(42:3)	10.4%	12.1%	20.0%	19.0%	17.3%	21.6%	13.2%	12.8%
PC(42:4)	6.5%	6.9%	5.7%	5.9%	8.0%	4.8%	6.2%	6.3%
PC(42:5)	1.0%	1.1%	0.8%	1.0%	1.0%	0.3%	0.8%	0.9%
PC(42:10)	0.1%	0.1%	0.1%	0.1%	0.2%	0.1%	0.1%	0.1%
PC(40:2)	4.3%	5.8%	11.6%	11.5%	6.8%	13.9%	6.6%	6.6%
PC(40:3)	2.2%	2.5%	2.7%	3.1%	1.9%	2.1%	2.7%	2.4%
PC(40:4)	1.1%	1.2%	0.9%	1.0%	0.2%	0.5%	1.4%	1.3%
PC(38:1)	1.0%	1.5%	2.7%	2.4%	1.6%	1.5%	1.6%	1.7%
PC(38:2)	1.2%	1.5%	2.4%	2.3%	0.3%	1.6%	1.8%	1.8%

Table 4.4 Continued

	456MAD	456FAD	456MTx	456FTx	456FCrn	456FBt	BenMAD	BenFAD
PC(38:3)	0.3%	0.2%	0.2%	0.1%	0.1%	0.1%	0.3%	0.3%
PC(32:6)	0.2%	0.3%	0.3%	0.3%	1.0%	1.5%	0.4%	0.7%
PC(30:2)	0.1%	0.1%	0.1%	0.1%	0.8%	1.0%	0.2%	0.3%
PC(28:0)	0.1%	0.0%	0.2%	0.2%	0.5%	0.2%	0.3%	0.2%
PC(28:1)	0.4%	0.3%	0.1%	0.0%	0.0%	0.0%	0.1%	0.2%
PC(28:2)	0.3%	0.4%	0.3%	0.2%	0.5%	0.2%	0.5%	0.4%
PC(28:3)	0.3%	0.2%	0.4%	0.3%	0.6%	0.0%	0.4%	0.3%
PC(26:1)	0.1%	0.1%	0.1%	0.0%	0.3%	0.1%	0.1%	0.2%
PC(26:2)	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.2%
PC TOTAL	917310.4	163958.3	227061.1	352467	71813.64	687698.3	200184.6	197223.3
PA	1.8%	3.3%	0.9%	0.7%	0.8%	2.4%	2.0%	2.9%
PE	0.9%	1.2%	1.7%	1.3%	10.6%	5.7%	2.2%	3.4%
PI	2.6%	4.4%	5.5%	4.2%	31.8%	12.0%	5.9%	8.3%
PG	0.3%	0.6%	0.3%	0.3%	0.7%	0.4%	0.2%	0.3%
PS	1.9%	2.9%	3.9%	3.1%	23.5%	9.8%	4.5%	6.5%
PC	92.4%	87.5%	87.6%	90.4%	32.7%	69.6%	85.1%	78.5%
Total PLs	992342.3	187327	259098	389827	219722.3	988186.4	235097.9	251164.3

This table displays the percent values for each subclass of lipid, for each strain and diet combination tested, for each headgroup, adding up to 100% detection for each headgroup. These abundance percentages are given for a single EMU.

Vita

Lucas Hietala was born on October 30, 1991 in Knoxville, Tennessee. He received a Bachelor's of Science in Horticultural Science and Production. During his time as an undergraduate, he took a position in the Jurat-Fuentes insect physiology lab in order to work more closely with insects, his lifelong passion. After graduation, he began his Master's project as a student of Entomology.

During his time working as a Master's student, he has exposed himself to a large number of topics in entomology, ranging from urban pests to laboratory genetics. He has obtained a Tennessee pesticide applicator's license, presented a poster and a student competition talk at Entomological Society of America meetings, and presented a poster at the International Conference of Entomology, 2016. He has reared and cared for a dozen species of insects from the orders Coleoptera, Hemiptera, Lepidoptera, and Orthoptera, including a number of insecticide resistant strains of Lepidoptera.

Lucas's stated goals included the furthering of human understanding of and appreciation for insects of all kinds, but particularly in regards to urban pest insects.