Video Article

Measuring Exercise Levels in *Drosophila melanogaster* Using the Rotating Exercise Quantification System (REQS)

Louis P. Watanabe[1], Nicole C. Riddle[1]

[1]Department of Biology, University of Alabama at Birmingham

Correspondence to: Nicole C. Riddle at riddlenc@uab.edu

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Abstract

Drosophila melanogaster is a new model organism for studies in exercise biology. To date, two main exercise systems, the Power Tower and the Treadwheel have been described. However, a method to measure the amount of additional animal activity induced through the exercise treatment has been lacking. The Rotating Exercise Quantification System (REQS) fills this need, providing a measure of animal activity for animals experiencing rotational exercise. This protocol details how to use the REQS to assess animal activity during rotational exercise and illustrates the type of data that can be generated. Here, we demonstrate how the REQS is used to measure sex- and strain-specific differences in exercise induced activity. The REQS can also be used to evaluate the impact of various other experimental parameters such as age, diet, or population size on exercise induced activity. In addition, it can be used to compare the efficacy of different exercise training protocols. Importantly, it provides an opportunity to standardize exercise treatments between strains, allowing the researcher to achieve equal amounts of activity between groups if needed. Thus, the REQS is a notable new resource for exercise biologists working with the Drosophila model system and complements existing exercise systems.

Video Link

The video component of this article can be found at https://www.jove.com/video/57751/

Introduction

Recently, researchers have begun to use the fruit fly *Drosophila melanogaster* to study exercise biology. *D. melanogaster* has been a genetic model system for over 100 years[1,2]. However, Drosophila research has made contribution to not just genetics, but also to a variety of other disciplines including neurobiology, behavioral biology, and physiology[3]. In 2009, the Power Tower, the first exercise machine for Drosophila was described[4]. The Power Tower takes advantage of the animals' negative geotaxis response. When disturbed, Drosophila tend to move to the top of their enclosure. This response is well established and is the basis of the popular "RING" (Rapid Iterative Negative Geotaxis[5]) assay that is used to estimate climbing ability and/or physical fitness in Drosophila. The Power Tower uses a mechanical arm connected to a motor unit to repeatedly lift a set of animals within their enclosures by several inches and dropping them back to the ground to induce the negative geotaxis response (Tinkerhess *et al.* 2012[6] provide a video illustrating the use of the Power Tower). Prolonged treatment on the Power Tower thus increases the amount of physical activity (running or flying) the animals perform compared to untreated control animals and over time leads to improved performance in the RING assay for physical fitness[4]. Thus, this work demonstrated the feasibility of using Drosophila as a model for exercise biology.

To expand the repertoire of tools available for Drosophila exercise research, in 2016, Mendez and colleagues described a second Drosophila exercise machine, the Treadwheel[7]. Similar to the Power Tower, the Treadwheel exploits the negative geotaxis response of Drosophila. However, this response is induced by continued rotation of the animal enclosures, rather than by lifting and dropping them as in the Power Tower. This induction method is gentler and allows for a more endurance oriented exercise regime that avoids any physical trauma that might occur during exercise in the Power Tower (see Katzenberger, R. J. *et al.* 2013[8] for the impact of repeated physical trauma on Drosophila health). Similar to the Power Tower[4], exercise treatment of animals on the Treadwheel leads to a variety of physiological responses, including changes in physical fitness, triglyceride levels, and body weight[7]. Thus, two complementary methods are available for Drosophila biologists studying exercise.

One limitation of both the Power Tower and the Treadwheel is the inability to measure the amount of activity induced by the exercise treatment. Analysis of video-recordings taken from the Treadwheel demonstrated that there were significant differences among the various Drosophila strains in how they respond to the exercise treatment[7]. Specifically, the strains studied differed in how much additional activity the animals performed when stimulated[7]. This observation prompted us to develop a third exercise system, the Rotating Exercise Quantification System (REQS), that allows us to measure animal activity levels during rotation-induced exercise[9]. The REQS utilizes a commercially available activity monitoring unit that is installed on a rotating arm to stimulate exercise through rotation as in the Treadwheel. Initial work with the REQS confirms that genetically different Drosophila strains — and sexes - can have significantly different responses to the rotational stimulation and thus the

amount of exercise induced is not identical among different genotypes[9]. Thus, the REQS now enables Drosophila biologists to measure the amount of exercise induced by the treatment, opening a variety of new research avenues in the exercise field.

Here we describe in detail how to use the REQS for quantification of rotational exercise. The REQS induces rotational exercise and simultaneously measures the activity levels of the animals being treated. The REQS is able to accommodate a variety of exercise programs, ranging from the simple 2 h continuous exercise regime demonstrated here to more complex interval training methods as described by Mendez and colleagues[7], and stimulation can be adjusted via rotation speed (between approximately 1-13 rotations per min). Depending on the activity monitor unit used to produce the REQS, this method is adaptable to the analysis of single flies or large populations of animals. Due to this versatility, the REQS provides Drosophila researchers with an array of opportunities to study, for example, different exercise regimes, diet interventions, or impact of population density.

Protocol

The REQS consists of a Drosophila Activity Monitor unit (for source information, please see **Table of Materials**) mounted on a rotating arm that is controlled by a motor unit (**Figure 1**). The activity monitor determines how often in a given time span the array of laser beams dissecting the middle of the assay tube is disrupted. For detailed drawings and an in-depth characterization, see our previous publication[9]. While our system uses the LAM25H unit, the REQS can be modified to accommodate other Drosophila Activity Monitor units as well.

1. Testing the Setup of the REQS

1. Load test flies into glass specimen tubes by anesthetizing the animals with CO_2 or other chosen method for handling, moving them into the empty tubes, and capping the tubes.
2. Insert capped glass specimen tubes into the slots of the activity monitor unit. Secure the tubes using rubber O-rings (17-18 gauge) and/or rubber bands on both sides of the unit to ensure the tubes do not move during rotation:
    1. Slip one O-ring over the specimen tube and position the O-ring close to the midpoint of the tube. Insert the specimen tube into a slot of the activity monitor from the front and center it.
    2. Move the O-ring as needed and ensure that once the specimen tube is centered, the O-ring is slid as close to the activity monitor unit as possible. Fix the specimen tube in place by slipping a second O-ring over the glass tube from the back, again moving the O-ring as close to the activity monitor unit as possible.
3. Initiate rotation of the REQS by flipping the power switch on the front of the unit. Adjust the rotation speed with the dial on the front of the unit to the desired speed (4 rpm (rotations per min) in this example).
   NOTE: The rotational speed in this example was chosen to match a previously published study[7]. As different rotational speeds will result in different animal activity levels, when initiating a new study, optimization of rotational speed for the specific experimental set-up (genotype, length of exercise bout, *etc.*) may be necessary.
   NOTE: It is important to ensure that the tubes are properly positioned so that they do not drag along the bottom of the REQS unit during rotation.
4. Test the connection to the data collection system provided with the Drosophila Activity Monitor unit by opening the DAMSystem308 software and ensuring that the connection light remains green for several rotations. The software will begin recording data into a text file "monitorX" (X being the unit number if multiple monitors are in use) immediately upon initiation. Recording frequency can be adjusted in the "Preferences" tab; we typically record in 5 min intervals.
5. Investigate the text file generated by the DAMSystem308 software to ensure that the data connection is functioning properly. Problems with the connection result in cutouts, time points where 0 activity is recorded for all positions in the REQS (see **Representative Results** and **Table 2**). If cutouts occur, adjust data connections leading to the rotating phone jack, as during rotation, this connection can become loose or twisted. We find that stabilizing the connection with tape helps to prevent this problem.

2. Preparation of Animals

NOTE: All animals were raised and tested under standard conditions in an incubator (25 °C, 60-70% humidity, 12 h light/dark cycle) on molasses/cornmeal media[10].

1. Two weeks prior to the planned experiment, set up vials with controlled animal density to collect the experimental flies from; we typically set up vials with 7 males and 10 females. With a healthy stock, from a single vial, approximately 15 virgin males and 15 females can be collected over a 4 day time period. Adjust the number of vials set up based on the number of animals needed for the assay; a typical assay in our laboratory includes 10 sets of 10 virgin males and females per genotype (100 males and 100 females).
   NOTE: Non-virgin flies can be used depending on the specific experimental question. If conducting longer-term experiments with non-virgin animals, crawling larvae may interfere with accurate activity monitoring.
2. Remove the parent flies one week after setting up the vials.
3. Starting on day 10 after setting up the vials, collect virgin flies from the vials and store them separated by sex.
4. Collect sufficient animals for the experiment and age them as needed, 3 days in this example.
   NOTE: We typically anesthetize the animals with CO_2 for handling. CO_2 is known to impact animal activity levels for extended periods of time after treatment (for example, see Bartholomew *et al.* 2015[11]). If the CO_2 effect interferes with planned downstream assays or analyses, use a different anesthesia method such as ice anesthetization.

3. Data Collection with the REQS

1. Anesthetize the animals to be used for the exercise quantification study with CO_2 or another anesthesia method. Divide them into groups as needed and load the groups of animals into the empty glass tubes of the activity monitor; in this example, 10 animals of the same age are loaded per glass tube, with 10 replicates for each animal type (sex/genotype). Make sure to note which animal type is loaded in each tube.
   NOTE: For longer experiments, food can be included in the specimen tubes. In this case, it is essential that the food is secure and does not become dislodged during rotation.
2. Load the glass assay tubes into the REQS unit and secure them with the rubber O-rings. If working with more than one sex/genotype, using a randomized block design or randomization of the position of the animals in the REQS will eliminate any potential position effects. Randomization can be achieved by assigning each vial a random number, for example from a web-based random number generator (*e.g.,* https://www.randomizer.org/) or in a spreadsheet, and then ordering the vials based on the random number.
3. Place the REQS into the incubator to ensure constant temperature, humidity, and light conditions. Ensure that both the data and power cables are properly connected.
4. Allow the animals to recover from anesthesia and to acclimatize to the new environment for 1 h.
5. Begin the experiment by initiating rotation of the REQS at the desired speed.
   NOTE: Another option is to first collect baseline activity data from the animals before the initiation of rotation, followed by recording activity levels in response to rotational stimulation.
6. Initiate data collection by opening the DAMsystem308 software (simply opening the software initiates data collection). Data are written into a text file at the set interval (here, 5 min); if needed, change interval settings in the "Preferences" tab (see step 1.4).
7. To ensure that the data are successfully transmitted to the computer, open the text file generated by the DAMsystem308 software after one or two time intervals have passed and confirm that data are being written to the file. Close and re-open this file to see data from any additional time points. Collect data for the desired amount of time; in this example, 2 h.
   NOTE: Do not open the incubator during the assay period, as the animals are very sensitive to any disturbance and will likely respond with increased activity.
8. At the end of the exercise assay, terminate data collection by closing the DAMsystem308 software, then turn off the REQS. Remove the animals from the glass assay tubes and clean the tubes. The animals can be moved back into food vials if repeated measures at different ages are needed.
   NOTE: If deaths occur during the exercise regime, it should be recorded, as the presence of dead flies can impact activity counts. In our experience with the DGRP2 fly lines[12,13], exercise at 4 rpm for 2 h did not result in any deaths, but weaker strains might respond differently.

4. Data Analysis

1. Open the .txt file labeled "Monitor1" produced by the DAMSystem308 software.
2. Inspect the data file for any problems that might have occurred during the data acquisition (missed time points, *etc.*; **Table 1**). If necessary, censor the file by removing data points at the beginning and end of the recording.
3. Using the statistical software of your choice (*e.g.,* R), analyze the data collected. Generate descriptive statistics and carry out analysis of variance (ANOVA) to investigate effects such as sex and genotype, if the data are normally distributed. If the data are not normally distributed, use non-parametric methods, such as Kruskal-Wallis tests, to compare groups. The specific analyses required will depend on the specific scientific question and the experimental design.

Representative Results

The output from an individual run with the REQS is a data table produced by the DAMSystem308 software, which will be labeled "Monitor1.txt" by default (for an example see **Supplemental File 1**). An excerpt from such a table is shown in **Table 1**. Each column contains the data from an individual assay tube, while the rows contain the activity measured in each time interval from the beginning of the experiment (top) to the end (bottom). The first three data points should be monitored in the event that there are data cutouts (such as those seen in **Table 2**), in which case the cable must be adjusted.

Figure 2 shows the results from an experiment comparing four genetically distinct Drosophila lines (DGRP2 lines 371, 703, 810, and 897[12,13]). For each of the four lines, ten replicate measurements from groups of ten virgin flies of both sexes were collected as described in the Protocol section. From the output file of the DAMSystem308 software, "Average activity per 5 min per 10 flies" was calculated by averaging the activity measures across the entire 2-hour time span. This average from each column thus produces a single measure for each assay chamber. The summary table that **Figure 2** is based on is provided in **Table 3**.

The data provided in **Table 3** were analyzed by ANOVA, testing for effects of genotype, sex, and the interaction between sex and genotype. As only the effect of genotype was significant ($p < 2 \times 10^{-16}$), the two sexes were combined in the graph shown in **Figure 2**. The ANOVA detected a strong effect of genotype, which is evident in the graph. The mean exercise activity levels between all four genotypes are significantly different from each other ($p < 0.05$; Tukey's HSD), and thus, **Figure 2** illustrates how the REQS can be used to detect an impact of genotype on exercise activity levels.

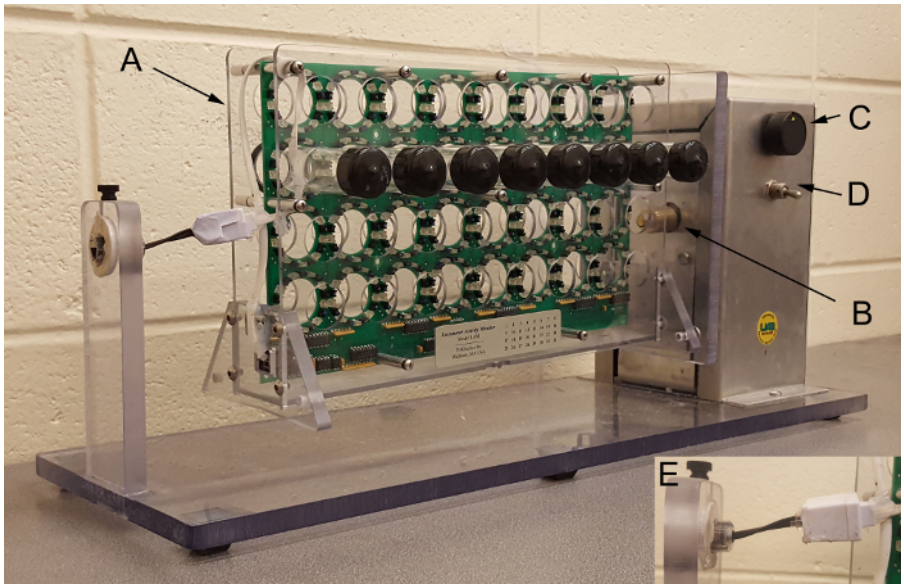

Figure 1: The REQS. Shown in this photograph is the REQS used in this procedure. The activity monitor unit (**A**) rotates around its horizontal axis driven by a rotating arm (**B**). The rotation speed is adjustable via a dial (**C**), and the operation of the machine is controlled by an on/off switch (**D**). The inset (**E**) shows a close-up of the data connection between the activity monitor unit and the rotating phone jack. Please click here to view a larger version of this figure.

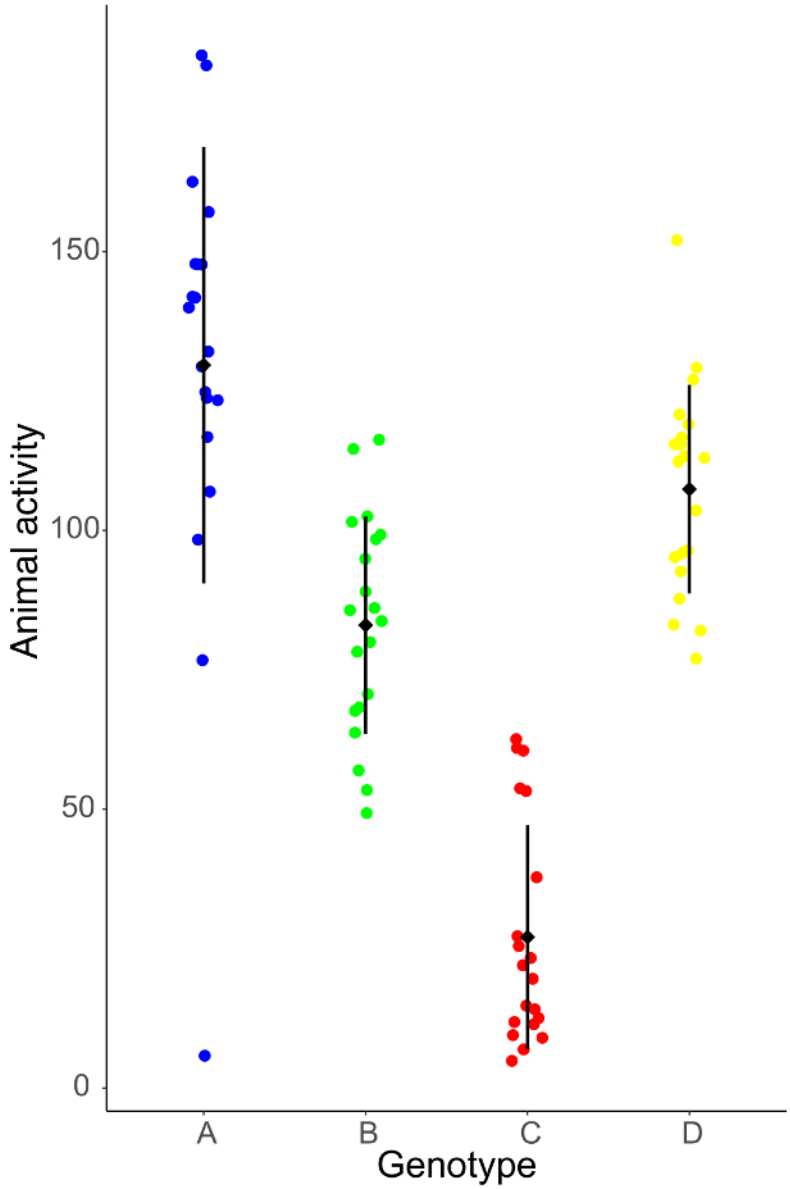

Figure 2: Comparison of exercise activity as measured by REQS in four different DGRP strains. X-axis: animal genotype. Y-axis: animal activity measured as beam crossings per 10 animals per 5 min. A: DGRP2 line 897; B: DGRP2 line 810; C: DGRP2 line 703; D: DGRP2 line 371. The graph shown here combines data from males and females, as there was no significant sex effect for these specific lines (ANOVA; p = 0.557). However, there is a strong genotype effect (ANOVA; p <2 x 10^{-16}), and all individual comparisons between the four genotypes are highly significant (Tukey's HSD; p <0.05). Black diamond: genotype mean; black line: +/- one SD (standard deviation). Please click here to view a larger version of this figure.

Time	Activity(1)	Activity(2)	Activity(3)	Activity(4)	Activity(5)
12:25:00	21	86	48	32	76
12:30:00	31	55	58	74	119
12:35:00	27	45	47	80	125
12:40:00	28	55	34	83	91
12:45:00	36	56	45	67	103

Table 1: Example output of accurate data from the DAMSystem308 software. Each "Activity(#)" column represents one vial of 10 flies, with activity recorded at 5 min intervals.

Time	Activity(1)	Activity(2)	Activity(3)	Activity(4)	Activity(5)	Fault?
12:00:00	0	0	0	0	0	yes
12:05:00	98	1	36	0	8	no
12:10:00	0	0	0	0	0	yes
12:15:00	88	24	44	1	9	no
12:20:00	0	0	0	0	0	yes
12:25:00	106	51	41	0	15	no

Table 2: Example output of faulty data with packet loss from the DamSystem308 software. Each column represents one vial of 10 flies, with activity recorded at 5 min intervals. Rows 12:00:00, 12:10:00, and 12:20:00 show "0" activity recordings across all columns, indicating a problem with the data connection (Fault? "yes" in the last column).

Table 3: Example data used to generate Figure 2. Please click here to download this file.

Supplemental File 1: Unprocessed output file as produced by the DAMSystem308 software. Column 1 records the time point number. Column 2 records the date of the experiment, and column 3 records the time each time point is recorded. Columns 4-10 are not used, and columns 11-42 represent the recordings from the 32 slots of the activity monitor. Please click here to download this file.

Discussion

As the representative results illustrate, the REQS is capable of accurately measuring the activity of exercising Drosophila. The REQS is flexible and allows researchers to address a variety of research questions related to exercise biology or exercise interventions. There are two critical steps in the protocol to highlight. First, it is essential to test the setup of the REQS to ensure that data transmission from the REQS to the DAMSystem308 is working properly. If not properly set up, the data cable can become tangled during the rotation, and sometimes the connection between the REQS and the DAMSystem308 is disrupted at the rotating connection due to wear and tear (although, with almost daily use of the REQS, we have had to replace the connector once in two years). It is prudent to keep replacement parts on hand. Second, the consistency of environmental parameters is essential to the success of the experiments. Drosophila are very sensitive to noise or vibration, and thus, it is important that the experiment is not disturbed during data collection. Thus, ideally, experiments are run in a dedicated incubator that is not accessed during an experimental run. Attention to these two critical steps will ensure that high quality data is collected from the REQS.

While we demonstrate here how the REQS can be used to measure animal activity during a 2-hour continuous exercise treatment from groups of animals, the REQS is flexible in that the time and intensity of exercise can be adjusted for a variety of exercise treatments. In addition, depending on the activity monitor unit used, it can be modified to measure activity from single flies or very large populations of animals. Additionally, the REQS can be used to test the design and optimization of exercise regimes. It can also be used to measure the effects of additional variables such as time of day, animal age, diet, population size, or drug treatment on induced activity and exercise responses. Depending on the exact experimental setup, *i.e.,* length of exercise regime and timing, the REQS can also be used to interfere with the natural sleep patterns of Drosophila. These examples illustrate the versatile nature of the REQS and some potential uses in Drosophila research. Other small animal research communities might also be interested in adapting the REQS for their purposes, thus broadening the utility of this new tool.

Currently, one limitation of the REQS is the limited number of samples that can be processed at a given time, which is dictated by the activity monitors used, which assay a maximum of 32 samples. While the use of multiple REQS units is possible, to allow the REQS to be used for large-scale genetic screens or similar applications, development of a higher throughput version of the REQS would be ideal.

Due to its ability to measure activity levels induced in an identical manner to the Treadwheel, the REQS can be used in combination with the Treadwheel, which allows for somewhat higher throughput (48 samples can be processed at a time). Exercise protocols can be optimized using the REQS and then implemented on the Treadwheel for further studies. Thus, the Treadwheel and REQS can be used complementary as needed for specific study designs.

The REQS is an important step forward in Drosophila exercise research as it allows for quantification of the induced activity. Having a machine that can simultaneously induce exercise and measure this exercise was a clear need in the Drosophila exercise field, as a German group of scientists independently developed a very similar device, termed "swing boat," that also utilizes an activity monitor to measure activity during exercise induced by rotation[14]. The "swing boat" does not use complete rotations, but instead swings the activity monitor unit back and forth approximately 30 degrees around a rotational axis. Thus, the "swing boat," like the REQS, uses the rotation to continually induce a negative geotaxis response and increase animal activity. The REQS and "swing boat" complement existing video-tracking methods used to assay Drosophila after stimulation, such as the Drosophila ARousal Tracking system (DART)[15]. Both the REQS and the "swing boat" improve upon systems like the DART, which track activity only after the cessation of the stimulus. Thus, the REQS and "swing boat" are important new tools for researchers in the Drosophila exercise field, which can be used in conjunction with the existing Treadwheel and Power Tower devices.

Disclosures

The authors have nothing to disclose.

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