**Response to Reviewers (Re: Manuscript by Verwey, Robinson & Amir entitled Recording and analysis of circadian rhythms in running-wheel activity in rodents; Submitted to JoVE)**

We would like to start by thanking both reviewers for their valuable feedback on this manuscript. We are confident that we have been able to address most of their concerns, and that these changes have produced many tangible improvements in the paper. These changes are detailed below, itemized according to the points of each reviewer.

**REVIEWER #1**

**Point #1-1:** It would be helpful to give at least some details on the light-tight boxes used to house the animals, as there obviously must be a certain sophistication in ventilation system to avoid light leaks. Some type of schematic would do it, just to sensitize the reader that the ventilation system needs proper consideration.

**Authors’ response to point #1-1:** We have added a section entitled “Ventilation”, which appears as section 1.4 in the revised manuscript and reads as follows. “Ventilation: Adequate air flow is imperative to making the boxes a comfortable home environment for rodents. The fan in each box should be hooded, so as to prevent light from outside the box from reaching the inside. Also, fans will typically remove air from the box and blow it into the room. Small light-tight vents allow air to enter the isolation boxes from several points, and help to avoid uncomfortable breezes. In order to verify that there is adequate ventilation, the temperature inside the isolation box (when closed for several hours, with the lights on) should be virtually identical to the temperature in the room where it is housed.”

**Point #1-2:** The authors should mention which specific software they used to calculate the periodograms

**Authors’ response to point #1-2:** We have added a section entitled “computer programs “, which appears as section 1.4 in the revised manuscript and reads as follows: “Computer programs: Specialized computer programs are typically used in the generation of actograms and the calculation of circadian period. These programs include, but are not limited to, Actiview (Minimitter, Bend, OR) and Circadia.“

**Point #1-3:** "The periodogram on the right confirms that this arrhythmic pattern exhibits roughly equivalent power for all circadian periods." The authors should change to something like "The periodogram on the right confirms the observed arrhythmic behavior showing equivalently low power for all periods in the circadian range (20-30hrs)"

**Authors’ response to point #1-3:** This statement was changed to “The periodogram on the right confirms this arrhythmic pattern of activity by showing equivalently low power for all periods in the circadian range (20-30h). “

**REVIEWER #2**

**Point #2-1:** When applied to the real world, some statements and strategies are oversimplified and require a better description. Specifically the strategies for analyzing data are very basic and will be useful for a baseline, however it is not explained how to analyze running-wheel activity after pharmacological or other environmental modifications. It is importamt to include advise and straegies for specific manipulations, specifically it will be useful to advise how to analyze behavioral changes elicited by food anticipatory activity, which is mentioned in the abstract, but is not explained further on in the text.

**Authors’ response to point #2-1:** Because of the relatively brief JoVE formatting requirements, a lot of text had to be cut away from our first draft. We have limited the manuscript to the scope of this single technique and, as much as possible, avoided talking about “other” manipulations and techniques that might warrant their own JoVE articles. That being said, because of our keen interest and previous experience with restricted feeding schedules, we have added a small section on food-anticipatory activity at the end of the results section. It reads: “The amount of running, and the time of day it occurs, can also be manipulated by environmental factors. For instance, if rodents are fasted and given a temporally-restricted meal each day, this restricted feeding schedule will induce a daily bout of food-anticipatory activity. It is termed “anticipatory” because it occurs prior to the arrival of the daily meal, and is especially obvious when the meal is given in the middle of the day, a time when nocturnal rodents are relatively inactive. For instance, if an experiment provides a single 2h meal each day, food can be added to the cage at ZT 4 (4h after lights turned on) and removed at ZT 6 (2h later). Moreover, wire mesh flooring in the cages is also advantageous for this type of experiment because it makes it impossible for the rats to hide food and store it for later, thus ensuring that the rat is actually consuming all the food within the prescribed mealtime. Finally, one of the main advantages of an accurate running-wheel activity recording is that it allows for correlations to be made between running-wheel activity and daily oscillations in the expression of circadian clock gene expression throughout the brain and body.”

**Point # 2-2:** ABSTRACT: Some sentences are oversimplified and suggestive; however they are not accurate and are not supported by the cited references: The statement: "running-wheel activity serves as a particularly reliable and convenient measure of the output of the master circadian clock, the suprachiasmatic nucleus (SCN) of the hypothalamus" is partiallytrue, as indicated by the authors in lines 5-7 Page 4. There are other examples that clearly show that voluntary wheel-running can be a response to external stimuli and does not exclusively report the activity of the SCN (See Redkin and Mrosovsky 1999; Beyond the suprachiasmatic nucleus Mrosovsky 2003; and others).

**Authors’ response to point #2-2:** We now mention masking directly in section 1.6 “Darkness/Dim red lighting”, which now includes the sentence “The specific red light you use should be tested to ensure it does not alter running-wheel activity (e.g. negative masking) or adjust the circadian clock (e.g. produce a phase shift).” We also now mention non-photic phase shifts directly in section 1.2 “Bedding changes:” and state that “Animal handling as well as changes in cages or bedding can all have non-photic effects on circadian rhythms”.

**Point #2-3:** Authors state that "circadian oscillators in many other regions of the brain and body9-14 could also be involved in the regulation of daily activity rhythms" Non of the studies cited here have proven that brain or peripheral oscillators can determine daily activity rhythms; all of them have evidenced the existence of extra-SCN oscillators. Likewise references 17 to 20 have evidenced that food related cues can entrain extra-SCN oscillators, however they have not reported that such oscillators can regulate anticipatory activity.

**Authors’ response to point #2-3:** This distinction is certainly correct. This is why we state that those putative oscillators “could be involved….” instead of using the more causal statement that they “are involved….”. Moreover, this statement has no references because the evidence that we are aware of, like the reviewer points out, is largely correlative. References 17-20, are again correlative, and so we have changed the sentence to read “…are correlated with changes in the activity of extra-SCN oscillators 17-20”

**Point #2- 4:** In the last paragraph it is suggested that running-wheel activity can be used to study the output of extra-SCN oscillators, however further on no strategies for this analysis are provided.

**Authors’ response to point #2-4:** We have added a new paragraph at the end of our results section discussing food-anticipatory activity. We now end this paragraph with: “Finally, one of the main advantages of an accurate running-wheel activity recording is that it allows for correlations to be made between running-wheel activity and daily oscillations in the expression of circadian clock gene expression throughout the brain and body.”

**Point # 2-5:** 1.2 Bedding changes: a good suggestion to possibly avoid entraining signals by handling due to bedding cleaning is to perform this at random hours and to use special long lasting bedding that will allow sporadic cleaning.

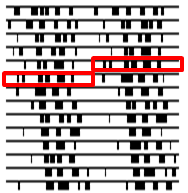
**Authors’ response to point #2-5:** This section now includes a sentence that reads “Alternatives include using longer-lasting bedding, which would allow for more infrequent cage changes, or changing cages on a pseudo-random schedule.”

**Point #2-6:** 2.2 Computer hardware: are there other companies providing hardware and software for running-wheel analysis? If so, they should be mentioned in order to avoid a biased advertisement.

**Authors’ response to point #2-6:** Both Vitalview and Clocklab were already listed in Section 2.3 “Computer hardware: “. In the Software section we mention both Actiview (Vitalview product) and Circadia, but have also added another reference to Clock lab. So, Section 3.1 “Files” now has a sentence that reads “Such data are best visualized and graphed with specially designed programs such as Actiview (Minimitter, Bend, OR), Circadia, or Clocklab (Actimetrics, Wilmette, IL) which can all produce periodograms and actograms”.

**Point #2-7:** 4.1 Actograms: the double plotted actogram represents in the same line day1 followed by day 2. The second line represents day 2 and day 3 and so on. A double plotted actogram does not "illustrate the same day twice" as stated in the manuscript.

**Authors’ response to point #2-7:** Double-plotted actograms are simply two single-plotted actograms put side-by-side and offset by 1 day. The actogram on the left contains the only plot of the first day, and the actogram on the right contains the only plot of the last day. Otherwise, all of the other data is duplicated in a double-plotted actogram. This duplication of a single day is what we were referring to by “illustrate the same day twice”, and is highlighted in red in the schematic below. In attempt to solve some of this confusion, we have rephrased the sentence to read “but double-plotted actograms plot two days on each horizontal line”



**Point #2-8:** 4.3 Results: (page 8 line 2) "the double plotted actogram on the left shows a rat?" needs to be rephrased: the actogram illustrates the ACTIVITY (running.wheel activity) of a rat?

**Authors’ response to point #2-8:** As suggested, this sentence has been rephrased to read “In Figure 2A, the double-plotted actogram on the left shows the running-wheel activity of a rat…”