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]

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: LabVIEW, Microsoft Excel, etc.

[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 development19. 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 tethered8,9 or untethered7 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](http://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/](http://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](http://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.