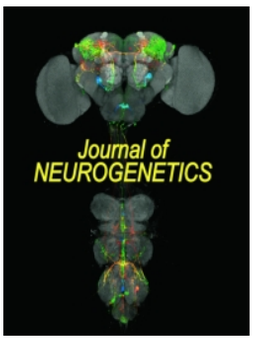

Journal of Neurogenetics

ISSN: 0167-7063 (Print) 1563-5260 (Online) Journal homepage: http://www.tandfonline.com/loi/ineg20

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To cite this article: Alex M. Willenbrink, Margo K. Gronauer, Leon F. Toebben, Daniel R. Kick, Madalyn Wells & Bing Zhang (2016): The Hillary Climber trumps manual testing: an automatic system for studying Drosophila climbing, Journal of Neurogenetics, DOI: 10.1080/01677063.2016.1255211

To link to this article: http://dx.doi.org/10.1080/01677063.2016.1255211

Published online: 21 Nov 2016.

JOURNAL OF NEUROGENETICS, 2016
http://dx.doi.org/10.1080/01677063.2016.1255211

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ORIGINAL ARTICLE

The Hillary Climber trumps manual testing: an automatic system for studying *Drosophila* climbing

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ABSTRACT

Climbing or negative geotaxis is an innate behavior of the fruit fly *Drosophila melanogaster*. There has been considerable interest in using this simple behavior to gain insights into the changes in brain function associated with aging, influence of drugs, mutated genes, and human neurological disorders. At present, most climbing tests are conducted manually and there is a lack of a simple and automatic device for repeatable and quantitative analysis of fly climbing behavior. Here we present an automatic fly climbing system, named the Hillary Climber (after Sir Edmund Hillary), that can replace the human manual tapping of vials with a mechanical tapping mechanism to provide more consistent force and reduce variability between the users and trials. Following tapping the HC records fly climbing, tracks the fly climbing path, and analyzes the velocity of individual flies and the percentage of successful climbers. The system is relatively simple to build, easy to operate, and efficient and reliable for climbing tests.

ARTICLE HISTORY
Received 22 September 2016
Revised 24 October 2016
Accepted 27 October 2016

KEYWORDS
Drosophila; climbing; automation; geotaxis; aging; neurological disorders

Introduction

Behavior is the functional readout of the brain and has been used extensively in both neuroscience and psychology to gain insights into the state or function of the brain. Fruit flies (*Drosophila melanogaster*) display a number of stereotypic and innate behaviors ranging from courtship to climbing. Climbing is governed by negative geotaxis, the tendency for flies to climb upward after being transiently forced downward. Coupled with sophisticated genetics this relatively simple behavior has been valuable for studying aging (Ganetzky & Flanagan, 1978; Gargano, Martin, Bhandari, & Grotewiel, 2005; Martinez et al., 2007; Rhodenizer, Martin, Bhandari, Pletcher, & Grotewiel, 2008) and neuronal development and dysfunction (Banerjee, Lee, Venkatesh, Wu, & Hasan, 2004; Nelson et al., 1997), including models of various human diseases (Haywood & Staveley, 2004; Liu et al., 2015; Martinez et al., 2007; Tauber, Vanlandingham, & Zhang, 2011; Watson, Lagow, Xu, Zhang, & Bonini, 2008).

At present, a typical climbing test is performed by first placing a small population (e.g. 10) of age-matched flies into a graduated cylinder (or a plastic or glass tube, covered by a piece of Parafilm to prevent fly escape), gently tapping the flies down to the bottom of the cylinder, then measuring fly climbing parameters such as climbing time or success rate (e.g. Martinez et al., 2007). Single sex flies are used to prevent complications from courtship or other inter-sex interactions. The climbing test is relatively simple to perform but there are two major concerns with this type of climbing tests. First, it is difficult for an experimenter to accurately record the time of fast climbers. Second, the result can vary considerably with different experimenters as they may tap the flies down with different amount of force. Several groups have since made significant efforts to improve the efficiency and accuracy of fly climbing systems. Video-based climbing assays such as the RING method afford more accurate analysis of climbing (Gargano et al., 2005; Nichols, Becnel, & Pandey, 2012; Rhodenizer et al., 2008). An automated system has also been developed to introduce a mechanical tapping system in the climbing assay (Podratz et al., 2013).

Here we present a new and simpler automatic climbing system, named the Hillary Climber (HC) in honor of the late New Zealand mountaineer Sir Edmund Hillary, that can lift and tap the flies down to a glass tube, record and analyze fly climbing. As a proof of concept we repeated previous published data by testing age-dependent changes in fly climbing, specifically in flies with loss of function of the fragile-X mental retardation (*fmr*) gene (Martinez et al., 2007; Tauber et al., 2011; Wan, Dockendorff, Jongens, & Dreyfuss, 2000; Zhang et al., 2001). Our data demonstrate that the HC is economic to build, easy to use, efficient for testing, accurate for data collection and analysis. We believe that the HC will provide a standardized method for testing climbing of *Drosophila melanogaster* and other insects.

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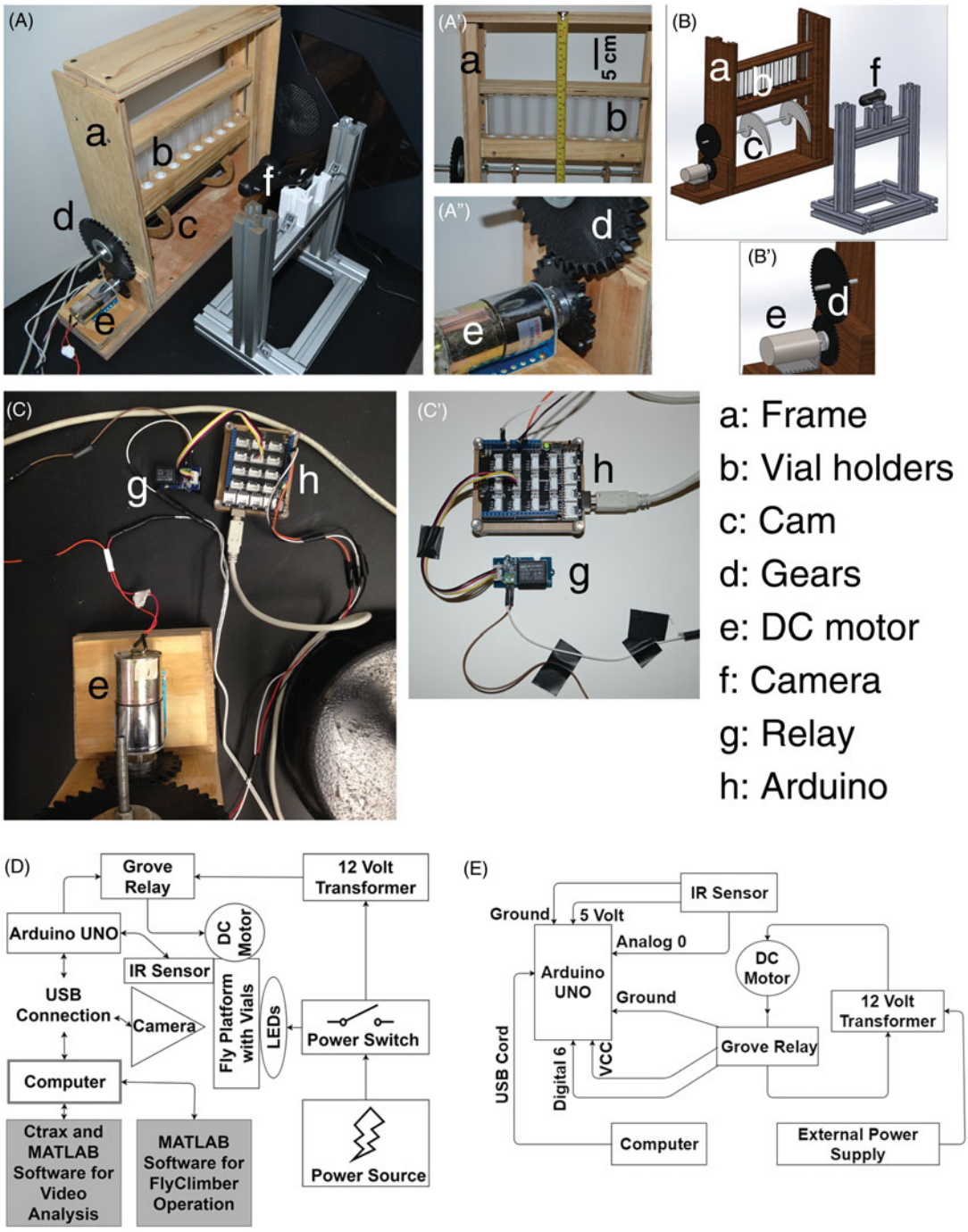

Figure 1. The Hillary Climber: mechanical, electrical, and software diagrams and components. (A) The complete mechanical part of the HC system. (A–A") different views of the mechanical and camera systems, including motor and gear. (B) Model of the complete mechanical set-up through SolidWorks software. (B') Close up of a SolidWorks model of DC motor and gear system. (C) Overhead view of the electrical components. (C') Close up of overhead view of the electrical components. Letters (a–h) label the key individual components of the HC system. (D) Schematic illustration of the full HC system, including the fly platform and vials, camera, electronic, motor, computer, and software systems. Lines and arrows indicate connections between the different components. (E) Schematic illustration of the electrical components. A computer powers and operates the Arduino, which is connected to all components except the USB cord through pins listed in the diagram (5 volt is marked 5V on the Arduino, Analog 0 = A0, Digital 6 = D6, Ground = Gnd, and VCC = VCC). The Arduino controls the motor via the relay.

Materials and methods

Manufacture the Hillary Climber: Construction of the HC is made with special attention to material design (Figure 1). Specifically, it is important that materials composing the HC are not only easy to find, but also simple to implement such that users in other labs can easily construct and use it. Most of the parts are made of wood, including the outer frame (Figure 1(a)), a vial holder (Figure 1(b)), and a double cam system (Figure 1(c)) of the HC. The gears to crank the cams were composed of ABS plastic, 3D printed in the Rostock MAX v2 printer (Figure 1(d)). Lastly, the camera stand was constructed of aluminum T-rods from 80/20 Inc. When fully assembled, the entire system can fit into a 50 L × 70 W × 50 H cm box. It is worth noting that another version (of similar size) of the HC was modeled (not implemented) in SolidWorks that consisted entirely of aluminum and ABS plastic (Figure 1(B)). The Solidworks engineering drawings

with corresponding STL files can be found on www.github.com under 'amwmv4/HC_Engineering_Models' in the search bar.

Mechanical functionality of the Hillary Climber: Flies are transferred individually into test tubes and secured into the fly platform (Figure 1). Two cams are rotated by gears driven by a DC motor (Uxcell Geargox Motor; Figure 1(e)). As the cams rotate past the edge of the fly platform, the fly platform will fall downwards along 2 metal rods anchored at the two ends of the frame. The lift height of the current design is 10 cm. As they reach the bottom of the fall, the force exerted on the platform pushes the flies to the bottom of the vials. This mimics the key, but not the entire, actions of a human exerting a tapping force to bring the flies to the bottom of a graduated cylinder or glass vials. Because this force is applied from a set height each time and the motor rotations per min (RPM) is set, the applied tapping force is consistent from trial to trial. This is important because changing the tapping force on the flies from trial to trial can affect the stress of the flies and consequently the climbing velocity. The cushioning material used at the bottom of the frame is Styrofoam, which may affect the tapping force. In our testing, flies fell to the bottom of the vial and did not bounce upward. In fact, it usually takes a brief moment (~500 ms) for flies to climb upward.

Electrical components and functionalities of the Hillary Climber: An Arduino Uno microcontroller (Figure 1(h)) is used to control all the electrical components except the LEDs. A grove shield with a grove relay (Figure 1(g)) is attached to the Arduino Uno (Seeed Grove Shield with Grove Relay) allowing the 12 volts power supply to reach the DC motor to spin the gears (Figure 1). An IR sensor (Model: GP2Y0A21YK0F, manufacturer: SHARP), located at the bottom of the HC base (not shown), tracks the height of the fly platform. To initiate a recording from the webcam (Model: Logitech Pro 9000, manufacturer: Logitech) the IR sensor must read at least 21 cm (i.e. the IR sensor is located on the bottom frame, about 15 cm below the fly platform before the experiment commences). At this point, the relay will supply power to the DC motor for 4 more seconds, which was carefully calibrated so that it reliably coincided with the dropping of the fly platform. Notice that if another system with different motor RPM is used one would need to recalibrate how long to supply power to the DC motor before the fly platform begins to drop and enter these new parameters. After the 4-s delay period the DC motor power supply draws to an end and the camera starts recording for the allocated amount of time defined by the experimenter. This process will repeat as many times as specified in the 'Rounds' parameter in the experimental start-up window. For a typical experiment, this 'rounds' is set at three, allowing the same flies to be tested three times in one trial.

Programs for running experiments: For recording experiments there is a program implemented within Matlab2014A with the Image Acquisition and Image Processing toolboxes, which utilizes the IR sensor, motor and relay system, and webcam (Figure 1(f)) to obtain fly climbing recordings. Additionally, the USB webcam support package and Arduino Hardware support package for Matlab 2014A were used for experiments. Parameters such as: webcam, Arduino, video name, video storage location, number of trials (denoted 'Rounds' in GUI), and recording time (denoted 'Recovery Time' in GUI) can all be changed by the user for optimal experimental settings (Figure 2). Note that the LED's are not connected to the Matlab system but are connected to an external power source, which can be controlled manually.

Programs for video analysis: Although the Ctrax software is excellent for tracking the movement of flies (Branson, Robie, Bender, Perona, & Dickinson, 2009), it was designed specifically to track fly walking. Thus, it makes two assumptions that may not be applicable to tracking climbing behavior. These assumptions (see below) will inevitably cause errors when analyzing the data. These errors, however, can be fixed manually or more easily automatically with a few custom Matlab programs.

Assumption 1: If a fly can be tracked, it should be tracked, regardless of position on the screen. Flies can demonstrate different climbing behaviors such as walking downward along the vial once they reach the top of the tubes and discover that they cannot escape. Therefore, climbing data for each fly is only collected until each fly crosses the 'finish line' or tracking height specified by the user (Figure 2(B)). The step where the user constructs the finish line for their set of flies is added after the region of interest selection step to further refine the individual fly paths.

Assumption 2: Every fly to be tracked will be viewable within every frame of the movie. In the movies generated by the HC, the flies start at the bottom of the tube where they cannot be tracked, and end up at the top of the tubes, where again they cannot be tracked. During the analysis portion of the data, a step where the user makes a region of interest selection around the tubes was added (Figure 2(C1,C2)). This allows the program to cut out any data points that are outside the selected regions of interest and furthermore deletes any extra data sets that may arise if the fly disappears briefly.

Custom-developed script: The HC works with Matlab and a custom-developed script. The code for the customer script can be found on www.github.com under 'amwmv4/HC_Code' in the search bar.

Fly stocks and genetics: We used two strains of flies as controls, *Canton-S* (*CS*) and w^{1118} in our climbing test and compared their performance with the loss of function *fragile-X* (*dfmr1*) mutant flies that we previously showed to be defective in climbing in an age-dependent manner (Martinez et al., 2007; Tauber et al., 2011). The mutant *dfmr1* was generated as *w*; $dfmr1^{83M}/dfmr1^{3}$ by crossing *w*; $dfmr1^{83M}$/TM6B, Tb with *w*; $dfmr1^{3}$/TM6C, Sb flies (Dockendorff et al., 2002; Wan et al., 2000; Zhang et al., 2001). All flies were cultured on a glucose-based cornmeal-agar food and raised at 25 °C. The flies (male) selected for the climbing test were kept and aged in an incubator at 25 °C under a 12 h/12 h light/dark cycle. Experimental flies were transferred to fresh food vials every 5 days and kept in the incubator during the aging process.

Statistics: Statistical analysis was performed with SPSS software. One-way ANOVA with a Tukey *post hoc* analysis was used on data for velocity and % flies to reach 50 mm in

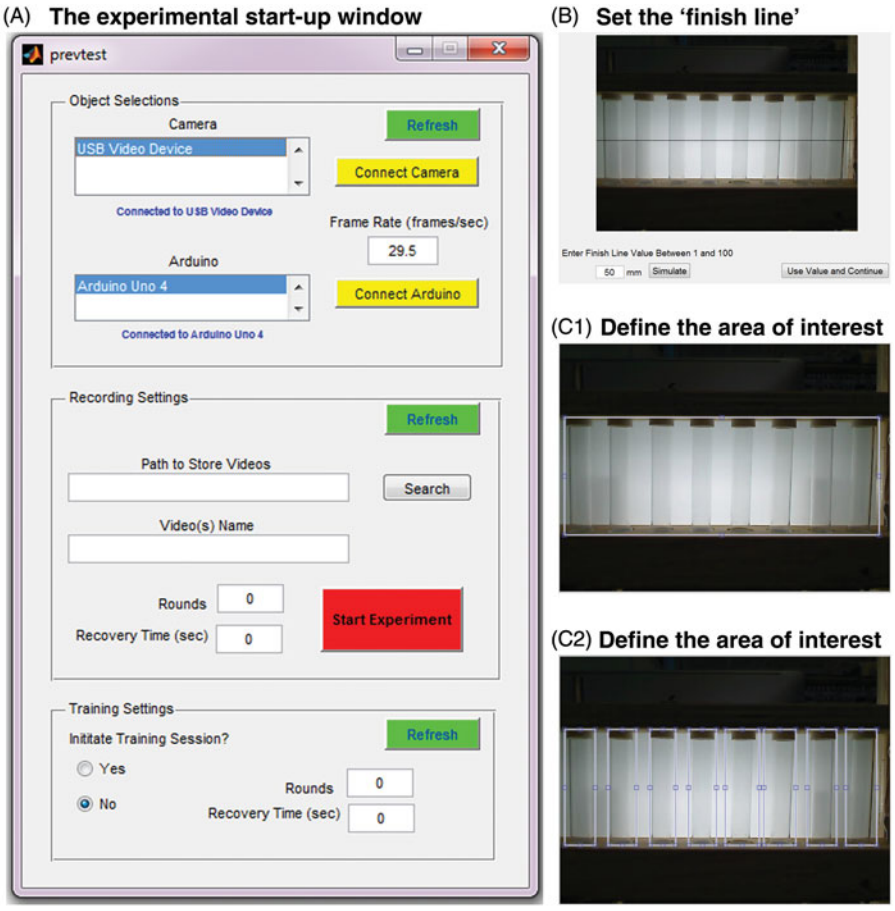

Figure 2. Key computer control panel and experimental steps. (A) The experimental start-up window allows one to start the experiment, save the video, and define the number of runs per experiment. (B) The user can determine the 'finish line' by typing in a specific number in the dialog box. (C) For fly tracking and data analysis the user will need to define the area of interest by drawing a large rectangular box around the glass vials (C1), which then automatically zoom in to individual vials (C2).

10 s data. Data for first fly time exhibited non-parametric qualities, thus a Welch One-Way ANOVA was used with a non-parametric Games–Howell *post hoc* analysis. All data are shown as mean ± SEM. Different levels of significance are noted as such: $^{*}p < .05$ (significant), $^{**}p < .01$ (very significant), $^{***}p < .001$ (extremely significant).

The experimental procedure: Age-matched male flies were placed individually into one glass vial (diameter 1.57 cm, height 10.3 cm) after briefly being anesthetized on ice and place in the vial holder. The flies were left in the vial to recover from the ice anesthesia for at least 5 min so that they were active and acclimated to the new environment. Flies were tested three times for climbing on the HC, videotaped, and analyzed using Matlab2014A-based software. A maximum of eight flies were used for each trial on the HC. Following the climbing test the flies were recovered by transferring them back to regular food vials and reused for later tests. The fly velocities were calculated by using the vertical linear distance over time, without considering the actual path along the curved vial.

Results

The design of the Hillary Climber

The HC system has three major components, the mechanical part, the electronic part, and the software. The key mechanical part is the motorized tapping mechanism that mimics the key action of human tapping of graduated cylinder in a climbing test. The motor-driven device that we have developed can lift up glass vials (secured in a holder) with a pair of cams and then suddenly drop the glass vials so that flies will fall to the bottom of the vial (Figure 1(A),(B)). Immediately following the fall of the holder flies began to climb up the glass vial and this climbing is recorded by a video camera placed in the front of the device. The motor is controlled by the Arduino (Figure 1(C),(C′)) via a PC computer to allow 'tapping' action to be repeated as many times as needed for the climbing test (usually three times). The diagram for the complete HC system is illustrated in Figure 1(D) whereas the wiring diagram is shown in Figure 1(E). Although the motor speed is set one can change the interval between runs.

Running an HC experiment

In an HC experiment the experimenter will first transfer the flies to the glass vials and then use a computer to run the experiment for data acquisition and analysis. In our experiments, we used ice anesthesia during the transfer of flies and thus avoided the complication by CO_2. A mouth-aspirator could also be used to transfer flies without using either CO_2 or ice. Some of the key steps of the computer operation are

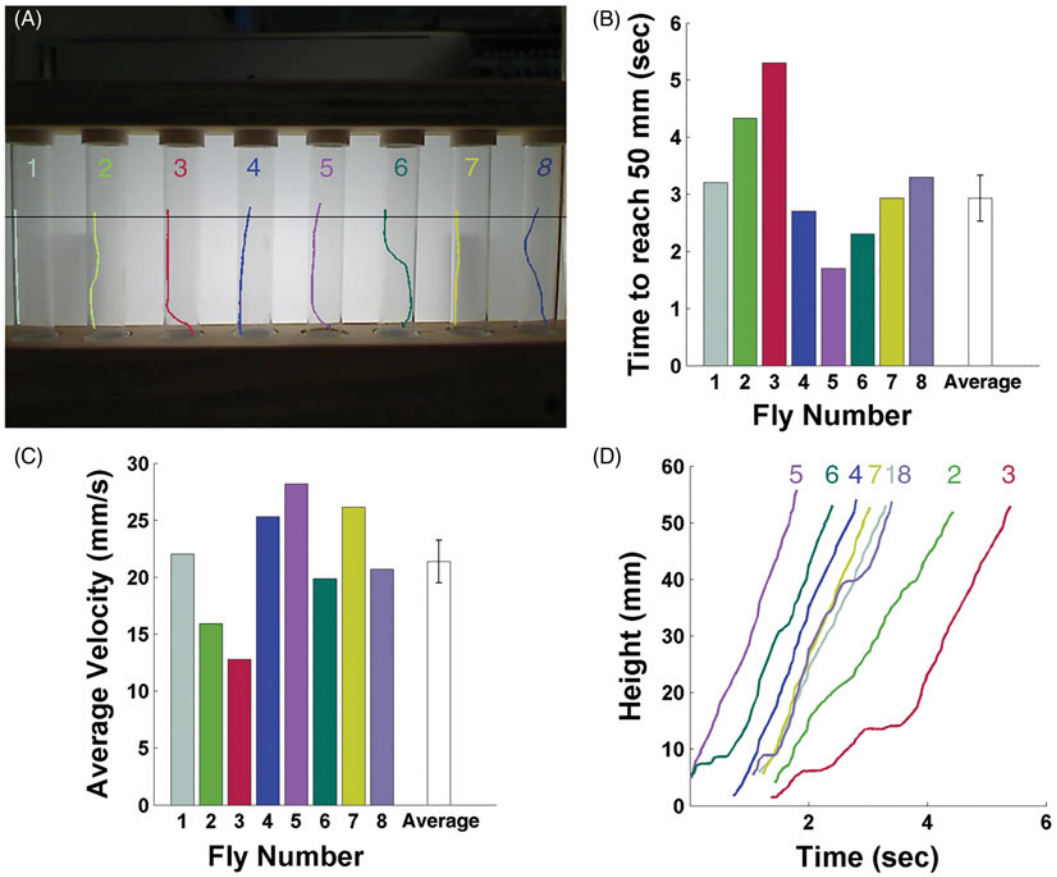

Figure 3. A Sample Graphical Output of One Climbing Trial. (A) Eight 5-Day old *CS* flies (male) were used in the climbing test, and their climbing paths are shown as colored lines along the glass vials. The finish line was set at 50 mm from the bottom of the vial. (B) This histogram shows the time taken for each fly to reach the 50 mm mark along the glass vial. The open bar column represents the average time taken for the population of eight flies to reach the 50 mm mark (with standard errors). (C) Histogram showing the climbing velocity of individual flies. The average velocity of the eight flies is shown in the open column, with standard errors. (D) The height vs. time plot shows the position of each fly along the glass vial over time recorded from the start of the experiment to 1 s after they have reached the 50 mm mark. Those flies that reached the finish line first are shown by lines on the left side whereas the slower flies are shown to the right side. The numbers in panel (A) match the fly numbers in panels (B-D).

illustrated in Figure 2. In the 'experimental start-up' window, under the 'Object Selections' dialog box, one will first connect the camera to the computer by clicking on the camera of one's choice (e.g. 'USB Video Device' in our case) and then connect to Arduino Uno ('Arduino Uno 4' is shown here as an example). In the 'Recording Settings' box, one will then determine how many rounds of test (repeats) will be performed by typing in a number in the 'Rounds' box and save the video with a specific name. The 'Training Settings' are optional, which gives one the option of doing some test runs first before commencement of the real test.

Once the experiment is completed, one can then upload the saved video and perform data analysis. First, one defines the 'finish line' by typing in a number (mm) between 1 and 100 (Figure 2(B)). This means that the software will only track and analyze flies climbing up the finish line and ignore all other information such as flies walking downward after reaching the top. The range itself will depend on the calibration setting and how many vials to track. To start tracking flies, one needs to draw a large rectangular box covering the vials of interest (Figure 2(C1)) and this will then automatically becomes smaller individual rectangular boxes representing 'areas of interest' surrounding each individual vial.

The function and performance of the Hillary Climber

We then tested the function of the HC using the wild type *CS* strain (5 days old, male) and showed that this simple device was useful and efficient for fly climbing tests. Coupled with Ctrax (Branson et al., 2009) and custom-developed scripts we were able to (a) track the pathway of fly climbing (Figure 3(A)), (b) calculate and plot the time that it takes for individual flies to reach a certain height (50 mm, Figure 3(B)), (c) the average climbing velocity (individual and group average, Figure 3(C), (d) the position of the flies at any given time (time vs. height plot, Figure 3(D)). As expected, each fly will take a different climbing path and climb at different velocity ranging from 12.8 mm/s to 28.2 mm/s, and an average of 21.4 ± 1.9 mm/s. The data shown in panel B and C also reveal the fastest and slowest climbers.

Application of the Hillary Climber for studying fragile-X flies

The ultimate goal of developing the HC is to aid behavioral studies of wild type and mutant flies, aging, models of neurological diseases or to screen for mutant flies with altered climbing behavior. Previously, we have shown that

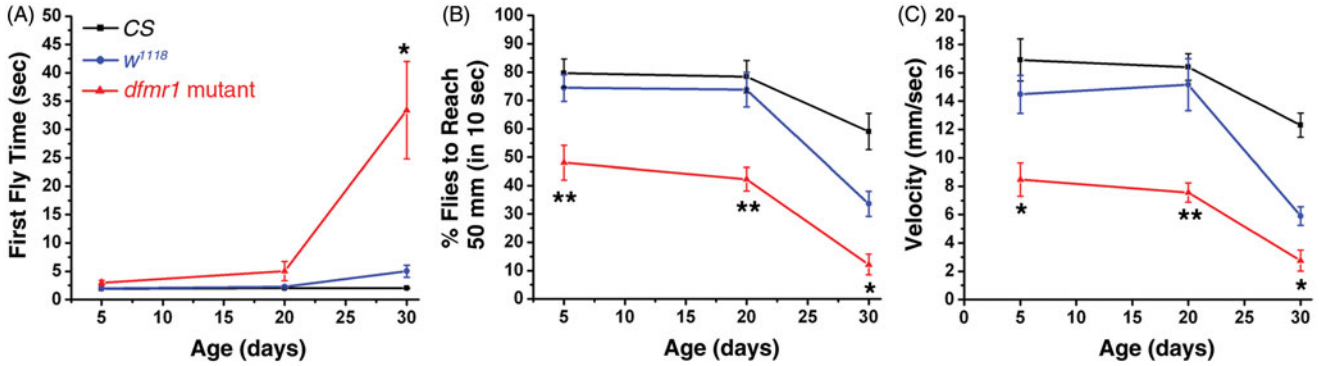

Figure 4. Comparison of climbing performance of control flies (*CS* and w^{1118}) and *dfmr1* mutant flies as they age. (A) The time taken by the first fly to reach the 55 mm mark is progressively increased for the for *dfmr1* mutant flies over a 30-day period in comparison to the control flies. Data are presented as the Mean ± SEM ($n = 5$–8 flies per trial, repeated three times, and a total of 40–50 flies). (B) The average percentage of flies to reach the 50 mm mark within a 10 s testing period is significantly lower for the *dfmr1* mutant flies compared to the control groups at all ages. (C) The average climbing velocity is also significantly lower for the *dfmr1* mutant flies compared to the control groups at all ages. Note that the climbing ability is weakened as flies age, even for the control groups. Moreover, w^{1118} mutant flies show a more rapid decline in climbing as they reach 30 days old compared to the wild-type *CS* strain. Statistically significant values are as follows: $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$.

the fly model of fragile-X syndrome, *dfmr1*, showed an age-dependent decline in climbing performance (Martinez et al., 2007; Tauber et al., 2011). Here, we used two control strains (*CS* and w^{1118}) and the *dfmr1* mutant to further test the HC. The results confirm previous findings (Figure 4), as *dfmr1* mutant flies show a dramatic decline in climbing ability over the 30 days period. The time it takes for the first fly to reach 50 mm is relatively similar to those of control groups at age 5 and age 20, averaging at 2–3 s. However, this time is much longer on day 30, with *dfmr1* flies taking 33 s compared to 2 s for *CS* and 5 s for w^{1118} flies at the same age. Consistent with previous findings, *dfmr1* mutant flies already show a poor climbing as a group at day 5, as only 48% of the population reached 50 mm in 10 s whereas nearly 74–80% of the control strains did (Figure 4(B)). As the flies aged, the percentage of flies that could reach 50 mm within 10 s was gradually reduced at day 20 but more dramatically reduced at day 30, when only 12% of the *dfmr1* mutant flies reached the finish line whereas the control groups were much better (59% for *CS* and 34% for w^{1118}). Not surprisingly, control strains also showed an age-dependent decline in climbing performance, presumably associated with normal aging (Gargano et al., 2005; Martinez et al., 2007; Rhodenizer et al., 2008). The velocity of fly climbing showed a similar trend as those for the graph showing the percentage of successful climbing (compare Figure 4(C) with Figure 4(B)). Notably, the *dfmr1* mutant flies climbed at 2.7 mm/s compared to the control strains (12.2 mm/s for *CS* and 5.9 mm/s for w^{1118}) at 30 days old.

Discussion

The HC is relatively economic to build, easy to operate, and efficient for collecting precise climbing data. Compared to the traditional manual climbing test, the HC has a number of advantages. First, the HC offers the ability to accurately measure climbing velocity and paths of individual flies as they make their assent of the vial. This ability offers personalized data for each fly as well as the group average. Second, the tapping force applied to the fly vials is consistent from trial to trial. Third, the experimenter does not have to be present after the experiment has commenced and one can test habituation and fatigue of fly climbing over repeated testing. Fourth, the experimenter can record fly climbing on a video and analyze it at a later time. Finally, one should be able to obtain a high degree of consistency through fewer trials.

Coupling videotaping with manual tapping has been used to study fly climbing and improve data acquisition and analysis (Gargano et al., 2005; Nichols et al., 2012). In this system group of flies are used and the analysis was performed using a custom-developed software. There is also an automated fly climbing system that enables mechanical tapping, videotaping, and data analysis using custom-made software (Podratz et al., 2013). The data analysis described by the authors was on the distribution of flies along the height of vials after tapping, but not climbing velocity or individual fly performance. Further, the motorized tapping system was not described to the detail level such that others can readily duplicate the system. We believe our HC device is complementary to the existing climbing systems in that HC records individual flies, follows their climbing paths, and analyzes the climbing performance.

One notable disadvantage of our HC system is that it is not for testing population of flies in one vial in the present design. This limitation, however, is not due to the mechanical setup, but rather the inability for the software to track multiple flies simultaneously. With development of new software, it is expect that the HC system will also be able to track and analyze more flies per vial if group test is needed. One may also note that in the present design the glass vial used in the HC system is shorter compared to a 250 ml graduated cylinder. Hence, the velocity it measures may not be suitable for direct comparison with the velocity obtained by the traditional manual method. For example, when we measured the velocity for the first (fastest) fly to reach the 17.5 cm mark on a 250 ml graduated cylinder, the velocity was 17.5 mm/s for the *CS* fly at 5 days old (Martinez et al., 2007). In the HC system the fastest fly climbed at 28 mm/s for the same genotype at the same age. Based on our observations, flies respond to the tapping force by first climbing rapidly and then slowing down the velocity further along the

climbing path. Hence, we speculate that the HC system captures the initial climbing phase and the velocity obtained from the HC may be more sensitive and valuable for separating minor differences between different genotypes. It is also expected that the arbitrary 'finish line' and duration (e.g. 10 s) could affect the velocity and percentage performance parameters. Alternatively, one could use longer glass vials in the HC system if the late phase of climbing is important for consideration (but it requires modification of the height of the frame). Another weakness is that the current system does not accurately measure the horizontal velocity when the fly climbs along the curvature of the vial. This will lead to underestimate of the real climbing velocity. One could increase the accuracy of velocity measurements by using a thinner and rectangular vial. Finally, the cushioning materials used and the drop height may influence the tapping force.

There are several other modifications that could be considered for improving the HC system. Our prototype of the HC was built primarily of wood. One future modification is to use aluminum and ABS plastic through 3-D printing. This would increase the ease of construction, as it would eliminate the necessity of having access to a machine shop and would increase durability of the HC. Furthermore, increasing the RPM of the motor would also be highly recommended, in part because in trials one fly may resist the tapping force by staying on the side of the tube. If a motor with a rotation speed greater than 60 RPM is used, it could easily be implemented to have the fly platform drop more than once per recording cycle, simulating more than one tapping applied to the flies, which more closely resembles the typical manual climbing test. Finally, the HC frame width can be expanded and the number of vials can be added to increase the capacity of the system so that more individual flies can be tested simultaneously or group of flies can be added to one vial for population testing.

Acknowledgements

This is an undergraduate honors research project and supported in part by the Life Sciences Undergraduate Research Opportunity Program (LS UROP) and Engineering Undergraduate Research Program at the University of Missouri (to AMW) and by a research grant from the National Science Foundation (NSF) (IOS-1354609 to BZ). We thank the members of the Zhang lab for discussions and support and the machine shop at the Bond Life Sciences Center at the University of Missouri for assisting us in the design and manufacture of the HC system. We also thank two anonymous reviewers for their helpful suggestions that have helped us improve the figures and text. This manuscript is part of the special issue honoring the lifetime achievements of Dr. Barry Ganetzky who recently retired from the University of Wisconsin-Madison. It is fitting to note that Barry was among the first persons to perform the fly climbing test (Ganetzky & Flanagan, 1978), and the method he developed was adopted later by many others, including the Zhang lab. BZ wishes to thank Barry for his mentorship, support, sense of humor, and friendship.

Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Funding

NSF [IOS-1354609].

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