

## **iLAM: imaging Locomotor Activity Monitor for circadian phenotyping of large-bodied flying insects**

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### **Running Headline**

imaging Locomotor Activity Monitor

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### **Conflict of Interest Statement**

None

### **Author Contributions**

J.N.D. and A.C.S.O. both formulated the study questions and methodology, collected data, analyzed data, and wrote the manuscript.

### **Data Availability**

iLAM documentation is available at <https://daytonjn.github.io/ilam/>. Data and scripts used for the analyses within this manuscript are available at GitHub repository [https://github.com/daytonjn/ilam\\_ms/](https://github.com/daytonjn/ilam_ms/).

## 1 **Practical Tools**

### 2 **Abstract**

- 3 1. Historically, most insect chronoecological research has used direct observations,  
4 cameras, or infrared beam-based monitors to quantify movement across timed  
5 intervals. Although some alternative DIY systems are cheaper than the current  
6 standard locomotor activity monitor, these options remain complicated to build and/or  
7 computationally intensive.
- 8 2. We developed the **imaging Locomotor Activity Monitor (iLAM)**, an affordable (~\$75  
9 USD/unit) system for activity quantification. The iLAM utilizes a Raspberry Pi Zero W  
10 computer and night-vision camera inside a flight cage to photograph a population of  
11 insects at user-defined intervals. Open-source, modular R-scripts process the images  
12 and output a file containing the number, size, coordinate location, and timing for all  
13 movements (blobs) identified between consecutive images. Output can be analyzed  
14 directly or converted into the standard TriKinetics DAM format.
- 15 3. We demonstrated the flexibility and power of the iLAM system by comparing diel and  
16 circadian activity of different insect species (fireflies: *Photinus marginellus*, *P. greeni*,  
17 *P. obscurellus*), ecotypes (moths: *Ostrinia nubilalis*), and sexes (moths: *O. nubilalis*).  
18 Data captured by only six iLAMs (\$450) identified that peak activity of *O. nubilalis*  
19 females (AZT: 19.2 hr) occurs significantly earlier than males (22.0 hr). Additionally,  
20 male moths from a univoltine population exhibited a significantly shorter endogenous  
21 period length (AZT: 21.3 hr) than males from a bivoltine genetic background (22.6  
22 hr).
- 23 4. The iLAM will serve as a valuable tool for future researchers seeking to measure  
24 locomotor activity across diverse species, sexes, and populations in constant and  
25 changing environments.

### 26 **Key-words**

27 chronoecology, circadian rhythm, locomotor activity monitor, Raspberry Pi

## 28 1. Introduction

29 Almost all organisms on earth perform various tasks at different times of day to take  
30 advantage of variation in resource availability. The evolutionary and physiological drivers of  
31 these diel activity rhythms, as well as their behavioral consequences, have been a focus of  
32 chronobiological research for close to three centuries (Pittendrigh, 1993). While some  
33 activity patterns are directly cued by external signals such as light or temperature, others are  
34 endogenously driven by a highly conserved, temperature-compensated circadian clock  
35 mechanism (Sauders et al., 2002; Brady, Saviane, Cappelozza, & Sandrelli, 2021). In  
36 insects and other animals, the circadian clock sits atop of a hierarchy of time-variant  
37 processes as diverse and fundamental as vision, navigation, learning, metabolism, and  
38 development (Tomioka, Uryu, Kamae, Umezaki, & Yoshii, 2012). The potential for  
39 anthropogenic stressors to alter diel activity rhythms to the detriment of affected taxa has  
40 become a focus of recent research in chronoecology (Gaynor, Hojnowski, Carter, &  
41 Brashares, 2018; Levy, Dayan, Porter, & Kronfeld-Schor, 2019; Gilbert et al., 2022).

42 As chronobiological research writ large requires sampling consistently over extended  
43 periods (e.g. counting firefly flashes “every half-hour or hour for 28 consecutive hours”; Buck,  
44 1937), much of which occurs outside of a typical workday, early advances in automated  
45 sampling were eagerly adopted (Konopka & Benzer, 1971). In the past two decades,  
46 *Drosophila*/Locomotor Activity Monitors (DAM/LAMs, hereafter referred to jointly as DAMs;  
47 TriKinetics, Waltham, MA) have become the primary method for recording the activity  
48 patterns of fruit flies and other small insects (Blanchardon et al., 2001). DAMs measure the  
49 timing and number of infrared beam interruptions made by individual flies moving across  
50 small glass tubes. DAMs have directly led to multiple breakthroughs in our understanding of  
51 the genetic and neuronal architecture of the circadian clock network in *Drosophila*  
52 *melanogaster* (Patke, Young, & Axelrod, 2020).

53 In addition to their high cost (\$525-\$3450), DAMs have several limitations that  
54 prevent their use with non-traditional model organisms (e.g., *Danaus plexippus*, Zhang,  
55 liams, Menet, Hardin, & Merlin, 2022). First, the small tubes (10-25 mm diameter) cannot

56 easily accommodate large-bodied insects; they limit movement and preclude ecologically  
57 relevant behaviors such as flight, orientation, mating, and foraging. Second, recording  
58 duration in DAMs is limited because these tubes lack space for water or nectar  
59 supplementation (but see Wang, Yang, & Chen, 2021). Third, they reduce the complexity of  
60 ecologically relevant behaviors into binary beam interruptions which removes information  
61 otherwise captured by live observations or recorded images.

62 In recent years, automatic processing of video or image time series has emerged as  
63 an attractive alternative to beam-crossing activity monitors. However, the majority of these  
64 approaches are intended not for activity monitoring *per se* but for automatically tracking  
65 individuals, recording movement paths (Panadeiro, Rodriguez, Henry, Wlodkowic, &  
66 Andersson, 2021), and classifying behaviors (e.g., Bohoslav et al., 2021). These programs  
67 can achieve impressive results, but many of their higher-level capabilities – although  
68 reducible to gross activity over a 24-hour period – exceed the demands of chronobiologists  
69 and behavioral ecologists. Furthermore, the data storage and supercomputing infrastructure  
70 needed to process days of video pose barriers to many researchers and limit the scope of  
71 experiments (i.e., number of samples and recording duration). A less technically demanding  
72 and computationally intensive system explicitly designed for automated activity monitoring  
73 would increase the accessibility of this technique and help bring chronoecological research  
74 into the modern era.

75 Our imaging **L**ocomotor **A**ctivity **M**onitor (iLAM) employs an infrared Raspberry Pi  
76 camera mounted on the bottom of a DIY flight cage. The camera periodically takes images  
77 of large-bodied insects flying or crawling overhead. Images are saved onto an SD card  
78 where they can be downloaded and automatically analyzed with scripts written in the  
79 programming language R. The scripts subtract consecutive pairs of images, identify  
80 differences between them, and then record where these differences – i.e., movements –  
81 occurred, how large they were, and when they took place.

82 In this paper, we describe how to build and operate an iLAM and demonstrate its use  
83 in three case studies: one on the temporal niches of *Photinus* fireflies and the other two on

84 sex-specific and ecotype differences in activity timing of the European corn borer (*Ostrinia*  
85 *nubilalis*). We conclude by comparing our iLAM system with other activity monitors currently  
86 in common use.

## 87 **2. Materials and Methods**

### 88 2.1 iLAM Design and Construction

89 Detailed step-by-step instructions for iLAM construction, image processing, activity  
90 analysis, and troubleshooting are provided online as Supporting Information  
91 ([https://\[xxx\].github.io/ilam/](https://[xxx].github.io/ilam/)). Briefly, the iLAM consists of three main components: (1) flight  
92 cages to contain insects, (2) single-board computers connected to night-vision cameras for  
93 imaging, and (3) customizable scripts to control imaging frequency, file export, and post-  
94 process image segmentation (Figure 1).

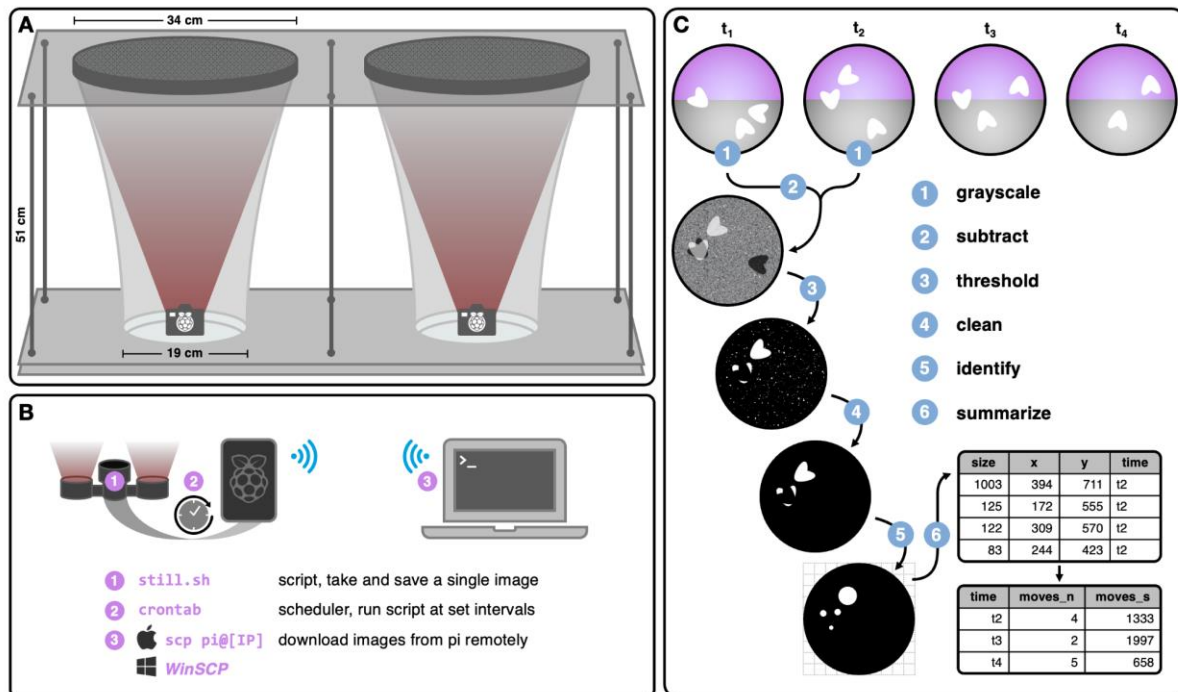
95 Although the flight cage design is flexible, we constructed side-by-side pairs of iLAMs  
96 from items commonly found at local hardware and art stores (\$150 USD/paired setup; Figure  
97 1A). This modular design facilitates disassembly and background customization to increase  
98 the detectability of black- (e.g., fireflies) versus white-bodied (e.g., moths) insects under  
99 infrared (IR) illumination. The tapered netting also increases detection by limiting movement  
100 outside the field-of-view of the camera. At the bottom of each flight cage, one Raspberry Pi  
101 Zero W computer and night-vision camera with infrared accessory lights photographs insect  
102 movement overhead (Figure 1A-B).

103 Custom scripts are scheduled with crontab (Reznick, 1993) to capture and save an  
104 overhead image (2592×1944 px) as frequently as every five seconds to a mounted micro-SD  
105 card. Saved images can be downloaded remotely for post-process segmentation and  
106 analysis.

### 107 2.2 Post-Process Image Segmentation

108 Briefly, all image segmentation is conducted in R (v.4.1.0, R Core Team) using  
109 `imager` (Barthelmé & Tschumperlé, 2019), an R wrapper that integrates the speed of C++  
110 image-processing library `cimg` (Tschumperlé, 2012) with existing R tools. The iLAM wrapper

111 function *find\_movements()* grayscales, optionally blurs, and subtracts consecutive images to  
112 identify the number, size, location, and timing of all prominent between-image differences  
113 (Figure 1C). Only pixel differences greater than a given quantile threshold (99.9%) are  
114 retained, as determined by *find\_threshold()*; these pixel differences correspond to animals  
115 that are absent from the first image ( $t_i$ ) and appear in the second image ( $t_{i+1}$ ). Filtered pixels  
116 are cleaned and clustered prior to segmentation into blobs, which are visual representations  
117 of movement (Figure 1C). The location and size of these identified movements are output  
118 into a CSV file for subsequent filtering. This CSV aggregates all movements identified by  
119 each iLAM in an experiment. The associated function *parse\_movements()* combines data  
120 from this CSV with metadata, including trial date, time, light:dark conditions, treatment, and  
121 species. To verify accuracy, iLAM identified movements can be visualized over the original  
122 images with *plot\_movements()* and *make\_gif()*. Finally, *make\_dam\_file()* converts these  
123 movement data from a CSV into the standard Drosophila Activity Monitor (DAM) format for  
124 activity analyses in *rethomics* (Geissmann, Garcia Rodriguez, Beckwith, & Gilestro, 2019).  
125 Users can choose whether to quantify activity as the total number of discrete movements or  
126 the summed size of all movements at a given time point.



127

128 **Figure 1.** Overview of iLAM design and workflow. (A) Schematic of DIY paired flight cage  
 129 setup with Raspberry Pi and infrared cameras installed at the base of each. (B) On-board  
 130 scripts to capture overhead images and remotely transfer them to a personal computer for  
 131 analysis. (C) Post-process image segmentation workflow to identify and summarize  
 132 movements between pairs of images.

### 133 2.3 Case Studies

134 As a proof-of-concept, we used the iLAM to measure diel activity and circadian  
 135 phenotypes in three case studies: interspecific variation in the temporal niches of male  
 136 fireflies (*Photinus greeni*, *P. marginellus*, *P. obscurellus*), sex-specific differences in moth  
 137 diel activity (*O. nubilalis*), and ecotype differences in moth circadian phenotypes (*O.*  
 138 *nubilalis*: univoltine, bivoltine). We also compared our measurements of *O. nubilalis* diel  
 139 activity with those obtained from two TriKinetics LAM25Hs (Waltham, MA), hereafter referred  
 140 to as DAMs. All experiments were conducted in a climate-controlled room (23.5°C, 40% RH).  
 141 Following a day of acclimation in the flight cages, insects were recorded for three full days of  
 142 16L:8D hours. For circadian phenotyping, *O. nubilalis* moths were additionally exposed to  
 143 four days of continuous darkness (DD). Further information is provided within the  
 144 Supplementary Material.

## 145 2.4 Data analysis

146 All analyses and visualization utilized the `rethomics` framework (Geissmann et al.,  
147 2017) in R (v. 2.4.2, R Core Team 2022). Activity was estimated as the sum, in pixels, of all  
148 movements identified between consecutive images taken two minutes apart. These data  
149 were combined into 30-minute bins and normalized across individual cages by setting the  
150 average activity level for all 30-min bins equal to one. Times were converted to Arbitrary  
151 Zeitgeber Time (AZT), where AZT 0 indicates when lights were turned on.

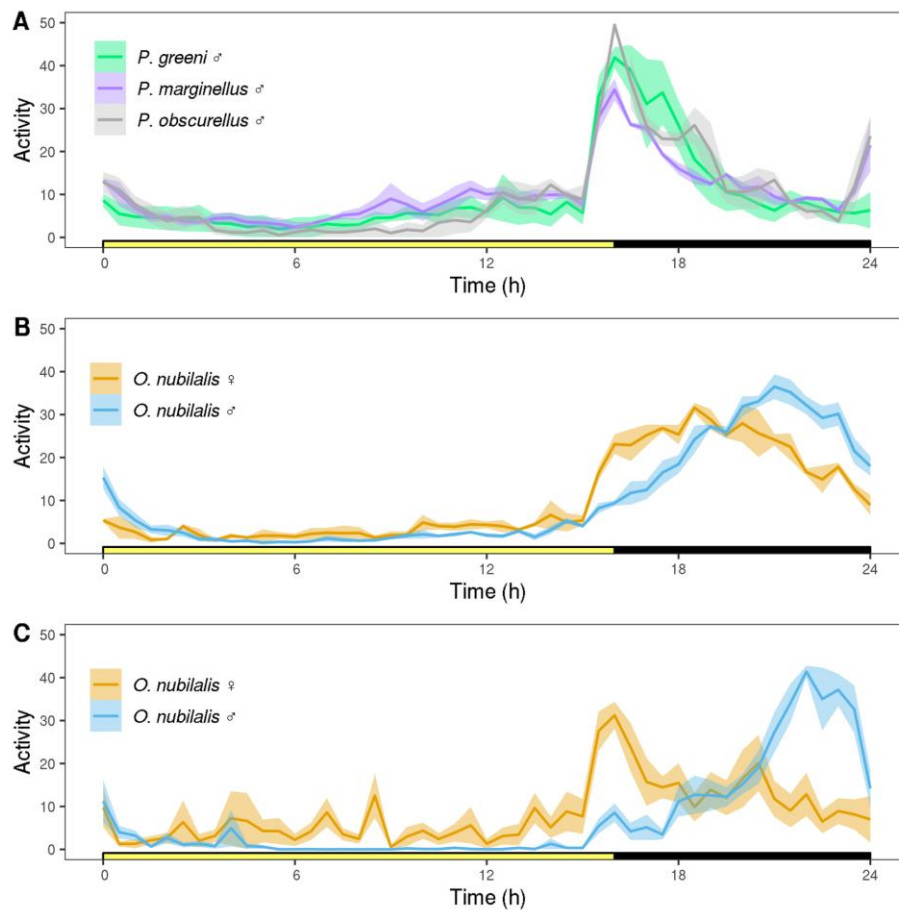
152 Per Kostadinov et al., 2021, daily activity was smoothed with a Butterworth filter and  
153 the `find_peaks()` function from `pracma` (Borchers, 2021) used to identify the timing of peak  
154 activity across three days of light:dark (LD) entrainment. We defined nocturnal activity  
155 (“nocturnality”) as the percentage of total activity that occurred during scotophase each day  
156 (Schlichting & Helfrich-Förster, 2015). Over four days in free-running continuous dark (DD)  
157 conditions, endogenous period length and rhythmic strength were estimated using a chi-  
158 square periodogram, where period length was the peak value above the significance  
159 threshold at  $\alpha = 0.05$  (for more details, see Cai, Hidalgo Sotelo, Jackson, & Chiu, 2022).

## 160 3. Results

161 Under 16L:8D, the timing of peak activity was similar across all three firefly species:  
162 *P. greeni* (AZT: 17.0 hr, 95% CI: 16.2–17.8), *P. marginellus* (17.0 hr, 95% CI: 17.0–17.0),  
163 and *P. obscurellus* (17.2 hr, 95% CI: 16.7–17.6). Similarly, there were no strong differences  
164 in nocturnality between *P. greeni* (0.66,  $N = 3$ ) or *P. marginellus* (0.52,  $N = 3$ ). However,  
165 there was weak evidence that *P. obscurellus* (0.75,  $N = 3$ ) exhibited greater nocturnality than  
166 *P. marginellus* ( $t = 3.1$ ,  $df = 2.4$ ,  $P = 0.07$ ).

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168

169 **Figure 2.** Diel activity of adult *Photinus* fireflies (A) and bivoltine *Ostrinia* moths as measured  
170 by iLAMs (B) and TriKinetics DAMs (C). Activity on the y-axis corresponds to the daily  
171 average ( $\pm$  SE) of normalized 30-min binned activity. The x-axis shows Arbitrary Zeitgeber  
172 Time (AZT); AZT 0 indicates when lights were turned on.

173 In contrast, *O. nubilalis* female (0.95, 95% CI: 91.8–99.0) and male (0.92, 95% CI:  
174 0.84–1.0) moths were almost exclusively nocturnal. Peak diel activity of *O. nubilalis* females  
175 (AZT: 19.2 hr) occurred substantially earlier than that of males (22.0 hr;  $t = 3.95$ ,  $df = 2$ ,  $P =$   
176 0.05; Figure 2B). This difference persisted when the activity of females (16.8 hr, 95% CI:  
177 16.2–17.4) and males (20.6 hr, 95% CI: 18.1–23.0) was measured with a TriKinetics DAM.  
178 However, the timing of females' peak activity significantly differed between the iLAM and  
179 DAM setups ( $t = 7.41$ ,  $df = 4.9$ ,  $P < 0.001$ ).

180 In complete darkness (DD), the rhythmicity of female moths markedly declined (Table  
181 1). In contrast, all male moths remained rhythmic. Univoltine males exhibited a significantly

182 shorter endogenous period length (21.3 hr) than males from a bivoltine genetic background  
183 (22.7 hr;  $t = 6.33$ ,  $df = 4$ ,  $P = 0.003$ ; Table 1).

184 **Table 1.** Activity rhythms of *Ostrinia nubilalis* moths from different ecotypes. Activity rhythm  
185 in constant darkness is given in hours  $\pm$  95% confidence interval.

| Population | Sex    | N | Rhythmic (%) | Period $\pm$ 95% CI | Strength $\pm$ 95% CI |
|------------|--------|---|--------------|---------------------|-----------------------|
| Bivoltine  | Male   | 3 | 100          | 22.8 $\pm$ 0.7      | 199 $\pm$ 119         |
| Univoltine | Male   | 3 | 100          | 21.3 $\pm$ 0.5      | 312 $\pm$ 249         |
| Bivoltine  | Female | 3 | 67           | 22.7                | 131                   |
| Univoltine | Female | 3 | 33           | 21.3                | 21                    |

#### 186 4. Discussion

187 The iLAM is an affordable and powerful system for monitoring the locomotor activity  
188 of large-bodied insects (Figure 1). Even with a small sample size – three paired cages with  
189 five to ten individuals each and recording durations of less than a week – our iLAMs proved  
190 capable of effectively capturing clear differences in activity timing between species, sexes,  
191 and populations (Figure 2, Table 1).

192 The iLAM produced strikingly similar results to a TriKinetics DAM, the current  
193 standard (Figure 2B-2C). The main difference is that the startle effect at the light:dark  
194 transition was more pronounced in DAM data (Figure 2C); this may be an artifact of the  
195 small diameter of the tubes. DAM data also had higher overall variance because insects  
196 were monitored individually instead of in small groups. Regardless, the circadian phenotypes  
197 captured here (Table 1) were remarkably consistent with those previously obtained by the  
198 same TriKinetics DAMs for bivoltine (period = 22.7 hr) and univoltine (period = 21.4 hr)  
199 males as reported by (Kozak et al., 2019).

200 Unlike existing movement trackers, including DeepLabCut (Mathis et al., 2018),  
201 MARGO (Werkhoven, Rohrsen, Qin, Brembs, & de Bivort, 2019), and others (Geissmann et  
202 al., 2017; Jolles, 2021), the iLAM is expressly intended to function as an activity monitor.  
203 Because it aggregates data from groups of insects and disregards individual movement  
204 paths, the iLAM is a highly accessible tool for all researchers with access to a personal

205 computer. Nevertheless, the iLAM can identify the location and size of all movements made  
206 by insects within the range of view of the camera. These data are much richer than the  
207 current standard: counts of beam interruptions made by insects in a tube (see also Sondhi et  
208 al., 2022). Our enclosures, which are large enough to accommodate flight, also permit a  
209 broader range of ecologically relevant behaviors. Although not reported here, future studies  
210 could easily connect the location and size of identified movements with particular behaviors  
211 (Gilbert et al., 2022) such as feeding (Fenske, Nguyen, Horn, Riffell, & Imaizumi, 2018),  
212 orientation (Wan, Hayden, Iiams, & Merlin, 2021), calling (Gao et al., 2020), or eclosion  
213 (Zhang, Markert, Groves, Hardin, & Merlin, 2017). Because the iLAM saves each image  
214 taken during the experiment, this leaves a strong record that can be checked for accuracy  
215 and revisited at any point for supplementary analysis.

216 In the 21st century, natural environments are changing at an unprecedented rate.  
217 Now more than ever, there is a critical need to document how organisms alter their activity  
218 timing in response to anthropogenic stressors across the seasonal cycle (Gilbert et al.,  
219 2022). Accessible, modular tools like the iLAM can catalyze faster adoption of automated  
220 activity monitoring, especially for novel study systems that require a more flexible approach.  
221 In this way, our simple system has the capacity to dramatically advance the growing field of  
222 chronoecology.

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## Supplementary Methods

### 2.3.1 Demonstration: Interspecific Differences in Diel activity

In summer 2022, male *Photinus greeni*, *Photinus marginellus*, and *Photinus obscurellus* fireflies were collected from the wild. *P. obscurellus* ( $N = 30$ ) were collected from Smith-Andover Field in Lincoln, MA, on June 14th (42.42568, -71.30675; sunrise: 5:08; sunset: 20:22), *P. greeni* ( $N = 30$ ) from Estabrook Woods in Concord, MA, on June 23rd (42.48515, -71.34635; sunrise: 5:10; sunset: 20:24), and *P. marginellus* ( $N = 46$ ) from Muster Field in Lincoln, MA on August 6th (42.407523, -71.330734; sunrise: 5:43; sunset: 19:57). Species identity was determined from body size, elytral pigmentation, and courtship flash pattern (Lloyd 1969); sex was determined from lantern morphology.

Males were haphazardly divided into groups of approximately five individuals and kept outside until the following day. They were then moved indoors to a climate-controlled room (23.5°C, 40% RH) and evenly distributed among three unpaired flight cages, each supplied with an *ad libitum* source of water and two cups of moist sponges for shelter. To remain consistent with the natural timing of sunrise and set, overhead lights automatically turned on at 05:00 and off at 21:00. Diel activity was recorded over three full days of 16L:8D hr, beginning the day after males were introduced to flight cages.

### 2.3.2 Demonstration: Sex and Population-Specific Differences in Diel activity

Bivoltine European corn borer (ECB) adults were collected from a laboratory population at Tufts University (Medford, MA) that has repeatedly been selected and studied for bivoltine characters (i.e., short post-diapause development timing) and studied previously (Levy et al. 2018 ; Kozak et al. 2019). This population was originally derived from larvae collected in Geneva, NY (42.8680° N, 76.9856° W) and out-crossed with bivoltine ECB from Hollis, NH (42.7668° N, 71.6000° W). Similarly, univoltine ECB adults were sourced from a laboratory stock population, founded from Bouckville, NY (42.8892N, 75.5513), and selected for univoltine characters (Levy et al. 2018 ; Kozak et al. 2019). ECB larvae were reared on artificial corn borer diet (Southland Products, USA) under 16:8 LD at 23.5°C. Pupae from each population/sex ( $N = 15-22$ ) were isolated into individual plastic cups with moist dental wicking. Following eclosion, 1-2 day old adults were randomly selected, sexed, and evenly distributed among three flight cages. All experiments were conducted in a climate-controlled room (23.5°C, 40% RH). Beginning the day after cage introduction, insects were entrained for three full days of 16L:8D hours. *O. nubilalis* were subsequently exposed to four days of continuous darkness (DD) for circadian phenotyping. Each cage contained one *ad libitum* water source and two cups of moist sponges.

### 2.3.3 Demonstration: Comparison with TriKinetics LAM25H

Bivoltine European corn borer adults were singly loaded into 30 TriKinetics LAM25H tubes (25mm diameter) and confined by cotton plug. For a water source, each tube contained a moist cotton ball and hydrated water crystals at the base of each tube. Activity was quantified as the number of beam interruptions that occurred within each 2-minute interval. Recordings occurred in a climate-controlled room (23.5°C, 40% RH) under a 16L:8D cycle. Beginning the day after tube introduction, insects were entrained for three full days of 16L:8D hours. *O. nubilalis* were subsequently exposed to four days of continuous darkness (DD) for circadian phenotyping. However, due to high mortality and low activity, circadian phenotypes could not be quantified from LAM25H insects.