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Using Flight Mills to Measure Flight Propensity and Performance of Western Corn Rootworm, Diabrotica virgifera virgifera (LeConte)

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Dr. Vineeta Bajaj
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Dear Dr. Bajaj,

On behalf of co-authors Eric Yu and Aaron Gassmann, I am resubmitting the written portion of our manuscript, "Using Flight Mills to Measure Flight Propensity and Performance of Western Corn Rootworm, *Diabrotica virgifera virgifera* (LeConte)," after revising based on your comments and those of the reviewers. The changes are visible in the tracked version of the manuscript, and are detailed in the rebuttal document. We appreciate all of the constructive comments and suggestions, they have helped us make this a better paper. I hope you will find our responses adequate, but if additional changes are needed, we will of course be happy to accommodate.

Thank you very much for considering this manuscript, and please let me know if we can provide you with anything else.

Sincerely,

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TITLE:

Using Flight Mills to Measure Flight Propensity and Performance of Western Corn Rootworm, *Diabrotica virgifera virgifera* (LeConte)

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Beetle, Coleoptera, dispersal, flight mill, insect flight behavior, tethered flight

SUMMARY
Flight mills are important tools for comparing how age, sex, mating status, temperature, or various other factors may influence an insect's flight behavior. Here we describe protocols to tether and measure the flight propensity and performance of western corn rootworm under different treatments.

ABSTRACT
The western corn rootworm, *Diabrotica virgifera virgifera* (LeConte) (Coleoptera: Chrysomelidae), is an economically important pest of the corn in the northern United States. Some populations have developed resistance to management strategies including transgenic corn that produces insecticidal toxins derived from the bacterium *Bacillus thuringiensis* (Bt). Knowledge of the western corn rootworm dispersal is of critical importance for models of resistance evolution, spread, and mitigation. Flight behavior of an insect, especially over the long-distance, is inherently difficult to observe and characterize. Flight mills provide a means to directly test the developmental and physiological impacts and consequences of the flight in the laboratory that cannot be obtained in field studies. In this study, flight mills were used to measure the timing of flight activity, the total number of flights, and the distance and duration of flights taken by female rootworms during a 22-h test period. Sixteen flight mills were housed in an environmental chamber with programmable lighting, temperature, and humidity control. The flight mill described has a typical design, where a flight arm is free to rotate about a central pivot. Rotation is caused by the flight of an insect tethered to one end of the flight arm, and each rotation is recorded by a sensor with a time-stamp. Raw data are compiled by a software, which is subsequently processed to provide the statistics for flight parameters of interest. The most

difficult task for any flight mill study is the attachment of the tether to the insect with an adhesive. The method used must be tailored to each species. The attachment must be strong enough to hold the insect in a rigid orientation and to prevent detachment during movement, while not interfering with the natural wing motion during flight. The attachment process requires dexterity, finesse, speed, and making video footage of the process for rootworms of value.

INTRODUCTION

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), was identified as a pest of cultivated corn in 1909[1]. Today, it is the most important pest of corn (*Zea mays* L.) in the U.S. Corn Belt, with larval feeding on corn roots causing most of the yield loss associated with this pest. The annual costs for management and corn production losses due to corn rootworm are estimated to exceed $1 billion[2]. The western corn rootworm is highly adaptable, and populations have evolved resistance to multiple management strategies including insecticides, crop rotation, and transgenic Bt corn[3]. Determining spatial dimensions over which tactics must be applied to mitigate local development of resistance, or a resistance hotspot depends on a better understanding of dispersal[4]. Mitigation measures will not be successful if they are restricted to too small of a spatial scale around a resistance hotspot because resistant adults will disperse beyond the mitigation area[5]. Understanding flight behavior of western corn rootworm is important to create effective resistance management plans for this pest.

Dispersal by flight plays an important role in adult western corn rootworm life history and ecology[6], and the flight behavior of this pest can be studied in the laboratory. Several methods may be used to measure flight behavior in the laboratory. An actograph, which restricts flight in a vertical plane, can measure the amount of time an insect is engaged in flight. Actographs have been used to compare flight duration and periodicity patterns of western corn rootworm males and females at different ages, body sizes, temperatures, insecticide susceptibility, and insecticide exposure[7,8,9]. Flight tunnels, which consist of a tracking chamber and directed air flow, are especially useful for examining insect flight behavior when following an odor plume, such as candidate pheromone components[10] or plant volatiles[11]. Flight mills are perhaps the most common method for laboratory studies of insect flight behavior and can characterize several aspects of flight propensity and performance. Laboratory flight mills have been employed in studies of western corn rootworm to characterize propensity to make short and sustained flights as well as hormonal control of sustained flight[12,13].

Flight mills provide a relatively simple way to study insect flight behavior under laboratory conditions by allowing researchers to measure various flight parameters including periodicity, speed, distance, and duration. Many of the flight mills used today are derived from the roundabouts of Kennedy et al.[14] and Krogh and Weis-Fogh[15]. Flight mills can be different in shape and size, but the basic principle remains the same. An insect is tethered and mounted on a radial horizontal arm that is free to rotate, with minimal friction, about a vertical shaft. As the insect flies forward, its path is restricted to circling in a horizontal plane, with the distance traveled per rotation dictated by the length of the arm. A sensor is typically used to detect each rotation of the arm caused by the flight activity of the insect. Raw data include rotations per unit time, and time of day flight occurred. The data are fed into a computer for recording. Data from multiple flight mills are often recorded in parallel, essentially simultaneously, with banks of 16 and 32 flight mills being common. The raw data are further processed by custom software to provide values for such variables as flight speed, total number of separate flights, distance and duration flown, and so forth.

Every insect species is different when it comes to the best method for tethering because of morphological variables such as overall size, size and shape of the target area for attaching the tether, softness, and flexibility of the insect, need and method for anesthetization, potential for fouling the wings and/or head with misplaced or overflow adhesive, and many, many more details. In the cases of visualized tethering of a plataspid bug[16] and an ambrosia beetle[17], the respective target areas for tether attachment are relatively large and forgiving of imprecise adhesive placement because the head and wings are somewhat well-separated from the attachment site. This is not to downplay the difficulty of tethering these insects, which is demanding for any species. But the western corn rootworm is a particularly challenging insect to tether: the pronotum is narrow and short, making very precise attachment with a minimal amount of adhesive (dental wax in this case) necessary to prevent interference with the opening of the elytra for flight and with the head, where contact with eyes or antennae can affect behavior. At the same time, the tether must be firmly attached to avoid dislodgement by this strong flyer. The demonstration of tethering of rootworm adults is the most important offering in this paper. It should be of help to others who work with this or similar insects where the method visualized here could be a useful option.

This paper describes methods used to effectively tether and characterize the flight activity of western corn rootworm adults that were reared at different larval densities. The flight mills and software used in this study (**Figure 1**) were derived from designs posted on the internet by Jones et al.[18] Tethering techniques were modified from the description in Stebbing et al.[9] An array of 16 flight mills was housed in an environmental chamber, designed to control lighting, humidity, and temperature (**Figure 2**). Using this or similar setup along with the following techniques allows for testing many factors that may influence the flight propensity and performance of western corn rootworm, including age, sex, temperature, photoperiod, and many others.

PROTOCOL

1. Rear western corn rootworm for flight tests

NOTE: If the adult's age must be controlled or known, adults must first be collected in the field followed by rearing their offspring to adulthood for testing. If the age of the beetle or a standardized rearing environment is not of concern, then directly testing field-collected adults may be possible, and the protocol can begin with step 2.

1.1. Collect at least 500 western corn rootworm adults from a cornfield of interest to ensure enough eggs are obtained for rearing adequate numbers of adults. Use a manual aspirator to collect adults from the field.

NOTE: It is recommended to collect adults during peak abundance, around late July in the U.S. Corn Belt, to ensure the collection of both sexes. Most adults will be males if collected earlier, whereas most will be females if collected later.

1.2. Place the collected male and female adults into a mesh cage containing chopped corn ear, corn leaf tissue, 1.5% agar solid, and an oviposition substrate. An 18 x 18 x 18-cm cage (mesh size 44 x 32, 650 µm aperture) can hold up to 500 adults at one time.

1.2.1. Use the corn grown in the field as a source of corn ear, which will be picked at the R3, or milk stage of kernel development[19] The R3 kernel is yellow outside, while the inner fluid is milky white due to accumulating starch. Corn ears can be frozen and stored for up to a year until they are needed. To feed the rootworm, remove the husk and chop the corn into horizontal cross-sections about 3 cm thick. Chopped corn is the primary diet for the adults and should be changed out twice a week.

1.2.2. Obtain leaves from greenhouse-grown corn plants of any age. Amount of leaf tissue will vary with the number of adults in the cage. Avoid using field plants, as they may introduce disease.

1.2.3. To make the solid agar, mix 15 g of agar powder with 1 L of DI water. Heat the mixture until boiling. Pour the liquid into Petri dishes (100 mm x 15 mm) while it is hot. Place a lid on the Petri dish once cool and place them into cold storage (6° C). Agar provides adults with a source of moisture and should be changed out twice a week.

1.2.4. To prepare an oviposition substrate, place 40 g of sieved field soil (<180 µm) into a Petri dish. Moisten the soil with deionized water. Ensure that the soil at the bottom of the Petri dish appear wet. Score the top of the moistened soil with a needle tool. Remove the oviposition substrate weekly and place in an incubator at 25° C and 60% RH for at least one month.

1.3. After incubating eggs for one month, wash the contents of the oviposition substrate through a 250-µm sieve until all soil has been removed. Quantify the eggs by placing washed eggs in a 10 mL graduated cylinder. There are approximately 10,000 eggs per 1 mL.

1.4. Place the quantified eggs into a 44-mL container and cover with sieved field soil (<180 µm). Western corn rootworm eggs undergo obligate diapause through the winter[20]. To break diapause, place eggs into cold storage (6° C) for at least 6 months.

NOTE: Eggs may be kept in cold storage for longer than 5 months, but egg viability decreases with time. After 12 months, there may be little to no hatch.

1.5. After a minimum of 5 months, remove eggs from the cold storage and place in an incubator at 25° C and 60% RH. Neonates hatch as early as 16 days after removal from cold storage.

1.6. Once the eggs hatch, place three germinated kernels at the bottom of a 44-mL plastic container with roots exposed (i.e., not covered with soil). Use a soft bristle brush to transfer 12 neonates to the surface of the roots.

1.7. Add 4.5 mL of DI water to 40-mL of sieved soil (<600 µm). Place the moistened soil over the germinated kernels that have been infested with neonates and cover the container with mesh fabric to prevent larvae from escaping.

1.8. On the same day that the 44-mL plastic container is set up with neonates, prepare a 473-mL container with corn kernels that have not germinated. The rootworm larvae will be transferred to this larger container later. The number of kernels determines the desired larval density per plant. Add 120 g of soil mixture consisting of 50% sieved field soil (<600 µm) and 50% potting soil moistened with 20-ml of deionized water.

1.9. After 7 days, transfer all contents of the 44-mL container to the 473-mL container. The larvae will be second instars at the time of transfer.

NOTE: This transfer to a larger container is necessary to supply larvae with enough root mass for feeding through pupation.

1.10. Observe the emergence of adults typically around 26 days after egg hatch. Adults are active fliers upon emergence and may escape the 473-mL container when attempting to collect them by hand. Instead, use a vacuum with an aspirator to collect adults.

1.11. Segregate adults by sex and/or date if needed for comparative testing. Sex of western corn rootworm can be determined by observing the morphology of the prothoracic basitarsi[21]. Males have broad, square-shaped prothoracic basitarsi, whereas those of females are narrow and conical-shaped.

1.11.1. Place beetle into a 45-mL clear polystyrene plastic vial and cover with a lid with 6 small (~1 mm diameter) holes.

1.11.2. Anesthetize the beetle. Place the end of a tube attached to a CO_2 tank regulator over the holes in the lid and allow a gentle flow of CO_2 to enter the tube for approximately 10 to 15 s until the adult loses its grip on the wall of the vial.

NOTE: The anesthetized insect will remain immobilized for at least 1 min.

1.11.3. Place the anesthetized beetle, ventral side up, on an inverted plastic petri dish bottom. Carefully place the non-inverted lid of the petri dish over the beetle. Ensure that the tarsi of the beetle press against the lid, allowing easy observation of the prothoracic basitarsi under a dissecting microscope.

1.12. If the experiment requires that beetles be mated prior to flight, then use males at least 5 days old to mate with the newly emerged females.

NOTE: Use of older males ensures that they are sexually mature upon their introduction to virgin females. Females are sexually mature upon adult emergence, whereas males require 5 to 7 days of post-emergence development to reach sexual maturity[22,23].

2. Start the flight mill software program prior to flight testing

NOTE: The flight mill program files (.vi file extensions which run in a commercial software platform, see Table of Materials) and details for their use are provided for download via links ("data analysis routine" and "Circular Flight Mill Instructions", respectively) in the "Flight mill wiring and software" section on the Jones et al.[18] website. If the programs no longer function in newer or future versions of the software platform, or if the user wants to add new capabilities, the routines provided by Jones et al. [18] can be modified by the user as needed.

2.1. Open the flight mill software program (**Figure 3**).

2.2. Enter the information under the **Initialization** tab.

2.2.1. Set the **Start Time** and **End Time** for the desired duration of the flight test.

NOTE: All adults should be tethered and mounted on flight mills by 30 min prior to the Start Time. It may take an experienced person 30 min to 45 min to tether and prepare 16 beetles for flight testing (see **Section 3**).

2.2.2. Set the **Min Threshold (min)** to 0. This ensures that any detection of the flight arm passing will be recorded, and is the default recommended by Jones et al. [18].

2.2.3. Set the **Max Threshold (min)** to 1. Here, 1 min was used. This value means that 1 min must elapse between sensor detection of the flight arm to "call" the end of a flight.

2.2.4. Enter a name for the file.

2.2.5. Set **Raw Data Log Interval (min)** to 1. This value controls the interval over which the raw data will be compiled for output reporting. Here, it is set to 1 min. Thus, the output of revolutions, for example, will be logged per minute.

NOTE: The actual time interval between electronic scanning of sensor activity is very short, but a 1-min interval allows logging at a fine enough scale for most research purposes, while restricting the number of lines in the spreadsheet output to a reasonable number for examining by eye.

2.3. Under the **Subject Information** fill in the columns labeled ID, diet, sex, species, and comments as desired.

2.4. Click on the **START** button located on the left side of the screen display. The program will begin collecting raw data once the **Current Time** matches the **Start Time**.

3. Tether western corn rootworm to flight mill

3.1. Bend a 40-mm length 28-gauge steel wire 90° at the center.

NOTE: The wire may also be of another metal such as copper.

3.2. Anesthetize the test adult with CO_2 as described above (see 1.11.1 and 1.11.2).

3.3. Place the anesthetized adult on a flat surface and position its dorsal side up. If the beetle does not lie completely flat on the surface, reposition the legs so that it does. It is important that the beetle lie as flat as possible on the surface to ensure the correct positioning of the wire.

3.4. Take a small amount of dental wax, slightly larger than a pinhead, and roll it between the fingertips until a ball is formed. Ensure that fingers are clean to prevent debris, dirt, and oil from incorporating into the wax, because it may prevent the wax from adhering to the insect.

3.5. Push one end of the 40 mm the bent wire into the center of the ball of wax.

3.6. Briefly, for 1 to 2 s, heat the dental wax on the wire with a butane lighter. If the wax is heated for too long, the melted wax will drop off of the wire. Do not reuse the wax if it has fallen off from the wire as it will not effectively adhere to the insect cuticle.

3.7. Carefully place the end of the steel wire with the melted dental wax on the dorsal surface of pronotum, while pointing the other end of the wire, (i.e., the end without dental wax), along the midline of the abdomen. Alternatively, point the end of the wire without the dental wax toward the head if desired. In that case, ensure that a flying beetle push the flight arm instead of pulling it. Be sure that the melted wax does not get on the elytra or its sutures, as it may prevent or hinder flight.

3.8. Place the free end of the wire into the opening of the hollow metal tube of the flight mill arm. Ensure that the wire fits tight enough to hold in place without slipping, simply by friction. The tethered beetle may be positioned to fly either clockwise or counter-clockwise.

3.9. Immediately after mounting a beetle, tear a small piece (~1-cm dia) of tissue paper from a larger tissue. Offer the tissue piece to the tethered beetle hanging from the flight mill for tarsal contact; most beetles will grasp the tissue and hold it against gravity until they release it at the beginning of their first flight activity. This will greatly reduce initial escape or landing flight behavior.

NOTE: Human presence in the flight-testing room should be limited to attaching and removing adults from the flight mills. The test period usually does not begin until at least 30 min have elapsed since attachment (see Note under 2.2.1), and humans should not be present in the flight room during this time or during the test period itself.

3.10. Remove all flight-tested adults after completion of a flight mill test. Remove the wax bead connecting the tether to the pronotum by gently peeling the wire away from the pronotum. The wax will separate easily without damaging the cuticle, making the insect available for further experimentation if desired.

4. Save the data collected from the flight mill program.

4.1. The program may be set to either **MANUAL** or **AUTO**. If the program is set to manual, then the user must end the program by clicking the **START** button. If the program is set to **AUTO**, then the program will stop collecting raw data once the **Current Time** matches the **End Time**.

4.2. Click **EXIT** after the flight-testing period has ended.

4.3. Ensure that a TDMS file is saved under the file name entered during program initialization (step 2.2).

4.4. Click on the TDMS file and save the document as a spreadsheet (.xlsx).

5. Retrieve flight parameters from the saved spreadsheet (.xlsx)

NOTE: A spreadsheet can be custom designed to manipulate the raw data output from the flight mill software. Here, the software program was the same as described by Jones et al. [18], but an additional routine was added to recognize and summarize the longest uninterrupted flight by an individual insect during the test period.

5.1. For each individual that engaged in flight activity, the spreadsheet will include the following information: flight number, total revolutions, start time, end time, and flight duration in minutes.

5.1.1. To calculate the total distance flown during the test period, sum the column labeled 'Total Revs' and multiply it by the distance flown per revolution. Distance per revolution depends on the length of the flight arm from the central pivot to the attached insect. For example, if this distance is 15.9 cm, each revolution is equivalent to one meter flown. The total number of revolutions may also be found in the 'Test Stats' tab.

5.1.2. To calculate the total duration flown during the test period, sum the column labeled 'Flight Duration (min)'.

5.1.3. To determine the distance and duration of the longest uninterrupted flight, go to the 'Test Stats' tab and look under the column labeled 'Longest Flight #'.

5.1.4. Flight speed can be calculated by dividing distance flown by flight duration. For insects, speed is commonly expressed in m/s or km/h.

REPRESENTATIVE RESULTS

Figure 4 shows representative examples of outputs expected after flight testing. Flight data were obtained from experimental work conducted in the Department of Entomology at Iowa State University. Six-day-old, mated female western corn rootworm adults were tethered to flight mills and placed in a controlled environmental chamber set at 14:10 L:D, 60 % RH, and 25° C. The beetles were left on the flight mills for 22 consecutive hours beginning 30 min before initiation of simulated dawn, and their flight activity was recorded (**Figure 4**). Dawn and dusk were simulated by a programmed, gradual change in light intensity from full-off to full-on at dawn (or vice-versa at dusk) over a 30-minute period. The first tab in the resulting spreadsheet summarizes the individual adults that were tested, using information entered from step 2.3. The subsequent tabs include flight data for each individual. The last two tabs are labeled 'RAW DATA' and 'Test Stats'. 'RAW DATA' includes time of flight activity for all individuals. 'Test Stats' indicates the longest uninterrupted flight for each beetle, and summaries of the duration of the longest uninterrupted flight in minutes, the total time spent in flight during the test period in minutes, and the total number of revolutions during the test period. Time stamps for beginning and end of each independent flight allow analyses of flight periodicity.

For the female beetle tethered to flight mill #2 (**Figure 4B**), the spreadsheet displays the number of flights, total revolutions per flight, start and end time of each flight, and the duration of each flight. This female engaged in several independent flights, most of which were very short. However, in flight #5 the female traveled 1,258 m (which equals the number of revolutions in this case, because the distance per revolution was 1 m) over a 37.8-min period of uninterrupted flight. The female beetle tethered to flight mill #1 (**Figure 4C**) did not engage in flight during the test period, so a blank spreadsheet is displayed.

As an example, results are presented from a simple comparison of flight characteristics between two groups of female western corn rootworm. Adults were collected in commercial cornfields from two locations in Iowa and allowed to oviposit in the laboratory. Eggs were collected, and offspring reared as described in Step 1 of the protocol at a post-neonate density (step 1.9) of 12 larvae per 36 seedlings. The resulting adult females were tethered and tested as described in Steps 2 and 3. **Table 1** shows a summary of the flight parameters from the raw data retrieved from the flight mill software as described in Steps 4 and 5. Total flight parameters refer to the sum of all flights of an individual during the 22-h test period, whereas the longest flight parameters refer to the longest uninterrupted flight during the test.

FIGURE AND TABLE LEGENDS

Figure 1. Insect flight mills used for tethered experiments. (A) Entire insect flight mill and (B) working portion of the flight mill. (A) Working portion of the flight mill is circled, **(B) (1)** 1 m hypodermic tube flight arm, **(2, 3)** repelling ferrite ring magnets, **(4)** digital Hall effect sensor, **(5)** small nickel ring magnet used to trigger the sensor, and **(6)** hypodermic thin wall tube ("central

pin") that separates the repelling magnets **(2,3)**. Flight mills modified slightly from the original design of Jones et al.[18]

Figure 2. Components of the flight mill environmental chamber. (A) Exterior chamber features include **(1)** Intellus controller, **(2)** control panel, and **(3)** main power disconnect. **(B)** Interior chamber features include **(1)** unit coolers (behind ceiling panel), **(2)** LED modules, **(3)** shelving units, and **(4)** pan-type humidifier.

Figure 3. An interface of the flight mill software program. (A) The first tab, labeled 'Initialization', requires information including start and end times, and the file name. **(B)** The second tab, labeled 'Subject Information', does not require any information to be entered, but is used to differentiate between multiple individuals evaluated in a single flight test.

Figure 4. Representative flight data from 6-day-old female western corn rootworm beetles. (A) The first tab of the output summarizes the information on seven individuals flight tested on a particular day. **(B)** Flight data for the female on flight mill #2 (FM#2), which engaged in multiple independent flights during the 22-hour test period. **(C)** The female placed on flight mill #1 (FM#1) did not engage in flight during the 22-hour test period, resulting in a blank spreadsheet.

Table 1. Mean (± SE) performance on flight mills of female western corn rootworm from two locations in Iowa. Longest flight refers to the longest uninterrupted (i.e., continuous) flight performed by each individual during the test period.

DISCUSSION

Characterizing western corn rootworm flight behavior is important for devising effective resistance management plans. Flight behavior of this pest has been studied in the laboratory using various methods including actographs, flight tunnels, and flight mills. Flight mills, as described and illustrated in this paper, allow insects to make uninterrupted flights so that researchers can quantify flight parameters such as distance, duration, periodicity, and speed of individual flights, over an entire test period.

The most challenging step in the protocol for flight mill experimentation with western corn rootworm, as it is for most insect species, is properly applying a tether to the adult (Step 3). This can be a difficult task due to the small amount of surface area available on the pronotum for attachment of the wire, as well as the copious amount of natural waxes on the cuticle surface. The task is made more difficult by the limited time available to apply the tether before the insect begins to stir as it emerges from CO_2 anesthetization. It is important that the tether is lined up correctly and adheres to the beetle's pronotum throughout the testing period. If the tether is misaligned, the beetle may have a difficult time engaging in flight while on the flight mill, resulting in the artifactually lower distance, duration, and speed. The beetle may escape during the test period if the dental wax does not adhere the wire strongly enough to the pronotum. Therefore, it is important to have clean, steady hands, a good sense for warming the wax to a workable temperature, and confidence while tethering beetles, all of which are attainable with adequate practice.

A decision must be made about what constitutes an independent flight event so that the Max Threshold value can be set (Step 2.2.3). An individual may make no flights, one flight, or dozens of flights during a test period, depending on its stop-and-go activity, but also on the assigned Max Threshold value. The default value reported by Jones et al.[18] is 5 s. In this study of western corn rootworm, the Max Threshold was set at 1 min. The most appropriate setting is a judgment call based on the insect species and the goals of the researcher. There are trade-offs. An insect that quits flying but continues to circle for one or more revolutions because of momentum will have those revolutions incorrectly counted as part of the previous flight when the value is set to 1 min. If the value is set at 5 s, most of the extra non-flight revolutions will not be counted and logging of that flight will be correctly terminated. On the other hand, sometimes an insect slows its flight substantially in an effort to control its direction, to land, or for other reasons, then resumes flying at higher speed without ever having stopped active flight. Such behavior on flight mills is common and has been observed in western corn rootworm; it would often be logged as two separate flights when the maximum threshold is set to 5 s, but would be correctly recorded as an uninterrupted flight when the threshold is 1 min. Under the 1-min threshold, however, the flight of an insect that truly stops flying then resumes flight within 1 min would be incorrectly recorded as not having stopped.

A minimum flight threshold (e.g., at least one flight of at least one minute) may be used to exclude from further analyses any adults that may have been damaged during handling or were otherwise in poor health. The trade-off of protecting against such false-zeros (or false very-short flights) is the possibility of excluding true-zeros (or true very-short flights), i.e., individuals that were healthy but were not motivated to fly. The researcher must decide how to handle zeros (or very short flights) based on the goals of the experiment, as well as which type of error is most likely and which is least desirable when it comes to interpreting the results. In addition, a common problem occurs when the position of the flight arm supporting an inactive beetle happens to be directly over, or very near, the sensor, where small movements of the arm caused by non-flight movements of the insect or slight air currents in the chamber may be falsely recorded as revolutions. To prevent this methodological artifact from inflating the frequency of shorter flight durations, it is recommended to exclude all flights lasting ≤1 min from analyses. This kind of artifactual reading, if it goes on for a longer time, can also result in a nonsensically high speed (e.g., ≥ 2 m/s) for a recorded "flight"; when detected, those "flight" data should be deleted for that individual.

Although flight mill studies have provided important insights into western corn rootworm flight behavior, as with any species there are complications in relating tethered flight to natural flight in the field[24]. An insect on a flight mill is suspended, which provides vertical support for its weight. Thus, the energy expended to provide lift during natural flight may not be invested by tethered insects on flight mills[25]. On the other hand, a tethered insect must provide more thrust than in free-flight to overcome friction at the pivot, the added weight of the flight arm, and aerodynamic drag from the flight arm[25,26]. Natural flight of western corn rootworm also sometimes occurs at altitudes above its flight boundary layer[27], where the distance covered during flight can be strongly influenced by wind speeds that are much greater than the insect's unaided flight speed[28].

Flight mills impose unidirectional flight, so that distance flown may overestimate total displacement in the field where the flight path may be meandering. Providing tarsal contact with a small piece of tissue after mounting the insect on the flight mill (step 3.9) reduces initial escape flight as well as flight activity associated with an attempt to land. However, once the beetle drops the tissue during an experiment, the same problem of inability to terminate flight by landing is encountered. Alternative actograph systems have been used in laboratory flight experiments with tethered[8,9] or untethered[7] western corn rootworm. While they alleviate the problem of flight termination by allowing spontaneous tarsal contact, the trade-off is the inability to measure flight distance or speed. Despite these limitations, the flight mill is very useful as a comparative tool for examining how a variety of developmental, biotic, and abiotic factors influence an insect's propensity to engage in flight, and how flight behavior itself is affected. When combined with other evidence, such as that provided by mark-capture experiments[29], trap data[30], and estimates of gene flow[31], the unique insights obtained from flight mill experiments contribute toward a holistic understanding of western corn rootworm dispersal in the field and its population-level consequences.

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DISCLOSURES

The authors have nothing to disclose.

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Figure 1

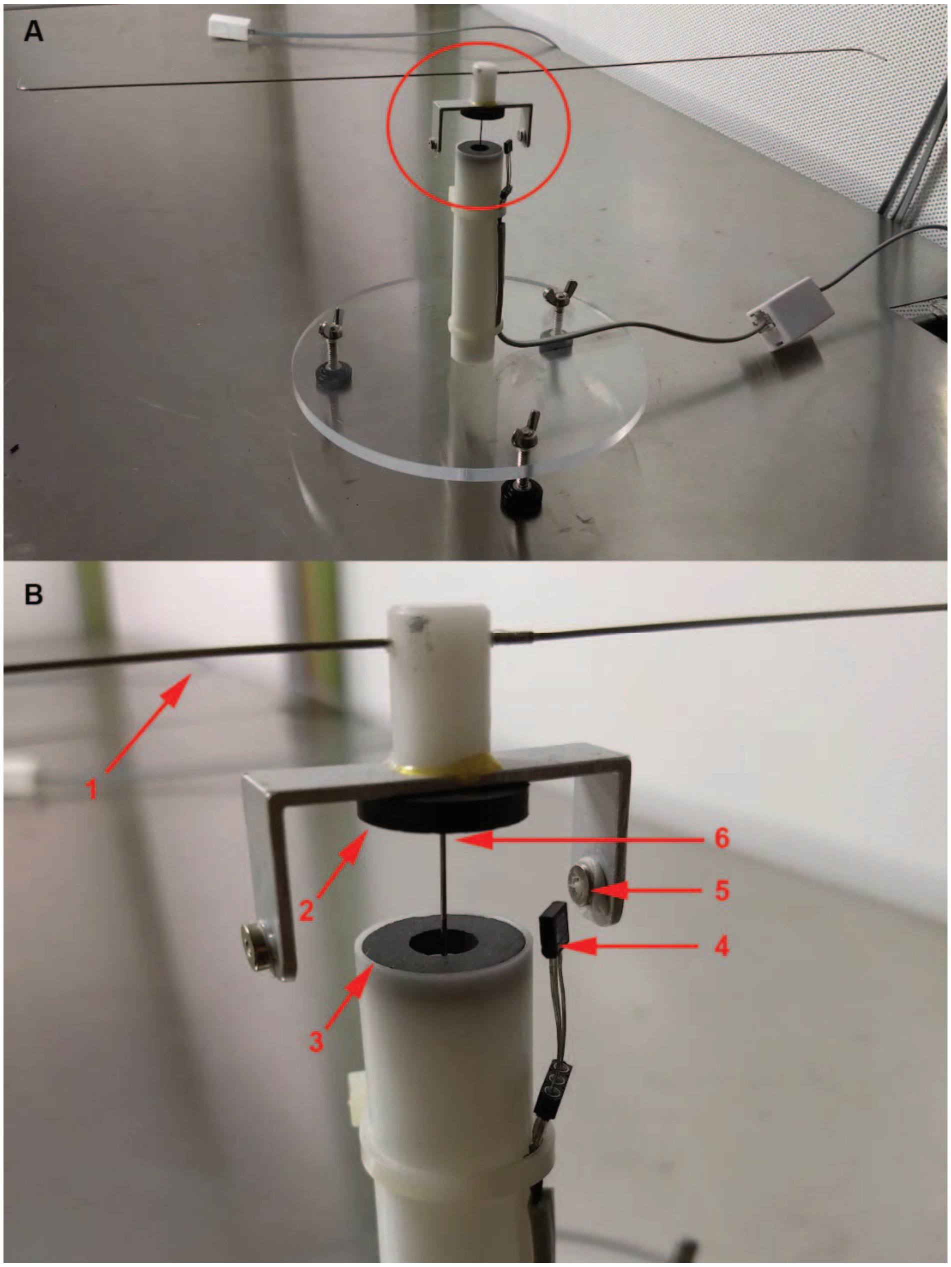

Figure 2

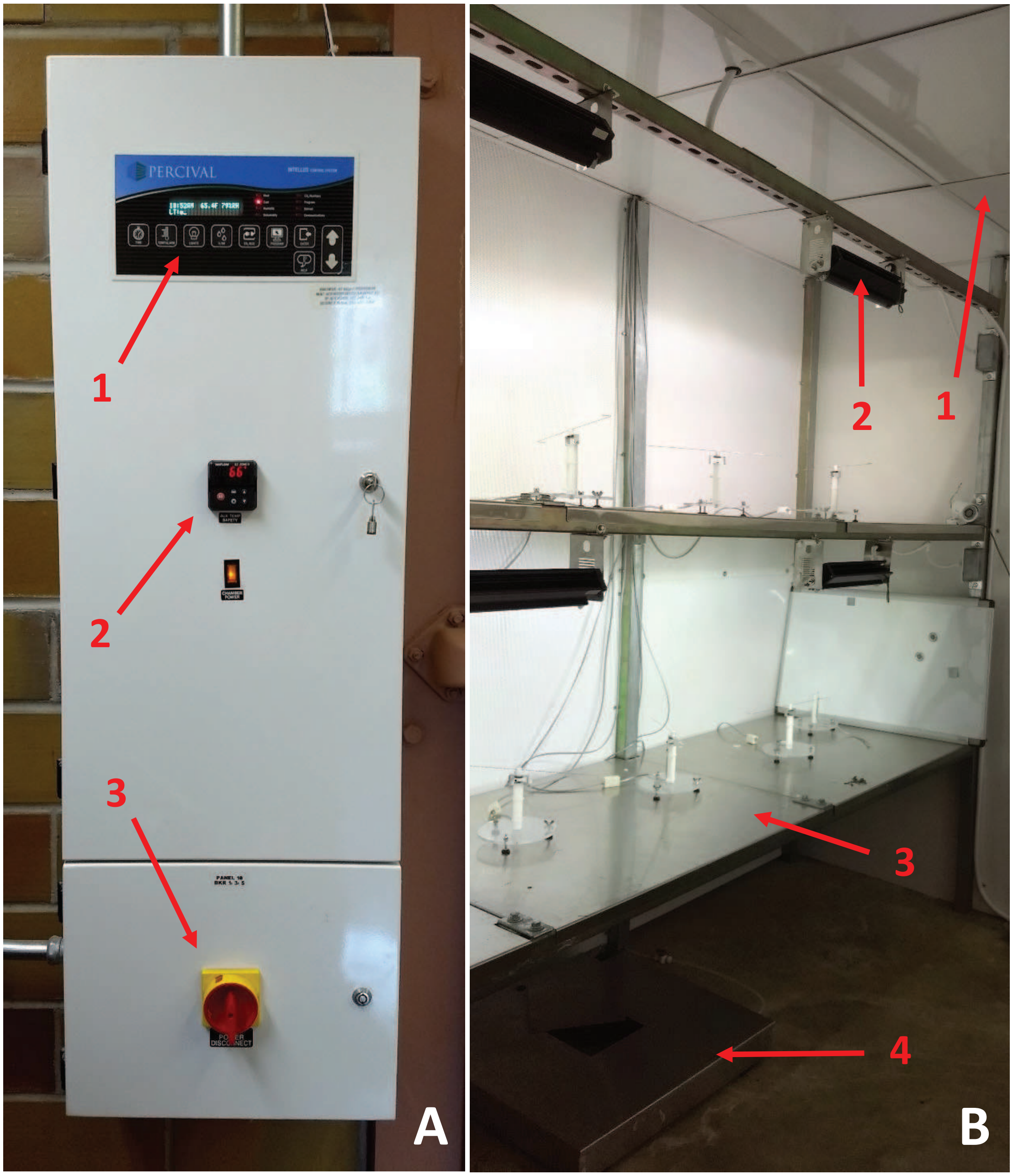

Figure 3

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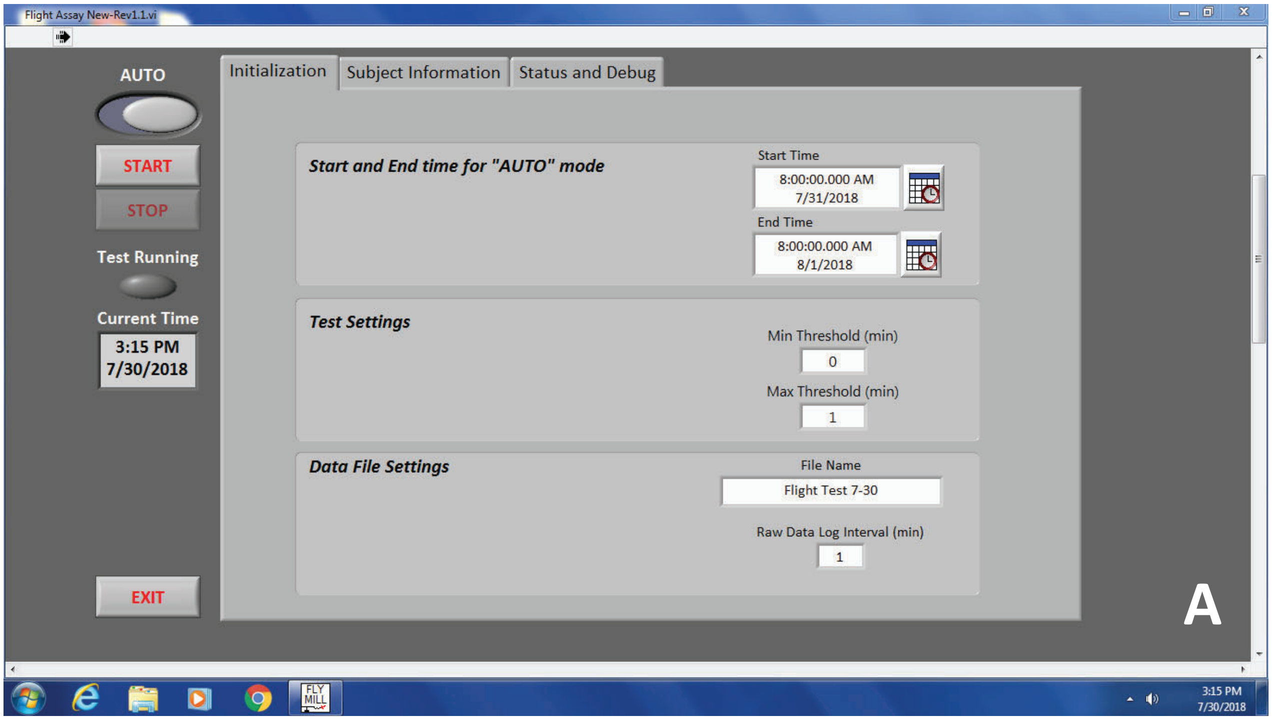


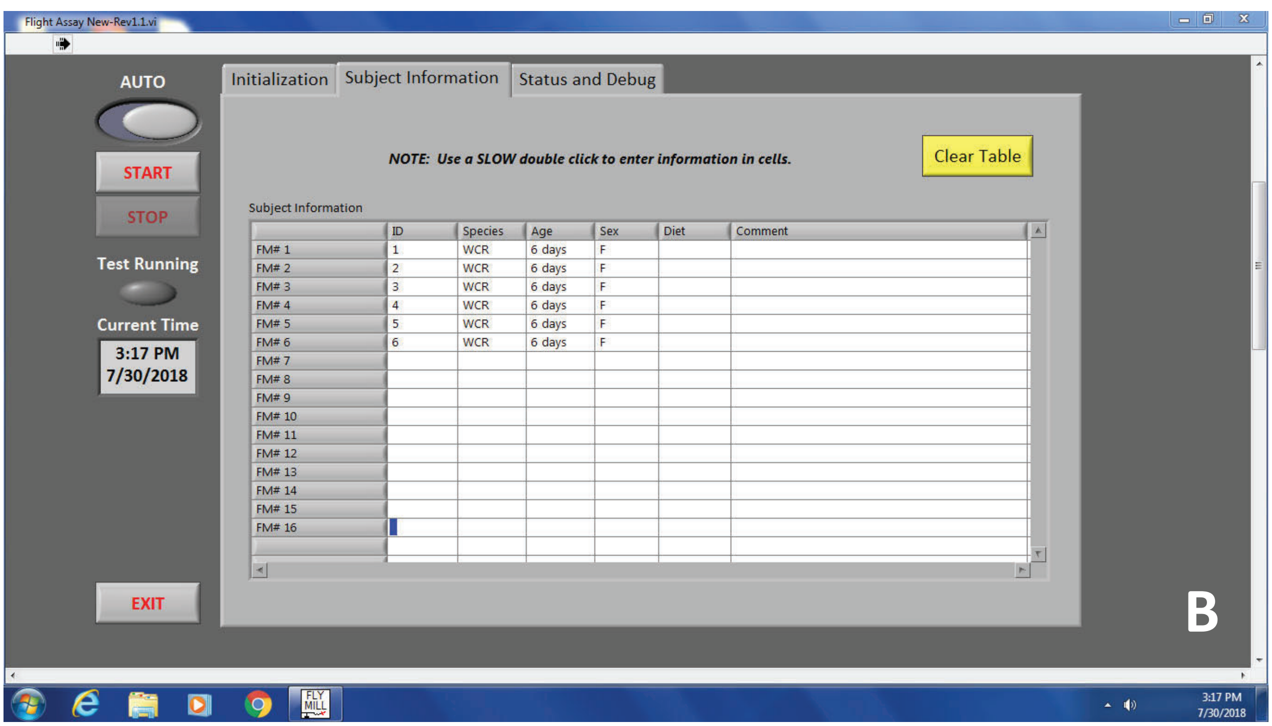
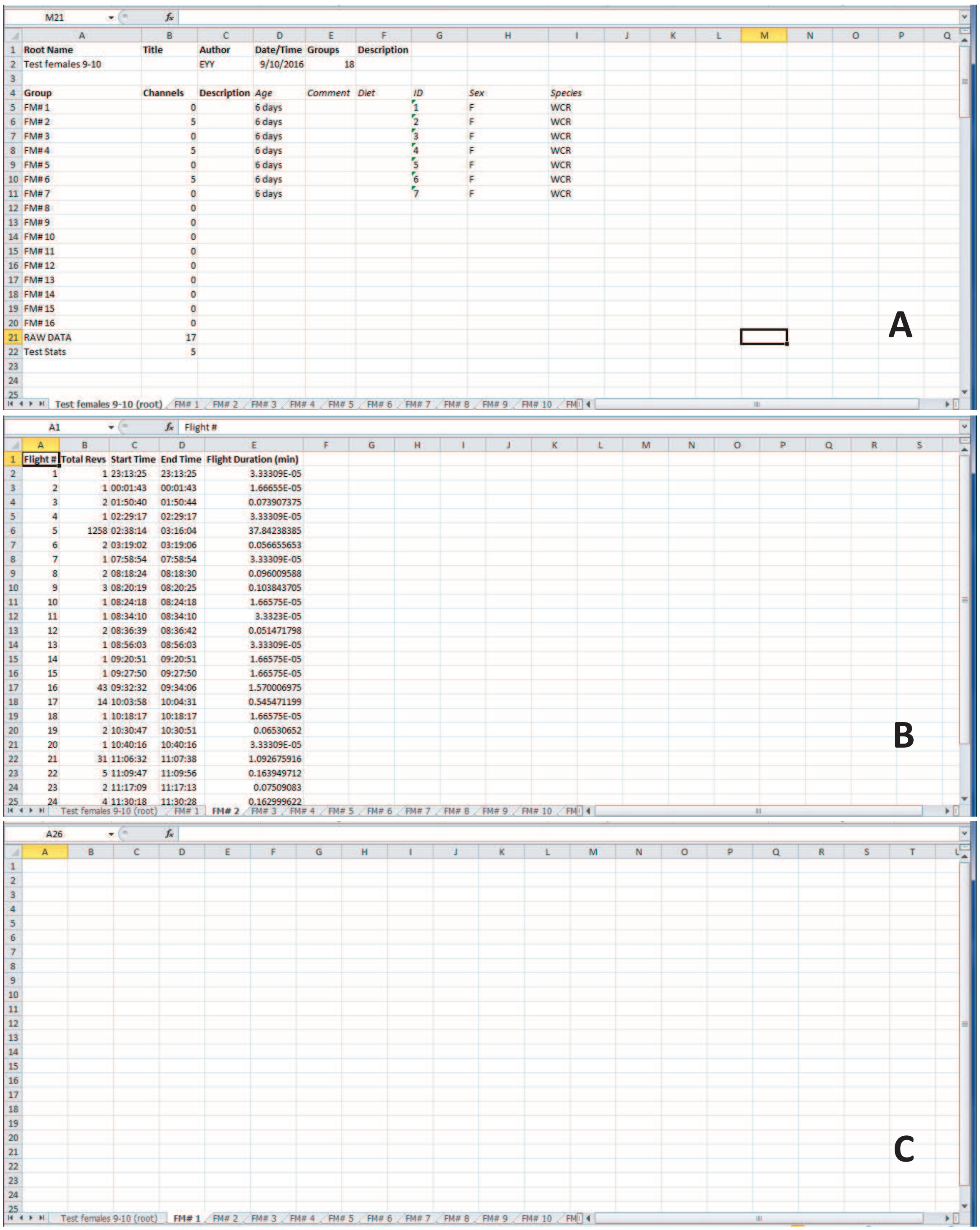


Figure 4



A

	A	B	C	D	E	F	G	H	I
1	Root Name	Title	Author	Date/Time	Groups	Description			
2	Test females 9-10		EYY	9/10/2016	18				
3									
4	Group	Channels	Description	Age	Comment	Diet	ID	Sex	Species
5	FM# 1	0		6 days			1	F	WCR
6	FM# 2	5		6 days			2	F	WCR
7	FM# 3	0		6 days			3	F	WCR
8	FM# 4	5		6 days			4	F	WCR
9	FM# 5	0		6 days			5	F	WCR
10	FM# 6	5		6 days			6	F	WCR
11	FM# 7	0		6 days			7	F	WCR
12	FM# 8	0							
13	FM# 9	0							
14	FM# 10	0							
15	FM# 11	0							
16	FM# 12	0							
17	FM# 13	0							
18	FM# 14	0							
19	FM# 15	0							
20	FM# 16	0							
21	RAW DATA	17							
22	Test Stats	5							

Test females 9-10 (root) / FM# 1 / FM# 2 / FM# 3 / FM# 4 / FM# 5 / FM# 6 / FM# 7 / FM# 8 / FM# 9 / FM# 10 / FM

B

	A	B	C	D	E
1	Flight #	Total Revs	Start Time	End Time	Flight Duration (min)
2	1	1	23:13:25	23:13:25	3.33309E-05
3	2	1	00:01:43	00:01:43	1.66655E-05
4	3	2	01:50:40	01:50:44	0.073907375
5	4	1	02:29:17	02:29:17	3.33309E-05
6	5	1258	02:38:14	03:16:04	37.84238385
7	6	2	03:19:02	03:19:06	0.056655653
8	7	1	07:58:54	07:58:54	3.33309E-05
9	8	2	08:18:24	08:18:30	0.096009588
10	9	3	08:20:19	08:20:25	0.103843705
11	10	1	08:24:18	08:24:18	1.66575E-05
12	11	1	08:34:10	08:34:10	3.3323E-05
13	12	2	08:36:39	08:36:42	0.051471798
14	13	1	08:56:03	08:56:03	3.33309E-05
15	14	1	09:20:51	09:20:51	1.66575E-05
16	15	1	09:27:50	09:27:50	1.66575E-05
17	16	43	09:32:32	09:34:06	1.570006975
18	17	14	10:03:58	10:04:31	0.545471199
19	18	1	10:18:17	10:18:17	1.66575E-05
20	19	2	10:30:47	10:30:51	0.06530652
21	20	1	10:40:16	10:40:16	3.33309E-05
22	21	31	11:06:32	11:07:38	1.092675916
23	22	5	11:09:47	11:09:56	0.163949712
24	23	2	11:17:09	11:17:13	0.07509083
25	24	4	11:30:18	11:30:28	0.162999622

Test females 9-10 (root) / FM# 1 / FM# 2 / FM# 3 / FM# 4 / FM# 5 / FM# 6 / FM# 7 / FM# 8 / FM# 9 / FM# 10 / FM

C

Test females 9-10 (root) / FM# 1 / FM# 2 / FM# 3 / FM# 4 / FM# 5 / FM# 6 / FM# 7 / FM# 8 / FM# 9 / FM# 10 / FM

Table 1

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	Location	
	Ames	Nashua
Sample size[1]	23	31
Total flight distance (m)	387.83 ± 146.21	949.10 ± 267.73
Total flight duration (min)	14.34 ± 5.06	37.01 ± 10.51
Total flight speed (m/s)	0.42 ± 0.04	0.44 ± 0.06
Longest flight distance (m)	184.48 ± 81.82	590.13 ± 186.01
Longest flight duration (min)	6.27 ± 2.26	22.15 ± 7.67
Longest flight speed (m/s)	0.46 ± 0.04	0.44 ± 0.03

[1] Flew at least 1 minute

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Butane multi-purpose lighter	BIC	UXMPFD2DC	To soften wax when tethering
Clear polystyrene plastic vial (45-ml)	Freund Container and Supply	AS112	To hold beetle while anesthetizing
Dehydrated culture media, agar powder	Fisher Scientific	S14153	To make agar for holding moisture for adults
Delrin rod (1" diameter, 3.75" long)	Many suppliers: can use cheapest on the internet.		For post of flight mill
Dental wax	DenTek	47701000335	Adheres wire tether to prothorax
Ferrite ring magnets (OD: 0.69”, ID: 0.29”, Thickness: 0.118”; 7oz pull)	Magnet Shop	63B06929118	Opposing - to generate the float.
Hall effect sensor	Optikinc	OHN3120U	Look under magnetic sensors on the left side of the Optekinc website then look for the part number. A link is given for current suppliers.
Hypodermic tubing (22 gauge; 0.0358” OD x 0.01975” ID x 0.004” wall)	Small Parts, Inc.	HTX-22T-12	Used for flight mill arms and main axis rod.
Incubator (104.1 x 85.4 x 196.1 cm)	Percival Scientific	I-41VL	
LabVIEW Full Development System software, system-design platform	National Instruments (See http://www.ni.com/en-us/shop/labview/select-edition.html)	LabVIEW 2018 (Full Edition)	Provides environment needed to run flight mill files (.vi extensions) available for download from Jones et al.[18] at http://entomology.tfrec.wsu.edu/VPJ_Lab/Flight-Mill. LabVIEW 2018 Full is compatible with Win/Mac/Linux operating systems.
Mesh cage (18 x 18 x 18 cm)	MegaView Science Co. Ltd.	BugDorm-4M1515	mesh size = 44 x 32, 650 µm aperture
Needle tool	BLICK	34920-1063	For scoring soil surface for egg laying in laboratory

Nickel ring magnets (3/16” OD x 1/16” ID x: 1/16” thick)	K&J Magnetics	R311	Used to trigger the digital hall effect sensor.
Petri dish (100 mm x 15 mm)	Fisher Scientific	S33580A	
Plastic container (44-ml)	Dart	150PC	For initial rearing of young larvae
Plastic container (473-ml)	Placon	22885	For rearing of older larvae
Round brush (size 2)	Simply Simmons	10472906	For transferring freshly hatched neonates to surface of roots
Sieve (250-µm)	Fisher Scientific	08-418-05	To separate eggs from soil
Steel wire (28-gauge)	The Hillman Group	38902350282	
Teflon rod (3/8" diameter, 3/4" length)	United States Plastic Corporation	47503	To accept the rotating arm.
Vacuum	Gast Manufacturing, Inc.	1531-107B-G288X	For aspirating adults in laboratory
White poly chiffon fabric	Hobby Lobby	194811	To prevent escape of larvae from rearing container

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Below are responses to each of the reviewers' comments in blue and in brackets [ ].

Editor's comments:
Changes to be made by the author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
[Done]

2. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
[Done]

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[Done]

4. 1.1: How many western corn rootworm adults are collected?
[Step 1.1, changed to: "at least 500". (L131)]

5. 1.2: What is the mesh size?
[Step 1.2, added: "(mesh size 44 x 32, 650 µm aperture)". (L138-139)]

6. 1.2.3: How large is the Petri dish?
[Step 1.2.3, added: "(100 mm x 15 mm)". (L153)]

7. 1.2.4: Please specify the amount of sieved field soil.
[Step 1.2.4, added: "40 g". (L157)]

8. Lines 219-233, 305-316, etc.: In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.
[Most of the material in the notes beginning at original Lines 219 and 337 has been moved to the Discussion (L435-451; and L453-467, respectively). Most of the material in the note beginning at original Line 305 has been moved to the Results (L357-364).]

9. References: Please do not abbreviate journal titles.
[Corrected]

10. Table of Equipment and Materials: Please sort the items in alphabetical order according to

the Name of Material/ Equipment.
[Done]

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This manuscripts describes provides written and video instruction for employing flight mills to investigate insect behavior and physiology using western corn rootworm as an example study organism. This work will be of significant interest to many other scientists that will want to use this valuable, yet often difficult to master, technique. I employ flight mills in my research often and I understand that there are steps that other are unable to overcome by just reading primary literature manuscripts. THis JOVE article will help those investigators significantly. I commend the authors for both undertaking this and doing such an excellent job with it.

Major Concerns:
I have none.

Minor Concerns:
I am unable to improve this manuscript further.

Reviewer #2:

Manuscript Summary:
In this study, the authors present technical method for measuring flight performance of Western Corn Rootworm by flight mill device. studying pest's flight propensity such as the Western Corn Rootworm, is important for applying effective pest management programs, and control its future distribution.

Minor Concerns:

1. Insect's flight mill studies have been around for long time and many papers had discussed and illustrated most of the technical details involved in building and operating this device. Additionally, two such papers have already been published in JoVE (1. Measuring the Flight Ability of the Ambrosia Beetle, Platypus Quercivorus (Murayama), Using a Low-Cost, Small, and Easily Constructed Flight Mill; 2. A Simple Flight Mill for the Study of Tethered Flight in Insects). My concern is that the necessity of the current paper is not well explained. The authors emphasized the delicate tethering method as a major reason for publishing this work, but it seems to be well described (not totally identical method though) in the previous works as well.
[We have now included these references (L100). What they show in the videos for attaching a tether will not work for the rootworm. See next comment.]

Is there anything more problematic in tethering Western Corn Rootworm than other insects? Is

there anything new in the flight mill design or any other variable which distinguishing the current method form previous works? The authors should emphasis this.
[Every insect is different when it comes to tethering, mainly because of overall size, size and shape of the available target area (such as degree of curvature) on the cuticle for attaching the tether, softness and flexibility of the insect, need and method for anesthetization, potential for fouling the wings and/or head with misplaced or overflow adhesive, and many many more details. In the case of the two JoVE videos that have been published, the first is of a plataspid (kudzu bug). It is an unusual heteropteran where the scutellum covers the entire abdomen of the insect and the wings stick out beyond it when in flight. This means that the entire "back" of the insect is available for attachment of the tether, and the large messy glob of hot glue applied in the video is fine, because it cannot gum-up the wings no matter how messy. This would not work for the rootworm. The situation for tethering the ambrosia beetle is similar in the sense that its pronotum is relatively large (and thus a large target for the tether adhesive) and well-separated from the base of the elytra and from the head which is largely tucked underneath and thus protected from excess glue. Many examples on the internet are of lepidopterans, which are quite different from beetles when it comes to tethering because the anterior-most sclerite on the abdomen (behind the wings) is usually a good option for attachment, getting the adhesive away from the head. In the case of western corn rootworm, the pronotum is narrow and short, making very precise attachment with a minimal amount of adhesive (dental wax in this case) necessary to prevent interference with the opening of the elytra for flight and with the head (eyes, antennae, mobility). So yes, the rootworm is a challenge to tether compared to most other insects for which there are visual examples available, whether in JoVE or on the internet. The flight mill design itself is not ours, and is one that has been used in a few other studies, in part because details of the design and program files are freely available (Jones et al. website, ref 18). Thus, it would not be appropriate for us to emphasize the design too much, but mainly to point the reader to that website and to demonstrate how we have used it productively. The primary emphasis we bring to the table is the tethering, which is different and should be useful to others for this insect in particular, and probably for other insects where this method could be a useful option. A full paragraph emphasizing this consideration has been added to the Introduction (L96-111).]

2. The origin of software used for monitoring the flight mill is unclear. Is it off-the- shelf product or a custom software built especially for this study? I followed the link from Jones et al. (2010) to LABView yet could not find the software in their inventory. All the flight mills systems I encountered so far used custom-built software, tailor made by programmers for flight mill needs. If that's the case, then it should be better explained. Additionally, if that's indeed a custom-built software, presenting all the functions and buttons would have no value for other scientists which have no access to this soft.
[The files provided by the Jones et al.[18] website are in a link called "data analysis routine", in a list of links under the sub-heading "Flight mill wiring and software". It is easily overlooked because the list contains several links that are not delimited by commas on the website. Clicking on the link unzips four files with a .vi extension readable by LabVIEW. Use of the files is described in a pdf document obtained by a link, "Circular Flight Mill Instructions" in the same paragraph immediately following the first link. The routines were written about 10 years ago, but should still work in newer versions of LabVIEW. Thus, the descriptions and figures we present are still relevant to the user. If the user wants to add new capabilities, the routines provided by Jones et al. can be modified by the user. Text explaining this has been added as a Note at the

beginning of section 2 of the Protocol (L223-228).]

3. Human presence in or out of the testing room is not mentioned. Such presence could add extra stress to the flying insect and impact their flight performance. for example, Once a beetle finished flying, was it taken off the flight mill while other beetles still flew in its vicinity or did they wait until all beetles stopped flying and only then took them off?
[We added the following note to section 3 of the Protocol (L299-302): "Note: Human presence in the flight testing room should be limited to attaching and removing adults from the flight mills. The test period usually does not begin until at least 30 min have elapsed since attachment (see Note under 2.2.1), and humans should not be present in the flight room during this time or during the test period itself."]

Specific comments:
L67-68: "Dispersal by flight plays an important role in adult western corn rootworm life history and ecology" - reference is absent.
[Now cite a review paper by Spencer et al. (new ref 6) (L68)]

L113: What is the preferred method to collecting this beetle? Traps? Pheromones? Hands?
[Added the sentence, "Adults may be collected from the field using a manual aspirator." (L132-133)]

L123: What is R3 stage?
[Added, "…should be picked at the R3, or milk stage of kernel development[19]. The R3 kernel is yellow outside, while the inner fluid is milky white due to accumulating starch." We added reference (19) which describes the stages of corn development. (L141-143)]

L354-358: I suspect pic 4B and pic 4C got switched
[The caption for Figure 4 was correct, but the text in the Results section had them mistakenly switched, as the reviewer noticed. The Results text has been corrected. (L366, 371-372)]

Figure 3: Poor quality picture.
[The figure has been redone, and quality is now good.]

Table 1: What is the meaning of longest flight speed? contentious flight speed?
[Added the following sentence to the Table legend: ' "Longest flight" refers to the longest uninterrupted (i.e., continuous) flight performed by each individual during the test period.' (L410-411) Like the other "Longest flight" parameters, in the case of flight speed it means the speed during the longest uninterrupted (= continuous) flight taken by each individual.]

Reviewer #3:

The Yu et al. manuscript (JoVE 59196), "Using Flight Mills to Measure Flight Propensity and Performance of Western Corn Rootworm, Diabrotica virgifera virgifera (LeConte)" provides detailed background and instructions for constructing an insect flight mill, rearing western corn rootworms (WCR), and using the device to measure WCR flight parameters in a laboratory setting.

Flight mills are tools to study aspects of insect movement, dispersal, and migration under controlled conditions. While there are certain compromises inherent in using tethered insects, these devices can be used to generate empirical data on a wide variety of questions relevant to insect flight and behavior. In the case of the WCR, a species with a great capacity for movement and one whose movement plays an important role in resistance and its spread on both a local and regional scale, there are few opportunities to observe and follow individual flights of any duration greater than a 0.5-1.0 minutes. Flight mills provide an environment where it is possible to compare flight performance of beetles from different treatments, locations, or lineages. While the superlatives of WCR flight derived from flight mill studies shouldn't be used as ecological absolutes, they provide us with valuable estimates/insights into the limits or potential for free flying WCR. Because directly measuring movement is hard (or practically impossible in many instances), flight mill data likely provide the best estimates of what WCR may be capable of in the field. These data are tremendously valuable in the study of insect resistance and its spread. Wildly optimistic expectations for WCR movement due to a lack of field-relevant movement and dispersal data likely contributed to the rapid evolution of WCR resistance to Bt corn hybrids.

Publishing a detailed methods paper on flight mill use with WCR is a great idea. Making schematics, parts lists, and instructions more widely available will help to broaden the base of research into WCR flight by reducing the significant 'activation energy' associated with designing a device from scratch. Prominently promoting a flight mill design also increases the chance that YOUR favorite design becomes a standard. Alternatively, sharing the details may prompt others to find ways to make significant improvements leading to even better and more versatile flight mills - this is not a bad thing!

I find it particularly interesting that the rearing of the WCR is included as part of the methods. This advances WCR science by suggesting how to standardize the test subjects and also provides a novice with instructions (and literature references) for rearing a WCR population.
This manuscript doesn't suffer from any major flaws; it is generally very well written and well organized. The title is concise and to the point. The abstract is also good, though being accustomed to research paper reviews, it was a little jarring to see that the results of the trial were not interpreted with respect to differences in beetle performance. The background information and use of the literature is accurate; in my opinion, the authors have provided just the right amount of detail to accomplish their "How-to" goal, but left good 'tracks' through the literature so someone wanting to learn more would know where to find it.

The steps are generally well described throughout. The key step - attachment of the WCR to the wire with dental wax - is emphasized, yet there are no pictures of that process or a diagram showing how a properly harnessed WCR would look in the system. A summary image would be very beneficial. It was not clear to me just how the mounted WCR (particularly the orientation of the attachment) was positioned with respect to the rotating arm. It prompted me to wonder if the flight mill system's computer interface would function the same if an insect was flying CW or CCW? In a later video element [which seems to be part of the next steps in this process?], I presume the attachment process in detail would be a high priority.

[We have elected not to add a figure of the attached beetle. The video will indeed emphasize and visualize the attachment of the tether to the beetle, and attachment of the beetle to the flight mill. We can add a figure if the Editor would like us to, but seems superfluous given the video to come. The new paragraph added to the Introduction (L96-111) (mentioned above under Rev 2 responses) indicates our emphasis on visualizing the tethering process in the video. We added the following statement to 3.8 in the Protocol: "The tethered beetle may be positioned to fly either clockwise or counter-clockwise." (L290-291)]

I expect that there would be an eager audience for this information. I expect that many scientists/students would be prompted to construct a flight mill after reading the publication/seeing the video. I recommend that this manuscript be accepted for publication after some minor revisions and corrections. I note those in detail below.

Other comments, suggestions and corrections (by line number):

Lines ca.70-76 have strings of words with different fonts or font sizes.
[Corrected]

Line 182: Wordy/awkward: "…5 or 6 small (~1mm dia) holes."
[Changed as suggested (L202-203)]

Line 219: How does the flight speed of a WCR affect the # of rotations that are completed once a beetle stops flying. Is 5s long enough to account for most end of flight coasting as the arm slows due to friction? What is the average time to complete an orbit at normal speed—is it very different from 5 s? Is there a slowest time that it takes for a flying WCR to complete one orbit?
[Based on average flight speeds in Table 1, WCR generally average a little over 2 revolutions per 5 sec, or 1 revolution per 2.5 sec. The flight arm has very little friction or drag, so in our experience if a WCR is going at a normal speed when it quits flying, it will take more than 5 sec for the arm to stop revolving, and probably will make 3 to 5 gradually-slowing revolutions before coming to rest. But if the WCR quits flying when it is barely moving (e.g., flapping furiously while trying to land but with little forward motion), the flight arm could quit moving in fairly short order, and perhaps no extra revolutions will occur in 5 sec or 1 min. There is not really a slowest time, because as mentioned the beetle may be torquing itself trying to land which could result in a quite slow m/sec speed over a short period – and even some of the 1-revolution "flights" on the spreadsheet (Fig. 4B) cannot be interpreted as a 1 revolution/sec speed, because the movement might have been only a few cm, but happened to pass over the sensor indicating a "revolution". Our goal in this passage (L435-451) (the original note has been moved to the Discussion) is to emphasize that there are trade-offs in what Max Threshold is selected, that it is a judgment call based on what the researcher deems is the least misleading kind of error to make given the particular insect and how the data are to be used, and in general just to make the researcher aware of this issue.]

Line 224: How long does a WCR continue to circle after it stops actively flying? Is there any way to measure the amount of force needed to overcome the inertia and initiate movement of the arm? This is more of a curious question related to the compromises that are part of using a flight mill. The recommended publication about flight mills (Minter et al. 2018) is highly appropriate!

[See comment above. We agree it would be good to know, but the answer will be variable between and within beetles, and probably would take some dedicated experiments to calculate some decent means.]

Line 221: Including "of course" in the text is too conversational; it may be how we talk, but it could be cut from the text.
[Deleted]

Line 233: Does it matter in what direction the flight mill spins?
[No. Added a line in the protocol to indicate this. (L290-291)]

Line 257: I think the section references need to be corrected from 1.10.11 and 1.10.2 to 1.11.1 and 1.11.2.
[Corrected]

Line 345: "those flight data". Data are plural, datum is singular.
[Corrected to "those"]

Line 354: It is not clear what is meant by "Simulated dawn". Please explain.
[Added the sentence, "Dawn and dusk were simulated by programmed ramping of light intensity from full-off to full-on at dawn (or vice-versa at dusk) over a 30 minute period." (L350-352)]

Line 366: Can you be clearer about the number of larvae per seed. Perhaps saying there were "12 larvae per 36 seeds"; this would be helpful to novices trying to figure out just how many seeds are set-up in the final rearing cup. I don't think that is very clear in that section of the methods. Is the density that is mentioned based on the number of survivors being transferred or on the initial number that there transferred?
[Changed to, "…at an initial density of 12 larvae per 36 seedlings." (L372-373)]

Line 383: We cannot see any details of (1) or (2) items in the figure. Do they need to be mentioned or could they be included in specifications the Percival unit? Also, the pan type humidifier is not visible in the picture; increase the brightness on shadowed area of the image?
[Items 1 and 2 of Fig. 2A have been removed (they are out of site on top of the panel) and items 3, 4, and 5 have been renumbered as items 1, 2, and 3, respectively. A photo from a slightly different angle has been substituted for the original Fig. 2B, and has been brightened slightly to make the pan humidifier more visible.]

Line 395: Do WCR lack a tarsal reflex? Is it possible for them to 'rest' when they are not touching a surface? Does the absence of a perch increase the probability of WCR flight? What do dangling WCR do between flights? Are there any data on WCR flight in some sort of vertically-oriented flight mills where they can perch when not flying?
[The reviewer brings up an important point (tarsal reflex and flight), and we neglected describing our approach for dealing with it. We provided each beetle with a small piece of tissue paper to cling to, which provides tarsal contact and reduces the kind of initial escape flight or other artifactual flight activity the reviewer is referring to. We added the following to the protocol as a new step 3.9 (L293-297): "3.9. Immediately after mounting a beetle, tear a small piece (~1-cm

dia) of tissue paper from a larger tissue. Offer the tissue piece to the tethered beetle hanging from the flight mill for tarsal contact; most beetles will grasp the tissue and hold it against gravity until they release it at the beginning of their first flight activity. This will greatly reduce initial 'escape' or 'landing' flight behavior." We also added the following text to the last paragraph of the Discussion (L480-486): "Providing tarsal contact with a small piece of tissue after mounting the insect on the flight mill (step 3.9) reduces initial escape flight as well as flight activity associated with an attempt to land. However, once the beetle drops the tissue during an experiment, the same problem of inability to terminate flight by landing is encountered. Alternative actograph systems have been used in laboratory flight experiments with tethered[8,9] or untethered[7] western corn rootworm. While they alleviate the problem of flight termination by allowing spontaneous tarsal contact, the trade-off is inability to measure flight distance or speed."]

Have you ever evaluated how much mass is loss (i.e. through dehydration) for an insect that has spent 22 h in the flight mill without access to food or water? I realize that any comparison with free flight is just not going to be possible.
[We did not test this directly, but in a companion study we set up females for oviposition after their flight, and any that did not survive at least 3 days or did not lay any eggs were discarded from the data set. The reasoning was that the flight test, or associated handling, may have injured or compromised their health in some way; dehydration while on the flight mill is one of many possible problems that would be accounted for in this way.]

Line 440: The data-hound in me very much longs for some explanation for the seeming differences between the flight parameters of the two WCR populations.
[Our understanding of the JoVE policy is that we are not to analyze this sort of thing, but just to use a real data set to show how it's done. We have a companion paper that will be published (accepted pending minor revisions), probably before this one, that makes and interprets comparisons of western corn rootworm flight behavior based on larval density treatments.]

Figure 3: the text is not sharp and is quite hard to read evena at 8.5 x 11 page size.
[Figure quality has been improved]

I noted that following the link to the software from the Jones et al. (2010) website takes you to an erroneous (?) address www.no.com. I was not able to get to the software or anything there from my computer. If you go to the National Instruments website first, you can find some FieldVIEW software; the correct National Instruments website address begins with www.ni.com/.... Perhaps linking this method through the Jones site is less than ideal? Given that the flight mills are modified from the Jones et al. (2010) model, is it wise to 'depend' on that website for background/reference when what is presented there is 'old'?
[See next comment.]
Is the LabVIEW software really as "turnkey" as it is presented in the manuscript? The source website seems to offer a fairly broad program suite that comes at several price points. The $300 yearly subscription doesn't support MacIntosh, the $3000 yearly subscription does support Win/Mac/Linux. Perhaps there is a more detailed link that takes one to the specific module that runs the flight mills with a page address that my MacIntosh computer couldn't open properly? The screen shots in Figure 3 suggest a fairly dedicated interface; however, I could not find it.

Details of the software that may present obstacles (annual subscription) or limitations (e.g. Windows only) should be noted.
[The link in the Materials List is meant to take the reader to the Jones et al.[18] web page where the link for the .vi program files are located (details have been added to the Protocol as noted above in a response to Rev 2 comments). However, we have also added the correct link to the National Instruments website to the second column of the Materials List. The LabVIEW Full Edition (the $3,000 option) is the necessary package for this application, and this should now be clear in the Materials List. It is compatible with Windows, Mac and Linux, and this information has been added to the Comments box in the Materials List. Also, please see response above to comment #2 by Reviewer 2.]

Reviewer #4:

Manuscript Summary:
This manuscript is very well written and exceptionally clear. I haven't done this particular type of tracking, but it seems to me as though they have thoroughly covered the subject and provided a great resource for those interested in this endeavor.

I do have just a few thoughts. One is that they cut off flights of less than one minute. I haven't spent a lot of time observing CRW but what I remember were many flights of relatively short duration covering perhaps 4-6m. If they are flying at 1-2m/s, these flights would not be considered by their software. I think it would be good to explain this decision better.
[The average speed of a rootworm is in the range of about 0.44 m/s as indicated in Table 1. Although they cannot fly the 1-2 m/s suggested by the reviewer, their point remains valid, because with a 1 min cutoff at normal speed, some flights covering less than about 25 m will be missed. If a researcher wants to capture short flights, the software can handle and report as short of an interval as desired (down to fractions of a second) by adjusting the 'Raw Data Log Interval (min)' (see Step 2.2.5). Using no cut-off at all is also possible, but there are trade-offs involved in managing the error-rates for accepting false zeros (or false short-flights) versus excluding true zeros (or true short-flights), which the researcher needs to be aware of. This issue is discussed in detail in the 4th paragraph of the Discussion (L453-467).]

The second thought is really a suggestion. In the tracking I've done, working with spreadsheets is rather cumbersome, and I've found the use of R scripts much more flexible. It easily deals with different length files, can produce graphics automatically and puts all your data in a format suitable for further analysis. Of course, if you don't know programming in R this might not be a useful suggestion. The people working on the open source project Ikhnaie (gitlab.com/Ikhnaie) have an example script for analysis of 3d tracks.
[Thanks for the suggestion, we may be able to convert to R in the future. The spreadsheets are indeed cumbersome.]

Overall I had a difficult time reviewing this manuscript simply because it was so clear and well written.