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A precise and autonomous system for the detection of insect emergence patterns
--Manuscript Draft--

1   **Title:**
2   **A Precise and Autonomous System for the Detection of Insect Emergence Patterns**
3
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19
20   **Keywords**:
21   Emergence, rhythms, falling-ball, Megachile, microcontroller, Arduino
22

23   **Summary:**
24   Measurement of insect emergence patterns requires precision. Existing systems are only semi-
25   automated and sample size is limited. We addressed these issues by designing a system using
26   microcontrollers to precisely measure the time of emergence of large numbers of emerging
27   insects.
28
29   **Abstract:**
30   Existing systems to measure insect emergence patterns have limitations; they are only partially
31   automated and are limited in the maximum number of emerging insects they can detect. In order
32   to obtain precise measurement of insect emergence, it is necessary for systems to be semi-
33   automated and able to measure large numbers of emerging insects. We addressed these issues
34   by designing and building a system that is automated and can measure emergence of up to 1200
35   insects. We modified the existing "falling-ball" system using Arduino microcontrollers to
36   automate data collection and expand the sample size through multiple data channels. Multiple
37   data channels enable the user to not only increase their sample size, but also allows for multiple
38   treatments to be run simultaneously in a single experiment. Furthermore, we created an R script
39   to automatically visualize the data as a bubble plot, while also calculating the median day and
40   time of emergence. The current system was designed using 3D printing so the user can modify
41   the system to be adjusted for different species of insects. The goal of this protocol is to investigate
42   important questions in chronobiology and stress physiology, using this precise and automated
43   system to measure insect emergence patterns.
44

Introduction:

Precisely measuring the timing of terrestrial insect emergence in experimental settings is notoriously difficult and requires some degree of automation. Several mechanisms have been designed in the past, incorporating either a "falling-ball" principle, using falling balls and sensors, or a "bang-box" using a funnel-type system[1,2,3]. There are two limitations with existing designs: 1) data collection is only partially automated, and 2) sample size or the number of emergent insects that can be detected is limited. These problems decrease the precision of data collection, which is important to studying timing of eclosion and/or emergence patterns. We addressed these problems by designing a system that is automated and not limited by sample size, enabling the user to better visualize emergence rhythms in response to environmental cues.

Our system is an improvement to the falling-ball principle, the most recent version of which used infrared sensors to detect insect emergence in six-minute increments[2]. Our system still uses infrared sensors, but also incorporates an Arduino microcontroller to record the date and time of each emergence event to the nearest second. Data is automatically stored to a secure digital (SD) card, which can be exported as a comma-delimited file for analysis. The analysis is automated by using a custom R script, which will graph the data as a bubble plot and identify the median time and day of emergence.

Multiple channels enable the user more flexibility in data acquisition. For example, our multiple channel design not only minimizes the impact of a "clogged" sensor, but also can be used to increase sample size. Furthermore, multiple channels allow the user to designate treatments to specific channels so they can run simultaneously in an experiment. Using all six channels allows for approximately 1200 emerging bees to be recorded in a single experiment. To our knowledge, this is the largest sample size of any current system measuring insect emergence and has allowed us to observe fine-scale emergence patterns in response to environmental cues. Finally, our system benefits from the fact that the majority of the parts are 3D printed. This creates precisely sized components, which reduces the likelihood of errors occurring (such as detector clogging) during operations. It also allows for customization for other research systems.

The goal of this protocol is to custom build a precise and automated system to measure insect emergence, to investigate questions in chronobiology and stress physiology. This system has been, and will continue to be, critical in investigating unanswered questions related to insect emergence patterns in response to environmental cues. Here we describe its assembly and use for emergence detection of the alfalfa leaf-cutting bee, *Megachile rotundata* in lab-based experimental settings. The system is automated using a programmable microcontroller and customizable using 3D printed parts. Printed racks hold in place tubes containing bee nest cells following a metal BB. Upon emergence, the metal BB is freed from the rack, passing through an infrared sensor recording the date and time of emergence to an SD card. The current design is optimized for *M. rotundata*, but with minor adjustments could be adapted for other species of insects.

Protocol:

1. System Construction

1.1. Using PLA filament, print the following number of parts for each channel being constructed: 1 collector manifold (collector_manifold.stl), 1 end cap (end_cap.stl), 6 platform supports (platform_support.stl), 4 tube rack base plates (base_plate.stl), and 4 tube rack face plates (face_plate.stl). Make sure printer bed is large enough to print an item before printing. All *.stl files are available in Supplemental data.

1.2. With 3 platform supports and a 33 x 30 cm piece of corrugated plastic, use hot glue to assemble 2 tube rack platforms per channel being constructed, as shown in **Figure 2**. The corrugated plastic can be scored on one side at each corner to allow for bending.

1.3. Install electronics into the collector manifold.

1.3.1. Solder a 120 Ω resistor to the anode (longer leg) of both the infrared emitter and the infrared detector, and a ~5 cm length of 22 GA wire to both cathodes. Use different colors of wires to avoid confusion in later steps.

1.3.2. Carefully insert the detector into one socket of the collector manifold (highlighted in blue in **Figure 3**) and the emitter into the second socket (highlighted in red). Both components should fit snugly.

1.3.3. Feed the detector wires through the cabling channel (highlighted in yellow in **Figure 3**) and pull all four wires through the access hole (highlighted in green). Ensure no bare wires are touching, using hot glue to secure them in place.

1.3.4. Solder all four wires to a RJ45 (Ethernet) jack, using the back row of pins. Both anodes should be soldered to the left-most pin, the cathode of the emitter to the right-most pin, and the cathode of the detector to either of the center pins (**Figure 4**).

1.3.5. Secure the RJ45 jack over the collector manifold access hole (highlighted in green in **Figure 3**) with hot glue, ensuring no bare wires are touching inside the manifold.

1.4. Construct falling ball collector (1 per channel being constructed) as shown in **Figure 5**

1.4.1. With one wired collector manifold, one end cap, and a 24 x 30 cm section of corrugated plastic, use hot glue to connect the base of the unit (red, green, and light grey components of **Figure 5**).

1.5. Use an 8 x 27 cm section of corrugated plastic to add a falling ball ramp to the collector (dark grey component of **Figure 5**). The end cap and collector manifold designs include ledges to ensure correct placement. Check for a smooth transition from the ramp to the collector to avoid jams during use.

133 1.6. Construct the central processor for the system (as detailed in **Figure 6**).
134
135 1.6.1. Print a custom printed circuit board for system construction. All files required for PCB
136 board printing are available in supplemental data.
137
138 1.6.2. Solder female headers on the through-holes labeled for the following installations: Arduino
139 Nano, temp, clock, SD module, and liquid crystal display (LCD) screen (unlabeled 2 x 5 through-
140 hole area in the upper left corner of the PCB board).
141
142 1.6.3. Snap in and solder six RJ45 jacks along the lower edge of the PCB board.
143
144 1.6.4. Solder six 470k ohm pulldown resistors into the through-hole sites located just above the
145 RJ45 jacks.
146
147 1.6.5. Install the Arduino Nano, DHT-temperature and humidity sensor, clock, and SD module
148 onto the PCB board. DHT-temperature and humidity sensor should be tested before use in
149 experiments to ensure accuracy.
150
151 1.6.6. Connect a 10-connector ribbon wire to the LCD screen connector of the PCB board. Solder
152 the other end of the ribbon wire to the LCD screen so that the screen pins correspond to the
153 Arduino pins, as noted in **Figure 4**. Further details on LCD wiring are available at
154 https://Learn.adafruit.com/character-lcds/wiring-a-character-lcd.
155
156 1.7. System programming
157
158 1.7.1. Download and install the latest version of the Arduino IDE for the correct operating system
159 from www.arduino.cc.
160
161 1.7.2. On first use, install Arduino libraries for the real time clock (github.com/adafruit/RTClib)
162 and the temperature/humidity sensor (github.com/adafruit/DHT-sensor-library). Set the clock to
163 the current local time by using the ds1307 script included with the library.
164
165 1.7.3. Upload the system Arduino script, available in supplemental data.
166
167 **2. System Use**
168
169 2.1. Assemble the system as shown in **Figure 7**. For each channel being used, one falling-ball
170 collector (assembled in step 1.4) should be flanked on either side by a rack platform (assembled
171 in step 5.1). Use packaging tape to hold together pieces and to create a smooth rounded edge on
172 the rack platform.
173
174 2.2. Configure unused channels to avoid false positive signals. Since the system relies on a low
175 signal to detect an event (infrared detector not receiving a signal from the infrared emitter),

176 unused channels must be configured appropriately to avoid false positive signals. This may be
177 accomplished by one of two ways.

179 2.2.1. Deactivate unused channels in the software by commenting out the loops corresponding
180 to the unused channels. In Arduino IDE, this can be accomplished by adding "/*" prior to the
181 unneeded loops and "*/" at their end.

183 2.2.2. Deactivate unused channels through a simple hardware accommodation. Simply solder
184 together wires #6 and #8 (usually the solid brown and solid green wires of a commercially
185 available cat 6 cable) and insert into the empty RJ45 jack on the central processor.

187 2.3. Load and place tube racks immediately prior to running an experiment.

189 2.3.1. Ensure that all holes contain a 0.5 mL microcentrifuge tube with the cap removed and that
190 the tubes fit snugly.

192 2.3.2. Fill each tube with one insect brood cell, pupal case or cocoon, one airsoft pellet, and finally
193 one metal BB. Make sure the flat edge side (cap) of the brood cell is facing toward the airsoft
194 pellet and metal BB. Affix the tube rack faceplate, with the rounded edge towards the bottom of
195 the rack, using ¼ inch nylon screws.

197 2.3.3. Place tube racks on the rack platform, with the opening facing towards the falling-ball
198 collector. Racks should be placed at the very edge of the platform so that a metal BB can fall
199 freely into the collector without bouncing against another portion of the structure (**Figure 7**).
200 When placing the rack, start with the opening facing upwards and then gently rotate into place
201 to ensure metal BBs are not released. The racks are designed so that the tubes will slant slightly
202 backwards when properly placed, reducing the chance of accidental release of the metal BBs.

204 2.4. Insert a SD card into the adapter and then start the central processor by plugging a micro-
205 USB connector into the Arduino, and the other end into any appropriate USB adapter. The LCD
206 screen will display numbers one through six when ready. Drop a single metal BB into the ball
207 collector of each channel and watch for the corresponding count to appear on the screen and for
208 the correct time to display at the bottom of the screen.

210 2.4.1. If the correct time is not displayed, repeat steps 1.6.3 and 1.6.4 to reset the clock.

212 2.4.2. If the test metal BB is not recorded, the collector is blocked. Check visually for blockage
213 and restart the system.

215 2.4.3. If a channel "counts" up one event every second, this indicates that the channel is not
216 properly connected. Check all connections and restart the system.

218 **3. Experiment End and Data Analysis**
219

220 <mark>3.1. After emergence has ended (see Results and **Figures 8 and 9** for examples of the time scale),</mark>
221 <mark>power down the apparatus by unplugging the Arduino. Racks may be disassembled and cleaned</mark>
222 <mark>for reuse.</mark>

224 <mark>3.2. During the experiment, data is stored on the SD card in a comma-delimited file (CSV)</mark>
225 <mark>accessible by the R programming language. Use the SD card to transfer data to the computer,</mark>
226 <mark>and RStudio to auto-generate bubble plots of the data.</mark>

228 <mark>3.2.1. Both event and temperature data are saved in the same file for data integrity. Hence, some</mark>
229 <mark>processing must be completed prior to analysis. Import the comma delimited file into a</mark>
230 <mark>spreadsheet program. Columns I and J are the date and time of emergence for bees; make them</mark>
231 <mark>columns A and B by cutting and pasting columns A-E into a second spreadsheet, and save as a</mark>
232 <mark>separate file, this is the temperature data.</mark>

234 3.2.2. Title column A with, "Date" and column B "Time," and sort the data by column A then by
235 B. Save as a CSV. file.

237 3.2.3. Download and install the latest version of the RStudio from https://www.r-project.org/.
238 Help with using RStudio for uploading and analyzing data can be found here at https://cran.r-
239 project.org/doc/manuals/r-release/R-intro.html.

241 3.2.4. Using the R script available in the supplemental data, upload the data into RStudio. Change
242 the working destination in the R script to match where the excel *.CSV file is located. Run the
243 script and select the data file to analyze. Type "plot" into the R console. The bubble plot will be
244 located in the working destination named "High-res;" rename this file to save as a high-resolution
245 tiff (300 dpi) file.

247 **Representative Results:**
248 Emergence of *M. rotundata* is asynchronous without exposure to an environmental cue, with
249 emergence occurring uniformly throughout the day[4]. However, when exposed to a square-wave
250 thermoperiod (4° C thermoperiod), emergence becomes synchronous to the thermophase[4,5].
251 This result is similar to other studies where insects have been found to use thermoperiod cues to
252 regulate emergence, including the flesh fly *Sarcophaga crassipalpis*[6], the onion fly *Delia atiqua*[7]
253 and the boll weevil *Anthonomus grandis grandis*[8]. One study has shown that stress during
254 development affects the synchrony of adult emergence in *S. crassipaplpis*[9]. Here, we present
255 results from *M. rotundata* that were exposed to a stress during development, to test the
256 hypothesis that this treatment causes the desynchronization of adult emergence.

258 *Successful run*
259 The user should watch the LCD screen before opening the incubator to make sure insects are no
260 longer emerging. Once the experiment is completed, the SD card is removed and the data can be
261 exported into RStudio as a comma-delimited file to be visualized as a bubble plot, as described
262 earlier. **Figure 8** displays bee emergence under a 4° C thermoperiod after exposure to a cold
263 stress during development. The red crosshairs indicate the median time and day of emergence

264 and the file name is the title. This R script should be used to visualize the data, but should not
265 serve as the sole analysis. To analyze emergence response to an environmental cue, the data can
266 be analyzed for rhythmicity (*see Analysis).*
267
268 *Complication*
269 When a sensor is clogged with metal BBs, the lack of a signal is repeatedly counted, giving rise to
270 multiple false data points. **Figure 9** demonstrates the same dataset presented in **Figure 8**, but
271 with one of the six channels clogged with BBs, therefore creating the large bubble on the graph.
272 In the event of a clogged sensor, data from this channel can be easily removed from the analysis.
273 Incorporating multiple channels in an experiment is beneficial in minimizing the impact of a
274 clogged sensor.
275
276 *Analysis*
277 Analyzing data for presence of synchronization can be done by calculating "parameter R," a scalar
278 statistic that identifies if emergence is rhythmic or arrhythmic[10,11,12]. This is done by calculating
279 the highest number of emerging adults in an 8-hour window, dividing this number by the number
280 of adults emerging outside the 8-hour window, then multiplying by 100. All individuals that
281 emerged should be pooled to calculate the number of emerging adults for each hour of the day.
282 The theoretical range of parameter R is from 0 (all emergence occurs within the gate) to 200
283 (emergence is distributed uniformly throughout the day)[10]. R values < 60 are considered rhythmic
284 emergence, 60 < R < 90 are weakly rhythmic, and R > 90 are arrhythmic. R values > 150 indicate
285 uniform distribution of emergence[10]. **Figure 8** shows that emergence is rhythmic with parameter
286 R = 20.21 < 60. Due to the fact that this type of data is distributed around a repeating 24-hour
287 clock, circular statistics must be employed for a more robust analysis (described in detail in
288 Bennett *et al.*, 2018[5]). This can be accomplished via circular statistics packages available for
289 RStudio (Package 'circular'- CRAN.R-project.org).
290
291 **Figure Legends:**
292 **Figure 1: Additive manufactured components.** Using PLA filament, 3D print the required parts
293 for the system. For each channel being constructed, parts needed are 1 collector manifold
294 (green), 1 end cap (red), 6 platform supports (orange), 4 tube rack base plates (purple), and 4
295 tube rack face plates (yellow).
296
297 **Figure 2: Tube rack platform assembly.** Use hot glue to assemble two tube rack platforms per
298 channel being constructed. Use three platform supports (shown in orange) with a section of
299 corrugated plastic (shown in grey).
300
301 **Figure 3: Collector manifold x-ray.** Insert one infrared detector into one socket of the collector
302 (shown in blue) and the emitter into the second socket (shown in red). Feed the detector wires
303 through the cabling channel (shown in yellow) and pull all four wires through the access hole
304 (highlighted in green). Ensure no bare wires are touching, using hot glue to secure them in place.
305

Figure 4: Connector wiring. Wiring diagram for the RJ45 jack prior to affixing to the collector manifold, as seen from the bottom of the jack and wiring table for connecting the LCD screen to the central processor.

Figure 5: Ball collector assembly. Using one end cap (shown in red), one collector manifold (shown in green) and a 24 x 30 cm piece of corrugated plastic (shown in light grey) assemble the shell of the ball collector assembly. Use an 8 x 27 cm piece of corrugated plastic (shown in dark grey) to add a ramp.

Figure 6: Central Processor PCB board. The PCB board for the central processor consists of a bottom layer (depicted in green), a top layer (depicted in red), and a silkscreen layer (depicted in blue). Solder female headers to all pass-through holes, except for those for the RJ45 jacks (along the bottom) and for the pull-down resistors (directly above the RJ45 pads).

Figure 7: Final assembly. When in use, the apparatus should be assembled with a tube rack platform on either side of each ball collector being used. Tube racks with attached faceplates should be positioned so that they are at the very edge of the Tube Rack Platform, reducing the possibility of falling BBs bouncing off of the apparatus. The footprint of the assembled apparatus is approximately 25 cm x 35 cm, with a height of 20 cm.

Figure 8: Graph of a typical experimental run after processing in R.

Figure 9: Graph of an experiment that suffered from a clogged detector, as shown by the relatively large bubble on day 4. The clogged channel can be removed from the analysis, thereby preserving the remaining data points.

Discussion:
We present a protocol for the assembly and use of a system that allows for precise measurement of the timing of insect emergence. This system solves two problems, which limited previous designs: partial automation and limited sample size. We solved these problems by automating data collection using microcontrollers, which also enabled us to increase sample size by using multiple channels. The current design has six channels that can hold a total of 1200 bees. Additional channels can be added or subtracted if needed, allowing not only for increased sample size, but also for simultaneously investigating the effects of multiple treatments. Critical steps, modifications, limitations and future applications are discussed below.

The only part of the system that is not automated is loading the racks with brood cells, metal BBs, and airsoft pellets at the beginning of the experiment. Although the racks are designed so they lean back slightly to prevent metal BBs from falling when the racks are standing upright, care must be taken when placing the racks to prevent the accidental release of metal BBs. Also, make sure the racks are flush with the edge of the shelf, so the falling trajectory of the metal BB aligns with the runway. Finally, leaf debris should be wiped clear from the runway, and the undercarriage holding the metal BBs from previous experiments should be cleared to prevent blocking of the sensor. Data is automatically recorded to an SD card as a CSV file, and the script

is written so that the Arduino will not run unless an SD card is present. The data file is manually imported into RStudio and visualized using the previously mentioned R script. This script will automatically graph the data as a bubble plot and identify the median time and day of emergence. The Arduino script is written to append event data to the end of the file, which prevents data loss in the event of a power failure. However, this also means that once data is extracted from the SD card, all files should be cleared prior to the next experiment.

Modifications to the SketchUp files can be made to adjust the size of the racks for insects of different sizes, with different sized tubes being used in the modified racks. Furthermore, the size of the airsoft pellet is important because it prevents the insect from leaving the tube, and pellets of different sizes may be needed as well. A wide variety of alterations can be made to the R script to change the appearance of the bubble plots, and other graphic parameters.

We reduced the risk of false positives by writing a debounce code that disables any given channel for one second after a metal BB is detected thereby preventing a single metal BB from being counted as multiple data points. Although, this creates the possibility of a data point being missed if many bees emerge at once, but the fact that the channels are independent reduces this risk. Another limitation of the current system is that individual data points are not discernable, *i.e.,* a falling metal BB cannot be traced back to a specific individual. Furthermore, the current system measures emergence but not eclosion rhythms in *M. rotundata*, but would measure eclosion rhythms in species where emergence and eclosion are synonymous. Finally, the current design is not weatherproof, limiting its use to controlled environments.

Future applications include examining the effects of other abiotic and biotic environmental cues for timing emergence of *M. rotundata*. Furthermore, because insects occupy diverse environments, relevant environmental cues vary between species. Thus, incorporation of more insect species is important to investigating how circadian systems evolved across taxa. Little is known about how developmental conditions affect the timing of adult emergence; therefore, our system can be used to decipher the effects of treatments on emergence. Furthermore, combinations of environmental cues can affect insect responses, thus future experiments should incorporate multiple environmental cues to understand their relative effects on emergence. Lastly, deployment in the field to observe how natural settings mediate emergence rhythms is of interest. The ease of use of this system, and its unique combination of additive manufacturing, open-source programming, and observable biological traits, make it a candidate for use in an educational setting.

Disclosures:
The authors have nothing to disclose.

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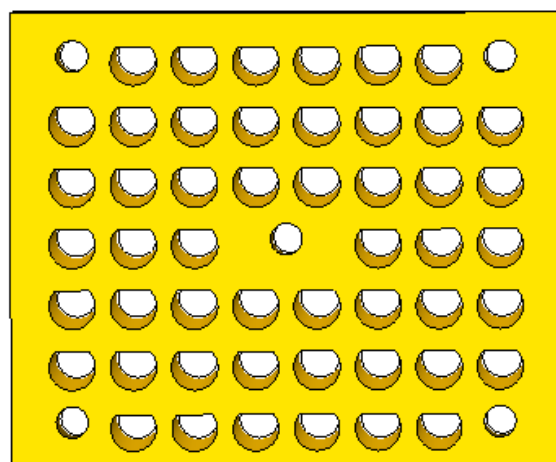425   *Insect Physiology.* **93–94**, 28–35 (2016).
426
427
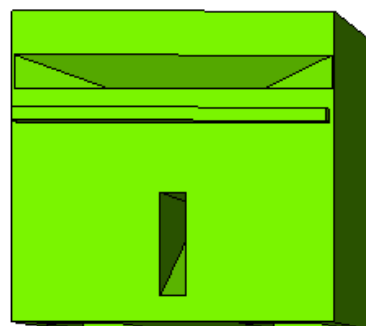
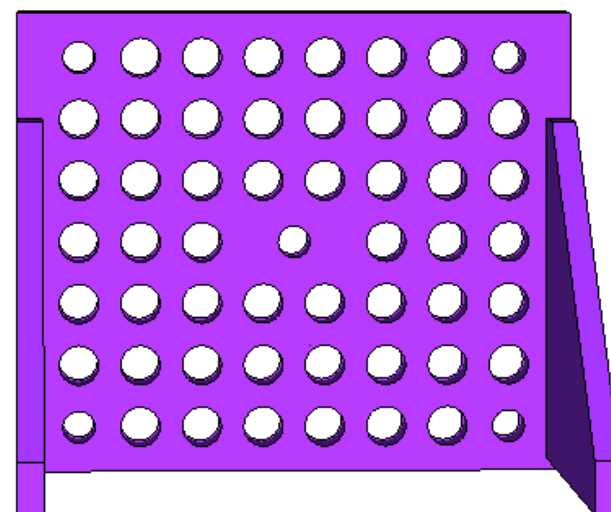Figure 1

collector manifold
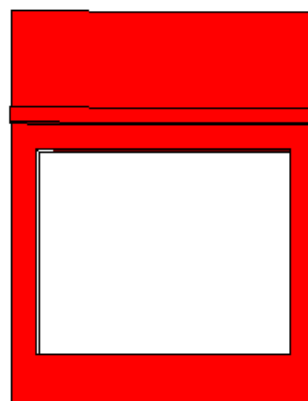
end cap
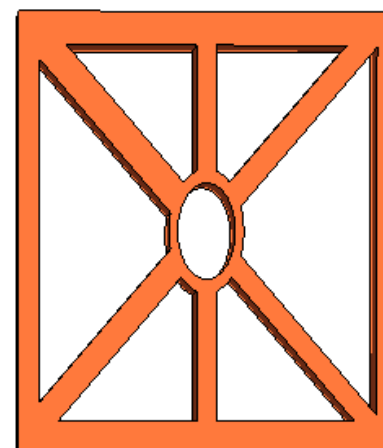
platform support

tube rack face plate

tube rack base plate

Figure 2

Figure 3

Arduino pin	LCD screen pin
3	4
4	6
8	11
7	12
6	13
5	14
5v	15
GND	16

Figure 5
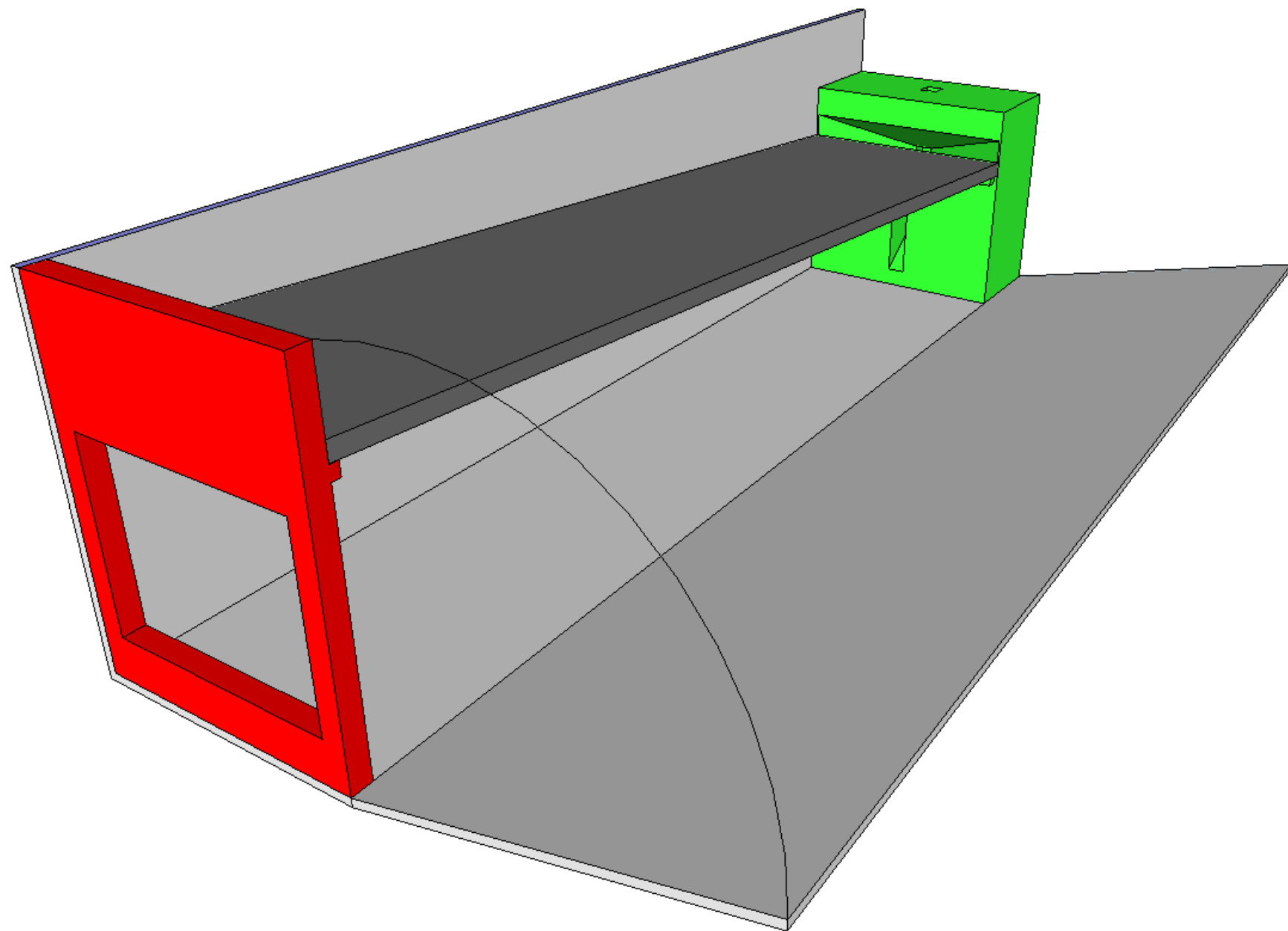
Figure 6
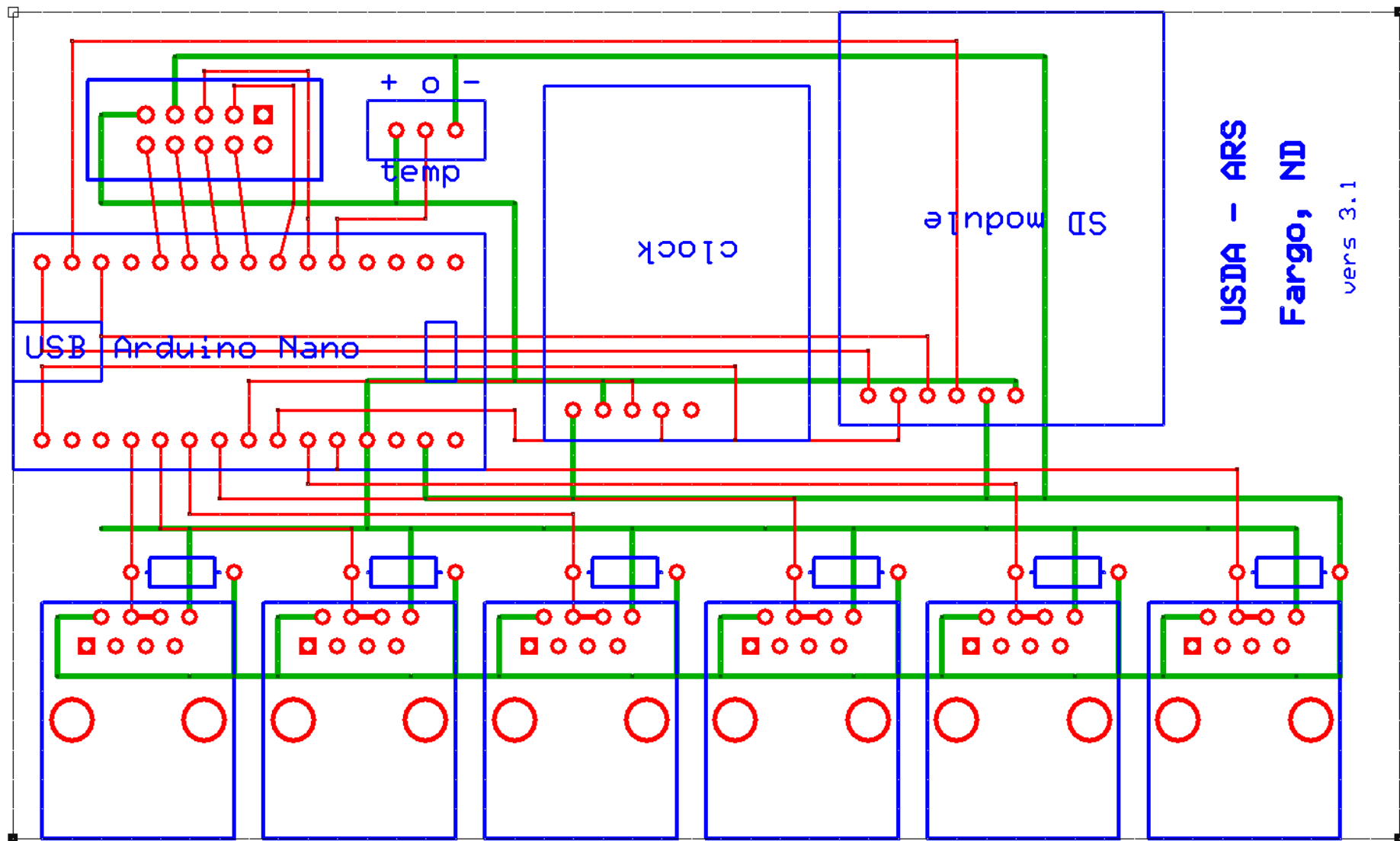
[Click here to download Figure figure6pdf.pdf](#)

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Fargo, ND
vers 3.1

Figure 7

Figure8 10-15-FTR

Figure 8-8-15-FTR
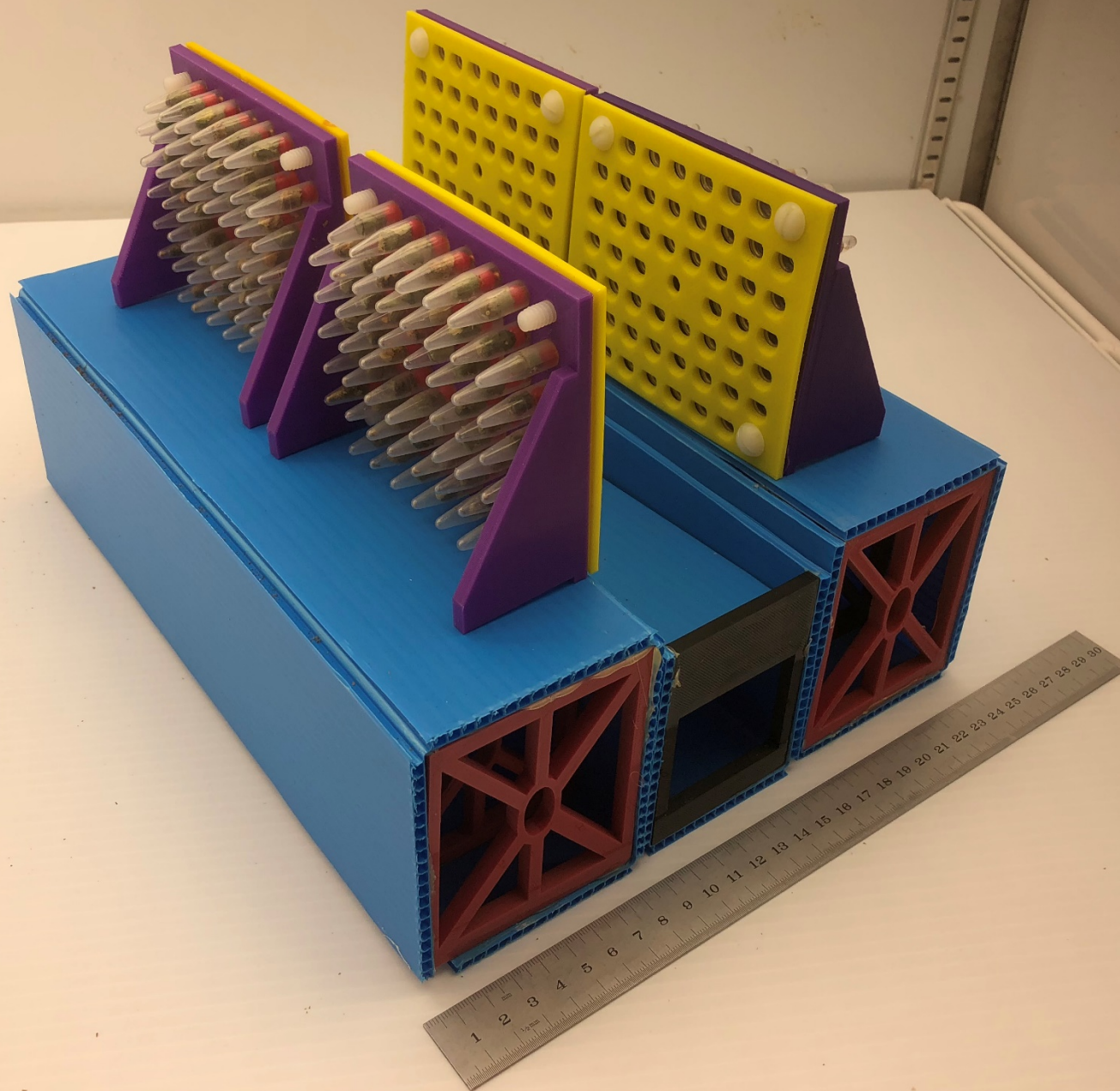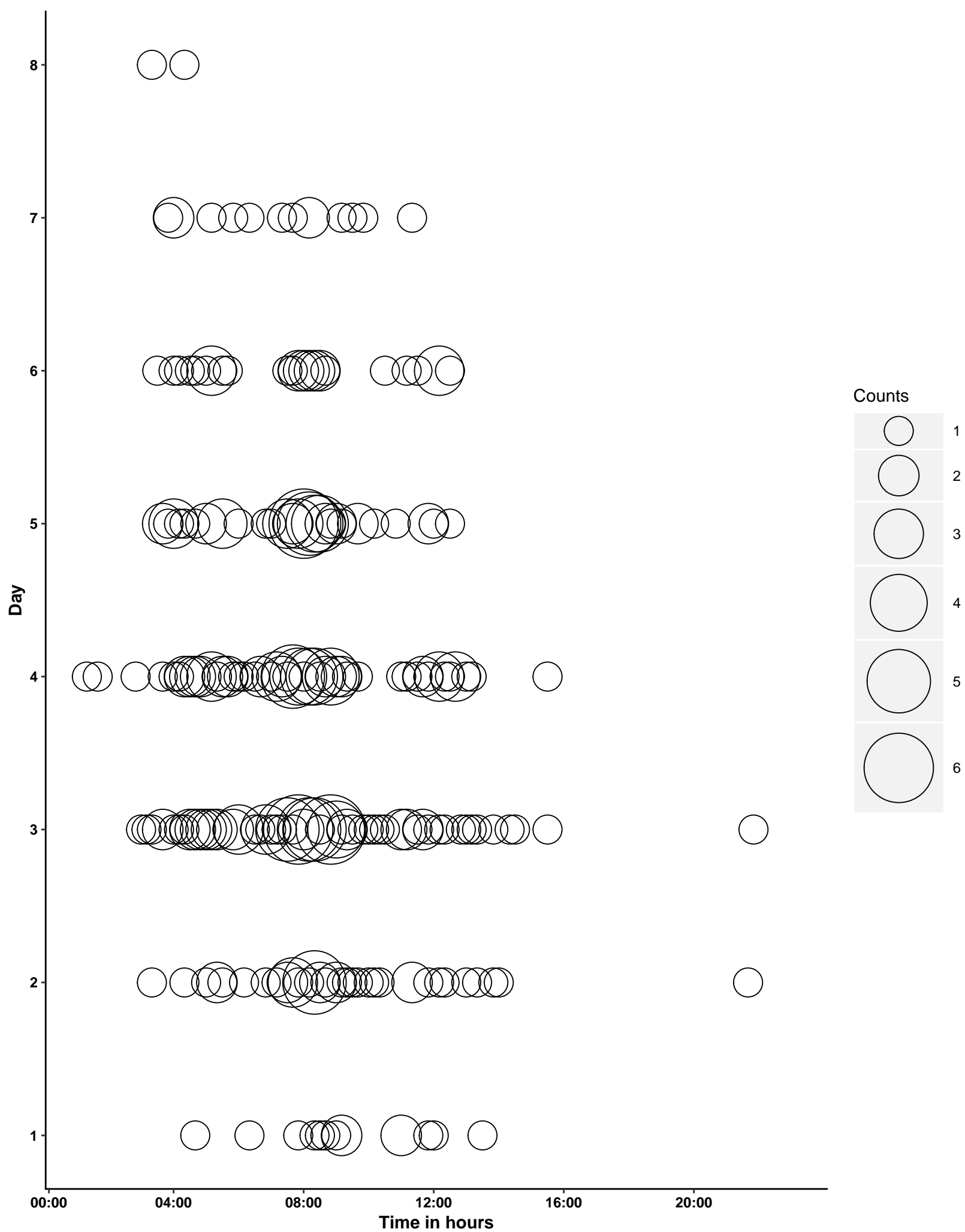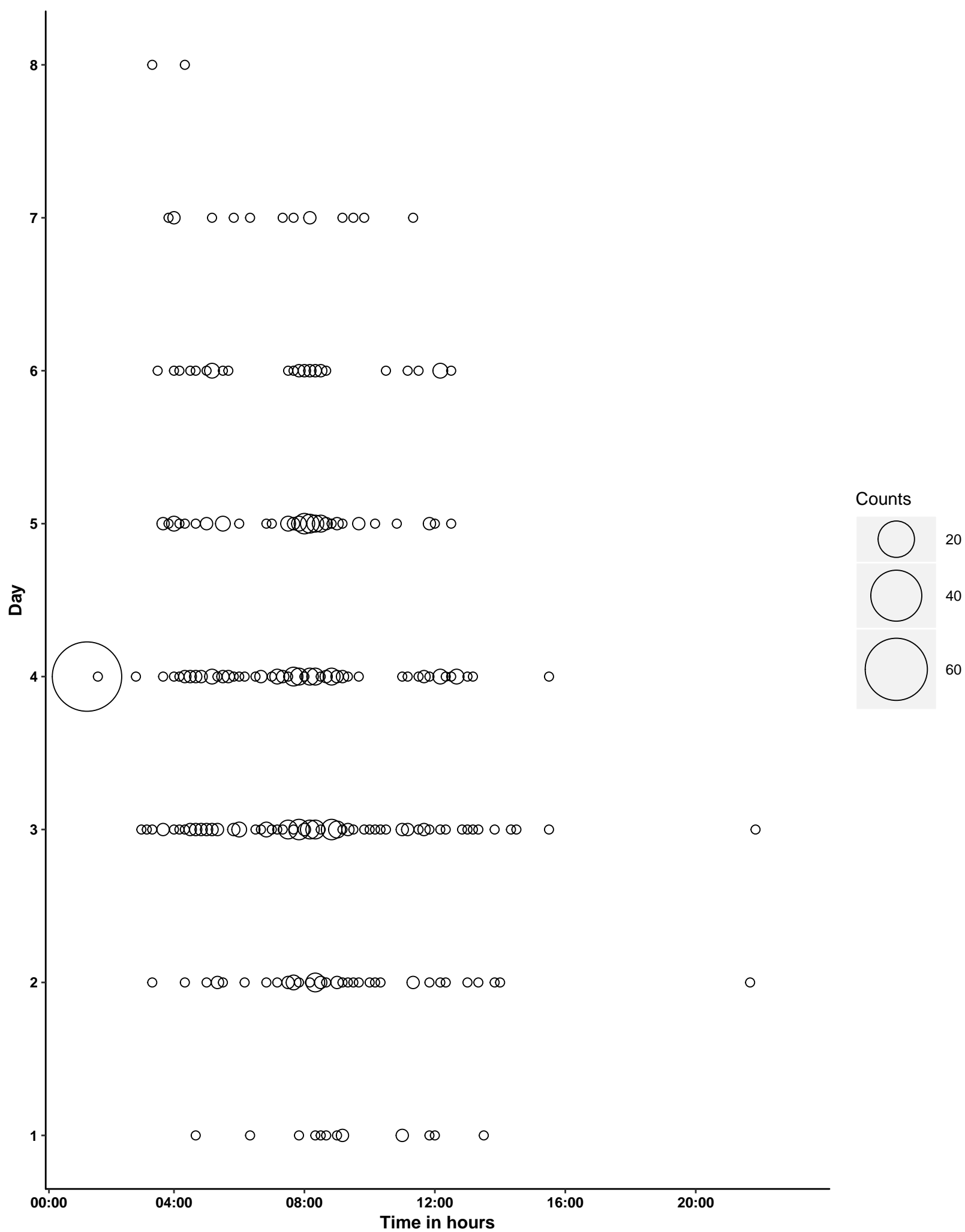
Table of Materials

Name of Material/ Equipment	Company	Catalog Number
PLA printer filament	www.lulzbot.com	various
0.5 mL microcentrifuge tubes	www.daigger.com	EF4254C
4.5 mm size "bb" metal pellets	www.amazon.com	B00419C1IA
6.0 mm plastic "softair" pellets	www.amazon.com	B003QNELYE
Plastic corregated sheet	www.lowes.com	345710
Infrared emmiter/detector pair	www.amazon.com	B00XPSIT3O
120 ohm resisitors	www.amazon.com	B01MSZK8DV
22 GA hookup wire	www.adafruit.com	1311
RJ45 jacks	www.sparkfun.com	PRT-00643
Custom PCB board	www.pcbexpress.com	n/a
Arduino Nano v 3.0	www.roboshop.com	RB-Gra-01
SD card module	www.amazon.com	DFR0071
Real Time Clock module	www.adafruit.com	264
Temperature/humidity sensor	www.tinyosshop.com	G4F4494F29ED05
470k ohm resistors	www.amazon.com	B00EV2R39Y
Female headers	www.adafruit.com	598
Male headers	www.adafruit.com	392
Ribbon wire	www.amazon.com	B00X77964O
LCD screen	www.adafruit.com	198
Cat6 cable	www.amazon.com	B00N2VISLW
SD card	www.amazon.com	B00E9W1URM

Comments/Description

Catalog number varies by color

Daisy 4.5 mm metal size bb pellets
Crosman 6 mm airsoft pellets
Corrugated plastic sheet
5 mm diameter, 940 nm wavelength
120 ohm, 1/4 watt

Can be printed from files included in the supplimental data

DFRobot SD card module
DS1307 real time clock breakout board
DHT11 temperature/humidity sensor on breakout board

Break off to desired length
Break off to desired length
10 wire ribbon wire with connectors

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Author(s):

Meghan M. Bennett, Joseph P. Rinehart, George D. Yocum, and Ian Yocum

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CORRESPONDING AUTHOR:

Name: Meghan M. Bennett1

Department: School of Life Sciences

Institution: Arizona State University

Article Title: A precise and autonomous system for the detection of insect emergence patterns

Signature: [signature]   Date: 4-23-18

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To whom it may concern,

Please accept our reviewed manuscript entitled "A precise and autonomous system for the detection of insect emergence patterns" for resubmission for publishing through the Journal of Visualized Experiments. The manuscript describes the construction and operation of a unique 3D printed, microcontroller-based system for the monitoring insect emergence patterns.

We independently addressed all four anonymous reviewers comments in a separate document and the changes are reflected in the manuscript. We were grateful for such helpful reviews and believe it improved the quality of the manuscript. Furthermore, we took additional care in proofreading and editing the manuscript.

Sincerely,

Dr. Meghan Bennett

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

2. Figures 8 and 9: Please capitalize the first word in the labels on the x and y axis (i.e., Day, Time).

MMB: The x and y axis titles were capitalized.

3. Please rephrase the Long Abstract to more clearly state the goal of the protocol.

MMB: The long abstract was reworded to clearly state the goal of the protocol.

4. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

MMB: The introduction was reworded to clearly state the goal of the protocol.

5. Please define all abbreviations before use.

MMB: All abbreviations were defined throughout the text.

6. Please include a space between all numbers and their corresponding units: 33 cm, 120 Ω, 15 mL, 37 °C, 60 s; etc.

MMB: A space was included between numbers and units throughout manuscript.

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

MMB: We removed any personal pronouns from the protocol text.

8. 1.7.2, 2.2, 2.2.1, 2.2.2: Please write the text in the imperative tense.

MMB: Text was written in an imperative tense at specified locations.

9. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

MMB: We corrected these mistakes in the references.

10. Please upload supplemental data that are mentioned in the manuscript. Please note that files uploaded to the "Supplemental Files (as requested by JoVE)" section of your Editorial Manager account are only for JoVE's internal use and will NOT be published with your article. If you would like your files to be available for download with your article, then please move them to the "Supplemental Code Files" section of your Editorial Manager account.

MMB: We will ensure that this issue is rectified in the final uploaded version.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors have developed an innovative system to measure insect emergence patterns in experimental settings. I would suggest revising the manuscript to provide additional context about the system. For example, I work with emergent aquatic insects and the purpose/potential uses of this system were not clear to me until I read most of the manuscript. In addition, the authors should provide more details about the setup and methods to help users new to this type of experimental system (like students) get started. I have provided several specific suggestions below. Good work.

Major Concerns:

Long abstract

1. 1st sentence, consider revising to "… they are limited in the maximum number of emerging insects they can detect."

MMB: This change was made to the first sentence.

Short Abstract

1. "multiple data points" is vague. Please define "data points." I think this is number of emerging insects.

MMB: This part of the sentence was removed to improve clarity.

Introduction

1. 1st sentence, explain what type of insect emergence is meant. i.e. terrestrial insects in experimental settings, this set up is not for aquatic insects or for use in field (i.e. non experimental) settings.

MMB: This sentence was reworded to be more specific.

2. explain the "falling-ball" principle at first mention. This design is based on falling ball so a brief explanation will help new users.

MMB: A brief description was added here.

3. define what is meant by "sample sizes" (i.e. number of emergent insects detected).

MMB: This was more clearly defined here.

4. What does "In our hands" mean? Couldn't anyone achieve similar results? I'd suggesting removing this statement or clarifying.

MMB: The words were removed here.

Protocol

1. Before the protocol section please provide a brief overview of the system and how it works. Without this it's not really clear how the systems works until one reads all the way through the protocol.

MMB: A brief overview of the system and how it works was added before the protocol section.

2. System use 2.3.2 and 2.3.3, "metal pellet" "metal ball" and "BB" are all used to describe the same thing. Please be consistent.

MMB: This inconsistency was corrected by using "metal BB" throughout the manuscript.

Representative results

1. Prior to explaining the results please provide a description of how the experiment was set up/methods. i.e. pupae were loaded into centrifuge tubes, next a plastic BB and metal BB were added to each tube, etc. Again, I think this will be beneficial to people new to the system (like a graduate student using this article/video to set up their experiment).

MMB: A further description of this process was added to section 2.3.2 of the protocol.

Figures

1. Please add labels to figures so that components can be identified without color (e.g. figure 1).

MMB: This change has been made to the figure.

2. Some actual images of system components with wiring, centrifuge tubes, etc along with renderings would be helpful. (e.g. figure 3)

MMB: In response to this statement and that of reviewer #4, we have changed the final figure to a photograph of the setup. We found wiring to be difficult to convey in a photograph, and are therefore reluctant to replace Figure 6 with a photograph. Additionally, we think that these issues will be addressed in the final video.

Minor Concerns:
Long abstract

1. 4th to last sentence. Replace "ran" with "run".

MMB: This mistake was corrected.

Limitations

1. two typos in this paragraph: "beingmissed" and "emergeat once"

MMB: These mistakes were corrected.

Figures

3. Figure 9 legend replace "overly" with "relatively"

MMB: This replacement was made.

Reviewer #2:

Manuscript Summary:

Bennett et al. have created an autonomous system to collect data on insect emergence. This system includes both a design that can be 3D printed and constructed from purchases pieces and R code to assist with the analysis of data from this emergence trap. The system is specifically designed to measure the of emergence of a leaf cutter bee.

Major Concerns:

It should be made clearer early in the manuscript both what types of insects this trap is optimized for and that it is for lab-based assays. As those that study insect emergence in the field (e.g. in aquatic environments) or in taxa that have a different life history (e.g. Drosophila) would be unlikely to use this trap.

MMB: This point was made clearer earlier in the manuscript, at the end of the introduction.

Minor Concerns:
Abstract:

Unclear what 'pretreatment' means in this context (and in the introduction).

MMB: We changed this to treatment to avoid confusion.

Should be clear in introduction that this apparatus is primarily for lab-based work on insect emergence-with a focus on Megachile rotundata.

MMB: A sentence was added to specify these points, at the end of the introduction.

Introduction:
Better to use 'exogenous factors' or 'environmental factors' than Zeitgebers to reduce sub-field specific jargon- or at least define with first use.
MMB: Zeitgeber was removed and replaced with environmental cues throughout the manuscript.
Protocol:
It would be useful to more thoroughly discuss how larval insects are arranged inside the apparatus so potential users working on different taxa can envision whether this design would be useful for them. What size range of insects are the sensors likely sensitive to (particularly on the small end of the spectrum)?
MMB: A further description of this process was added to section 2.3.2 of the protocol.

Reviewer #3:
Manuscript Summary:
This manuscript describes new apparatus for recording the timing of insect eclosion/emergence. By improving the existing system, the authors succeeded to improve the measurement accuracy, to expand the sample size, and to be automated further. I think the paper would be useful for those just starting the research in this field. The subject is suitable for the journal. Usability of this new device is guaranteed from the results obtained. I would recommend this manuscript to be accepted after minor revision.

Minor Concerns:
Is it possible to monitor the emergence status during the measurement? If so, the author should better explain this.
MMB: This point is discussed in the "limations" section.
References 3 'drosophila' should be 'Drosophila'
MMB: Drosophila was capitalized here.
References 4 '…. Emergence in the Alfalfa Leafcutting Bee' should be '…. emergence in the alfalfa leafcutting bee'
MMB: The case was fixed here in this reference.

Reviewer #4:
Manuscript Summary:
The paper describes a neat, efficient system for remotely measuring insect emergence. The instructions are easy to follow, and should be repeatable by anyone with basic technical knowledge of circuitry, or enthusiasm to learn the basic skills required.

Major Concerns:
1. In Section 1.1 and Figure 1 - The number of parts for each component don't match in the text and figure.
MMB: This change has been made to the figure.
2. I couldn't see any supplementary files, and I would have liked to look them over. For e.g., I couldn't assess whether the supplementary .stl files are well-named for easy reference, nor whether the R script is well annotated for easy use.
MMB: We will ensure that this issue is rectified in the final uploaded version.
3. The only mention of an incubator is in the results section under "Successful run". You may want to mention earlier in the text that the system can be placed in an incubator, and perhaps give some guidelines of the temperature range in which this setup would function.
MMB: The mention of an incubator was added in the description of the system before the protocol.
4. Provide the raw data files used in Figure 8 and 9 as supplementary materials. This way users building their own setups could check the downstream processing pipeline.
MMB: Raw data files will be provided in supplementary materials.
5. I would like to see a photo(s) of the completed setup included.
The final figure has been replaced by a photo of the assembled setup.
6. Adding dimension information to some of the figures would be helpful for readers to easily see how much bench/desk/incubator space it takes up. Some overall dimensions (length, breadth, height) in the text would also be worthwhile adding.
MMB: The dimensions of the assembled apparatus were added to the Figure 7 legend.

Minor Concerns:

Long abstract

-1st sentence: Strange wording - Replace "that" with "the"

MMB: This sentence was reworded for clarity.

Introduction

-Provide a short description of the "falling-ball" or "bang-box" systems.

MMB: A brief description was added here.

-Suggest you provide the common name for this species.

MMB: The common name was added to the introduction.

-The last two paragraphs of the introduction are a little repetitive. Consider revising the text to streamline the information.

MMB: The information was streamlined to avoid repetition.

Protocol

-Would any 3D printer be able to print the required components? If there are minimum requirements these should be specified.

MMB: Added this note to the 1.1 section in system construction section.

-It may be nice to include the actual filenames for printing in the text

MMB: This was added to the protocol section of the manuscript.

-Make sure to define abbreviations (where needed)

MMB: Abbreviations were defined throughout.

-1.7 Does this software work on all operating systems?

MMB: Arduino can be downloaded for both windows and mac operating systems, specification was added 1.7.1.

-2.1 Does the system just "click" together? OR is adhesive needed?

MMB: It was further specified in 2.1 how to assemble the rack platforms.

-2.2. Make sure it's clear in the manuscript what a channel is

MMB: Channels are discussed throughout the manuscript.

-3 How is temperature recorded and logged? I see there's a sensor in the breakout board listed, but does it need calibration?

MMB: In section 1.6.5 a note was added to test temperature sensor before use.

-3.2.4 Link to a "How to use R" type document/website for those not familiar with instructions like 'change the working destination"

MMB: In section 3.2.3 a link for help with R was added.

Results

-Figure 1 - I would find it helpful if the parts were labelled in the actual figure (not just the legend)

MMB: This change has been made to the figure.

-Figure 3 legend - "for" should be "four"?

MMB: This mistake was corrected.

-Figure 4 - A and B labels missing in figure

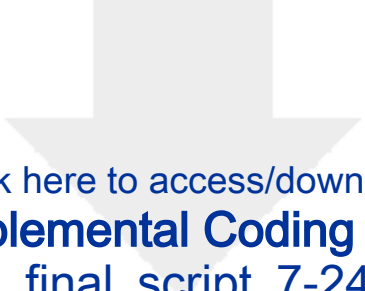*MMB: This issue has been corrected.*

-Figure 8 - I'm not sure why you need the "color" legend to be visible in the plot

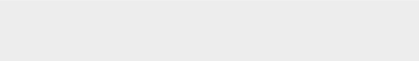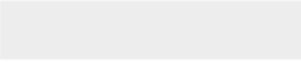*MMB: This mistake was corrected.*

Discussion

-Limitations section - Numerous spacing issues in the 2nd sentence.

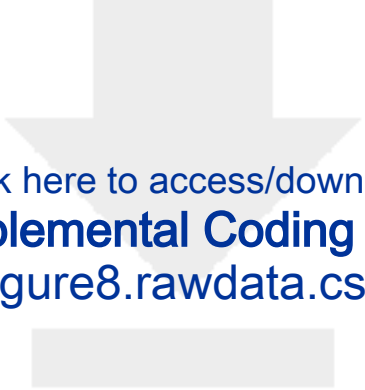*MMB: This sentence was adjusted to be more streamlined.*

R script

Click here to access/download
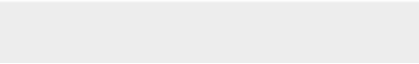**Supplemental Coding Files**
JOVE_final_script_7-24-18.R

Arduino script

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arduino code.docx

figure8 raw data

Click here to access/download
Supplemental Coding Files
figure8.rawdata.csv

figure9 raw data

3D printing file
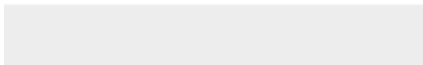
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