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TITLE:

An Automated Method to Determine the Performance of *Drosophila* in Response to Temperature Changes in Space and Time

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KEYWORDS:

Temperature-controlled arena, locomotor behavior, *Drosophila*, temperature performance, automatic heating mechanism, positional tracking

SUMMARY:

Here we present a protocol to automatically determine the locomotor performance of *Drosophila* at changing temperatures using a programmable temperature-controlled arena that produces fast and accurate temperature changes in time and space.

ABSTRACT:

Temperature is a ubiquitous environmental factor that affects how species distribute and behave. Different species of *Drosophila* fruit flies have specific responses to changing temperatures according to their physiological tolerance and adaptability. *Drosophila* flies also possess a temperature sensing system that has become fundamental to understanding the neural basis of temperature processing in ectotherms. We present here a temperature-controlled arena that permits fast and precise temperature changes with temporal and spatial control to explore the response of individual flies to changing temperatures. Individual flies are placed in the arena and exposed to pre-programmed temperature challenges, such as uniform gradual increases in temperature to determine reaction norms or spatially distributed temperatures at the same time to determine preferences. Individuals are

automatically tracked, allowing the quantification of speed or location preference. This method can be used to rapidly quantify the response over a large range of temperatures to determine temperature performance curves in *Drosophila* or other insects of similar size. In addition, it can be used for genetic studies to quantify temperature preferences and reactions of mutants or wild-type flies. This method can help uncover the basis of thermal speciation and adaptation, as well as the neural mechanisms behind temperature processing.

INTRODUCTION:

Temperature is a constant environmental factor that affects how organisms function and behave[1]. Differences in latitude and altitude lead to differences in the type of climates organism are exposed to, which results in evolutionary selection for their responses to temperature[2,3]. Organisms respond to different temperatures through morphological, physiological, and behavioral adaptations that maximize performance under their particular environments[4]. For instance, in the fruit fly *Drosophila melanogaster*, populations from different regions have different temperature preferences, body sizes, developmental times, longevity, fecundity, and walking performance at different temperatures[2,5–7]. The diversity observed between flies of different origins is explained in part by genetic variation and plastic gene expression[8,9]. Similarly, *Drosophila* species from different areas distribute differently among temperature gradients and show differences in resistance to extreme heat and cold tests[10–12].

Drosophila has also recently become the model of choice to understand the genetic and neural basis of temperature perception[13–17]. Broadly, adult flies perceive temperature through cold and hot peripheral temperature sensors in the antennae and through temperature sensors in the brain[13–20]. The periphery receptors for hot temperatures express *Gr28b.d*[16] or *Pyrexia*[21], while the periphery cold receptors are characterized by *Brivido*[14]. In the brain, temperature is processed by neurons expressing *TrpA1*[15]. Behavioral studies on mutants of these pathways are improving our understanding of how temperature is processed and give insights into mechanisms that vary among populations of *Drosophila* from different regions.

Here we describe a temperature-controlled arena that produces fast and precise temperature changes. Investigators can pre-program these changes, which allows for standardized and repeatable temperature manipulations without human intervention. Flies are recorded and tracked with specialized software to determine their position and speed at different phases of an experiment. The main measurement presented in this protocol is the walking speed at different temperatures, because it is an ecologically relevant index of physiological performance that can identify individual thermal adaptability[5]. Together with temperature receptor mutants, this technique can help reveal the mechanisms of thermal adaptation at cellular and biochemical levels.

PROTOCOL:

1. Preparation of Fly Food Medium

1.1. Pour 1 L of tap water into a 2 L glass beaker and add a magnetic stir bar. Put the beaker on a magnetic hot plate at 300 °C until boiling temperature is reached.

1.2. Stir at 500 rounds/min and add the following: 10 g of agar, 30 g of glucose, 15 g of sucrose, 15 g of cornmeal, 10 g of wheat germ, 10 g of soy flour, 30 g of molasses, and 35 g of active dry yeast.

1.3. When the mix foams vigorously, turn down the hot plate temperature to 120 °C while continuing stirring.

1.4. Turn the hot plate temperature further down to 30 °C after 10 min and continue stirring until the mix cools to 48 °C. Measure the temperature by inserting a thermometer directly into the food without touching the walls of the beaker.

1.5. Dissolve 2 g of p-hydroxy-benzoic acid methyl ester into 10 mL of 96% ethanol and add it to the mix, together with 5 mL of 1 M propionic acid. Continue stirring for 3 min.

1.6. Turn the hot plate off and pour 45 mL of food into the rearing bottles and 6.5 mL of food into the collection vials.

2. Preparation of Flies

2.1. Place 20 male and 20 female flies in the rearing bottles containing 45 mL of fly food medium. Transfer the flies to new bottles after 3 to 4 days by tapping them down and then tapping them into the fresh bottles. Discard the flies after three changes.

2.1.1. Place the bottles inside the incubator under 12-h light/12-h dark cycles with a constant temperature of 25 °C.

Note: A new generation of flies will eclose after three changes.

2.2 Anesthetize newly eclosed flies on carbon dioxide pads for a maximum of 4 min and collect them in 2.5 x 9.5 cm fly rearing vials with 6.5 mL of fly food medium using a paintbrush.

2.2.1 Collect only virgin flies and separate them by sex into groups of 20 flies per rearing vial.

2.2.2 Place the vials inside incubators for 5-7 days, changing the flies to new vials every 2-3 days and on the days before experiments.

3. Frame of Lights

3.1. Make a wooden frame of 10 cm length, 4 cm width, 4 cm height, and 0.5 cm thick.

3.2. On each of the short edges, create a border of 4 cm length, 4 cm height, and 1.5 cm width towards the inside area of the wooden frame. Leave the internal face of the border open.

3.3. Drill two holes of 0.5 cm diameter at the intersection of one of the long edges of the wooden frame and at each of the borders at the short edges.

3.4. Place 10 cm of a warm white LED strip inside each of the borders on the short edges. Peel the back of the LED strip to immediately glue it in place.

Note: For experiments in which illumination needs to be eliminated, the warm white LED strip can be substituted for infrared LED strips.

3.5. Connect one end of the LED strip in one of the borders to the switching power supply and its other end to the LED strip on the opposite border.

3.6. Turn the switching power supply on to verify that both LED strips turn on.

3.7. Cover the open side of each border with a white piece of paper.

3.8. Glue another piece of paper to each of the internal phases of the long edges.

4. Temperature-Controlled Arena

4.1. Turn on the temperature-controlled arena (**Figures 1A** and **1C**). Ensure that the fan starts running and the aluminum ring starts warming up.

4.2. Use a USB cable to connect the temperature-controlled arena to the control computer running the *TemperaturePhases* script with the temperature sequences.

4.3. Open the *TemperaturePhases* script in the control computer and verify that the temperature sequence is properly set up (**Video 1**).

4.3.1. Check that the duration of each experimental phase is set to 60 s by verifying that "par.StimulusDur" is equal to 60 s.

4.3.2. Check that the 1) number equal to 23 phases, 2) iterative ON/OFF set-up of the indicative red light emitting diodes (LEDs), 3) 2 °C temperature increase per phase, and 4) 16 °C as the starting temperature are all correct under the "Start the experimental block" section.

Note: Allow the flies to acclimate to the Fly Arena for 7 min at 16 °C to avoid an artificial increase of speed during the first experimental phases (**Figure 2**).

4.3.3 Run the *TemperaturePhases* script. The software will initialize for 5 seconds as determined in "arena.Wait", then stop press the keyboard key to continue.

4.3.4 Press the spacebar of the keyboard to begin running the experimental phases once a fly has been blown into the Fly Arena (step 5.3).

Note: The *TemperaturePhases* is the current script controlling the box; however, it is possible to create other custom scripts to use this device that adjust to the requirements of different experiments.

4.4. Connect the camera on top of the arena to the recording computer using the camera's USB cable.

4.5. Open the video recording program (see **Table of Materials**) in the recording computer by selecting “File | New Movie Recording”. A screen showing the image from the camera will open.

4.5.1 Ensure that the camera image captures all edges of the arena and the indicative red LEDs.

4.5.2 Start recording by pressing the red button in the middle of the screen’s bottom edge showing the camera image once the frame of lights is set around the arena (step 5.4).

Note: Small changes in lighting can affect accuracy of the tracking. It is recommended to keep the illumination of the temperature-controlled arena constant by fixing the location of the apparatus.

5. Temperature Behavioral Experiments

5.1. Prepare the Fly Arena (**Figure 1C**).

5.1.1 Place a strand of white conductive tape on the top of the copper tiles, ensuring all edges are covered.

5.1.2. Place the heated aluminum ring around the copper tiles. The edge of the ring fits perfectly around the copper tiles so it is always placed in the same location.

5.1.3. Clean the glass cover with a clean tissue and place it on the top of the aluminum ring, leaving a gap through which a fly can be blown in.

Note: Before the experiments, coat the glass cover with the siliconizing agent to create a slippery surface. Apply the siliconizing agent for 24 h and rinse it with water before use.

5.2. Run the *TemperaturePhases* script (step 4.3.3) and open the video recording program (step 4.5).

5.3. Blow the fly from a rearing vial (step 2.2.2) into the Fly Arena (*e.g.*, 1 male fly in **Figure 3**).

5.3.1 Take a vial of flies from the incubator, tap it twice to force them to go to the bottom, trap one fly with a mouth aspirator, and close the vial and put it back into the incubator.

5.3.2. Place the fly in the arena through the gap that has been left between the glass cover and aluminum ring (step 5.1.3).

5.3.3. Close the gap between the glass cover and aluminum ring by pushing the glass cover until it reaches the edge of the aluminum ring as soon as the fly is introduced to the Fly Arena.

5.4. Place the frame of lights around the arena to ensure symmetric illumination.

5.4.1. Mark the location (*e.g.*, using a permanent marker) of the frame of lights around the Fly Arena (**Figure 1C**) to ensure that the frame is always placed in the same location.

5.5. Start recording with the video recording program (step 4.5.2) and press the spacebar on the keyboard of the control computer to begin running the experimental phases (step 4.3.4).

5.6 After all experimental phases are done, save the video in .mp4 or .avi format and remove the fly from the Fly Arena with the mouth aspirator.

Note: The end of the experimental phases can be determined by both indicative red LEDs being turned off or by the *TemperaturePhases* script stopping.

5.6.1 Stop the video recording by pressing the stop button in the middle of the screen's bottom edge in the recording program. Press "File | Save us" to save the video.

6. Video Tracking and Data Analysis

6.1. Use the *FlySteps* tracking software (**Video 2**) to track the videos.

6.1.1. Open the "configuration_file.ini" inside the "FlyTracker" folder.

6.1.2. Set the location of the videos in "video_folder" and the names of the videos in "video_files".

6.1.3. Specify the borders of the Fly Arena in "arena_settings" based on (x, y) pixel coordinates of multiple points at the edge of the arena.

6.1.4 Specify the location of the indicative red LEDs in "led_settings" based on (x, y) pixel coordinates of the location of the center of the LEDs.

6.1.5 Check the location of the borders of the Fly Arena by setting "debug" to "true" in "arena_settings", clicking "Save", and running the script in the terminal. A screen capture of the video will appear with a blue square formed by the coordinates inputted in "arena_settings".

Note: This square surround the area to be tracked.

6.1.6 Change "debug" in "arena_settings" to "false", click "Save", and run the screen in the terminal once more.

Note: This will start the tracking process.

Note: Flies can walk out of the tracking area onto the heated aluminum ring. This happens during the first seconds of an experiment, after which flies stop touching the heated ring and remain inside the tracking area.

Note: Videos can be tracked with other tracking software according to the experimenter's preferences.

6.2. Use the (x,y) location of each fly provided by the tracking software to calculate the measure of interest for the temperature performance. Custom scripts (*e.g.*, *FlyStepsAnalysis* in **Supplementary**) can be used.

6.3. Compare the temperature performance curves of different fly groups using repeated measurements (RM) analysis of variance (ANOVA) and *post-hoc* multiple comparisons using statistical software (see **Table of Materials**).

REPRESENTATIVE RESULTS:

The temperature-controlled arena (**Figure 1A**) consists of three copper tiles whose temperature can be individually controlled through a programmable circuit. Each copper tile possesses a temperature sensor that gives feedback to the programmable circuit. The circuit activates a power supply to increase the temperature of each tile. Passive thermoelectric elements act as constant heating elements to maintain the desired temperature, while a heat sink cooled by a fan provides constant cooling. The magnitude of temperature change determines the speed of the process in a non-linear manner. An increase of 2 °C requires only 0.1 s, and an increase of 18 °C requires 4 s. A screen connected to the programmable circuit (**Figure 1C**) informs the user of the temperature measured by the temperature sensors in each of the tiles. The copper tiles are surrounded by an aluminum ring constantly heated to 50 °C (**Figures 1B** and **1C**) by semiconductors around the periphery. This ring forms the edges of the Fly Arena (**Figure 1C**), the area in which flies are to be placed. The Fly Arena is covered by a siliconized glass cover (**Figures 1A** and **1C**), which provides a 3 mm high space which ensures that flies can walk but not fly. Next to the Fly Arena are two red LEDs (**Figure 1C**) that can be programmed to mark different experimental phases. For example, for the results shown in **Figure 2A**, each LED is associated with a different temperature, while in **Figure 2B**, each LED indicates 60 s. The *FlySteps* software can register when each of the indicative LEDs is on, and the researcher can then use this information to automatically determine the experimental phases based on temperature or time.

The temperature-controlled arena can be used to compare the behavioral response of flies from different genetic backgrounds to dynamic temperature changes. For example, flies from different species can be exposed to gradually increasing temperatures (**Figure 3**) to compare differences in thermal performance. The speed of all species increases as temperature increases until reaching a point of maximum performance, after which it decayed and perished. However, each species has a particular response curve with specific maximum response speeds and thermal tolerances. Previous reports have shown that *Drosophila* from different species differ among developmental timing, longevity, fecundity, body dimensions, sexual communication, and temperature tolerance[3,6-8,22]. Thus, our description of species-specific locomotion in a temperature gradient adds to this body of work.

The temperature-controlled arena can also be used to explore the response to conditioning experiments based on temperature. The simplest form of this approach is an operant conditioning paradigm in which flies are trained to prefer one side of the arena over the other,

by warming up the side that will be avoided[23-25]. We exposed individual flies to 40 °C in the middle and one of the side tiles, while leaving the other side tile at a comfortable 22 °C (**Figure 4**). Wild-type flies quickly stopped moving along the arena and remained in the comfortable location. In contrast, the classic memory mutant *Dunce* kept exploring the arena and spent less time than controls in the comfortable location. The differences between performance of the wild-type flies and *Dunce* mutants became larger when all tiles were set to 22 °C and comparisons were made between the treatment groups. *Dunce* mutants also showed greater differences between training and test phases in comparison to the wild-type flies (**Figure 4**). These results suggest an effect of memory on remaining in the comfortable location.

Combinations of temperature and location are also useful to understand the function of different temperature receptors during dynamic temperature changes. We exposed individual *D. melanogaster Gr28b.d* and *TrpA1^{GAL4}* mutants to increasing temperatures (2 °C increase every 60 s) while providing a comfortable location at 22 °C (**Figure 5**). The comfortable location shifted from left to right, and vice versa, per iteration. Results show that the periphery temperature receptor *Gr28b.d* mutants behave as the control, as they spend more time in the comfortable location as temperature increases. However, brain temperature receptor *TrpA1^{GAL4}* mutants are not affected by increasing temperatures and do not change their locations in the arena. The increases and decrease in the curve of *TrpA1^{GAL4}* mutants show the effect in flies that were already sitting in the comfortable location before it became comfortable and remained there during that phase. The consistency of peaks and valleys of the curve of *TrpA1^{GAL4}* suggest that these flies remained still for most of the experiment; hence, they were constantly counted when their location was the one considered comfortable. This conclusion was confirmed by visual inspection of the recorded videos. These results support previous physiological reports suggesting that periphery perception of fast and large changes does not depend on *Gr28b.d*[17] and that flies possess a main central mechanism to sense temperature based on *TrpA1*[14,21].

FIGURE LEGENDS:

Figure 1: Diagram of temperature controlled-arena. (A) A lateral view of the temperature-controlled arena. A programmable circuit connects a power supply and temperature sensors to heating elements under copper tiles to control their temperature. Tiles are constantly cooled down through a heat sink connected to a fan. A heated aluminum ring over which a glass cover rests surrounds the tiles. (B) Thermal imaging showing the tiles set at 24 °C (top) and side tiles at 24 °C with a middle tile at 30 °C (bottom). (C) A top view of the arena. A camera records the copper tiles, aluminum ring, and red LEDs, then automatically determines experimental phases. A screen in the corner of the box, not recorded by the camera, displays the current tile temperature. (D) Ring of light: two warm white LED strips inside a wooden box covered in white paper ensure constant and symmetric illumination of the whole arena.

Figure 2: Flies must acclimate to the arena before starting the temperature protocol. (A) Single male flies were introduced to the arena and allowed to explore at a constant 16 °C for 1 min, after which the temperature started increasing. (B) Single flies exposed to 16 °C, 20 °C, or 24 °C (no group differences; two-way ANOVA $F(2,570) = 4.156$, $p = 0.162$) have a higher locomotion at the beginning of the experiment than after 5 min (two-way RM ANOVA $F(9,570) = 7.803$, $p < 0.0001$). Data are mean and standard error of the mean (± SEM) of 20

virgin female flies 5 to 7 days old tested over multiple days. Asterisk indicates significant difference among groups (****p < 0.0001; Tukey's multiple comparison test, p = 0.05).

Figure 3: Locomotion of 5 *Drosophila* species exposed to gradually increasing temperatures. Individual male flies from temperate (blue), tropical (red), and cosmopolitan (brown) *Drosophila* species were exposed to an increasing temperature gradient (2 °C every 60 s) between 16 and 46 °C. The first 7 min were constantly at 22 °C to allow flies to explore the arena. Species were significantly different (two-way RM ANOVA $F(4,70) = 28.46$, $p < 0.001$). (a) *D. melanogaster* (brown; filled circles) was faster when introduced to the arena. (b) *D. yakuba* (red; empty squares) was faster as temperature increased. (c) *D. suzukii* (brown; filled square) was slower than the other cosmopolitan flies at its maximum performance point. (d) *D. simulans* (brown; empty circles) was in decay at the maximum point of *D. melanogaster.* Each point represents the mean (± SEM) of 15 male flies 5 to 7 days old tested over several days. Significance indicated by symbols (◆ = difference from all, $p < 0.0001$; † = difference from all except *D. melanogaster,* $p < 0.0001$; • = difference from *D. melanogaster,* $p < 0.01$; ¢ = difference from *D. melanogaster,* $p < 0.001$; **** = difference between named groups, $p < 0.0001$; Tukey's multiple comparison test, $p = 0.05$).

Figure 4: The temperature-controlled arena can be used for operant conditioning. *D. melanogaster* Canton-S strain (wild-type; black border) and *dnc*^{1} (*Dunce*; red border) mutants were trained to prefer a lateral tile at 22 °C after warming the middle and opposite lateral tiles to 40 °C for 4 min (training, no pattern). Memory of the heated areas is then tested by setting all tiles to 22 °C (test; grid pattern). Flies were conditioned to prefer tiles on the left in half of the experiments, then tiles on the right in the other half. The percentage of total time inside the tile at 22 °C during training and testing was measured to compare performances. Groups were significantly different (one-way ANOVA $F(3,76) = 23.23$, $p < 0.0001$), with *Dunce* performing worse than wild-type overall. Data are mean (± SEM) of 20 virgin female flies 5 to 7 days old tested over several days. Asterisks indicate significance difference among groups (****p > 0.0001; ***p > 0.001; **p > 0.01; Tukey's multiple comparison test, p = 0.05)

Figure 5: Response of temperature mutants to increasing temperature when a comfortable location is provided. Temperature mutants *Gr28b.d* (green; squares) respond as controls (*w*^{1118}, black; circles) by increasing the percentage of time in the comfortable area as temperature increases (two-way RM ANOVA $F(1,38) = 0.5107$, $p = 0.479$). *TrpA1*^{GAL4} mutants (yellow; triangles) are different from controls (*w*^{1118}, black), as they do not increase the time in the comfortable area as temperature increases (two-way RM ANOVA $F(1,38) = 1.670$, $p = 0.019$). Data are mean (± SEM) of 20 male flies 5 to 7 days old tested over several days. *TrpA1*^{GAL4} is significantly different from *Gr28b.d* and the control ($p < 0.05$; Tukey's multiple comparison test, $p = 0.05$).

DISCUSSION:

Here we have presented an automated temperature-controlled arena (**Figure 1**) that produces precise temperature changes in time and space. This method allows exposure of individual *Drosophila* not only to pre-programmed gradual increases of temperature (**Figures 2** and **3**), but also to dynamic temperature challenges in which each tile of the fly arena was heated independently to a different temperature (**Figures 4** and **5**).

The temperature-controlled arena uses an innovative approach to the heating process. Instead of producing temperature changes in the tiles through thermoelectric Peltier heating elements used in traditional methods, the temperature-controlled arena uses current to warm up a copper mass with the copper tiles, and flies are placed at the top. The copper mass is constantly cooled down by a heat sink block connected to a fan. Peltier-like elements are used to maintain the desired temperature of the copper mass once it has been warmed up. Because these elements are not the main temperature generators, they suffer less stress, which extends their life span and permits faster temperature changes. A programmable circuit that receives feedback from temperature sensors under each of the copper tile, which can also activate the low voltage power supply, coordinates the heating mechanism. Researchers can specify when and where temperature changes occur and determine the intensity and direction of such changes. Furthermore, coupling the method with specialized tracking software, such as *FlySteps*, permits analysis of all aspects relating to *Drosophila's* movement, such as the overall speed at certain temperatures or time spent in certain locations (**Figures 2-5**). Nevertheless, all results must consider characteristics inherent to fly behavior that might affect their locomotion. For example, if flies are not allowed to explore the arena and settle before changing the temperature, speed measurements might be artificially high (**Figure 2**). Flies can also leave odorants that affect subsequent flies; hence, the glass cover must be cleaned, and tape covering the tiles must be changed between subjects. Given that locomotion declines as flies age[26], it is important that flies are standardized for age to avoid variation in results. In our arena, flies have also shown centrophobism, preferring edges over the middle area. Experimenters must control for this by changing the location of comfortable areas to prevent overestimating site preference.

The current characteristics of the arena and requirements of the tracking process could limit some experimental procedures. For example, the close environment of the arena does not include access points through which odours could be introduced, which prevents studies in which this stimulus is important. Similarly, the *FlyStepts* tracker necessitates videos with uniform backgrounds, which limits the possibility of adding food or other items to the fly's environment. The arena could be adapted to include a connection to a gas valve, and software developments exist that may allow for more objects to be present. Future projects may take advantage of these possibilities to adapt the temperature-controlled arena to specific experimental needs.

Finally, we have shown in the results that different species of *Drosophila* perform differently as temperature increases (**Figure 3**) and that temperature mutants do not respond in the same way as controls (**Figure 5**). This shows that this new method may be used to explore *Drosophila's* thermal behavior and how it is affected by natural selection and functional characteristics. Finally, it illustrates that our method may help further understanding of thermal adaptation and speciation as well as the interactions of temperature receptors with other stimuli in future studies.

DISCLOSURES:

The authors declare that they have no competing financial interests.

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Scripts *TemperaturePhases, FlySteps,* and *FlyStepAnalysis* can be found as supplementary information and in the following temporary and publicly available link:
https://dataverse.nl/privateurl.xhtml?token=c70159ad-4d92-443d-8946-974140d2cb78

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Figure 1

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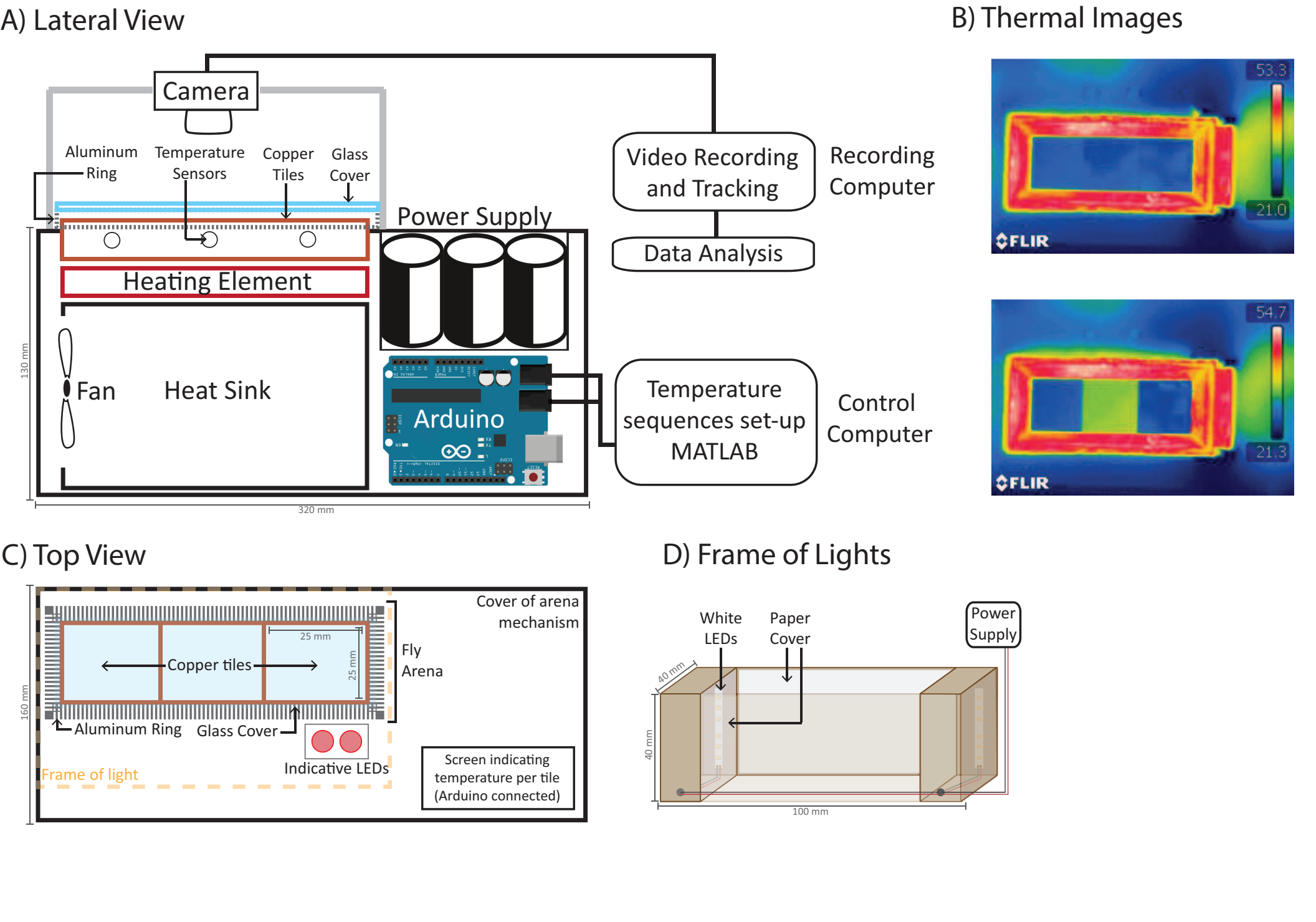

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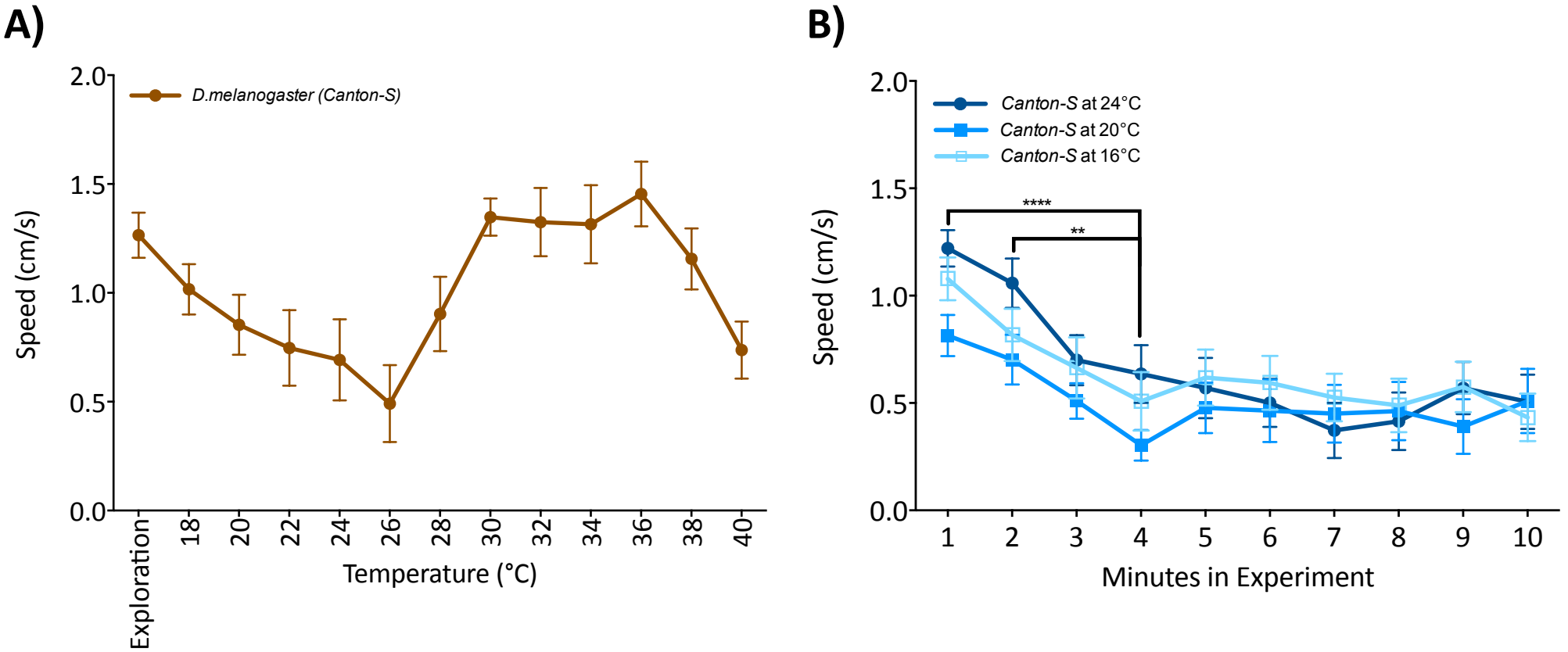

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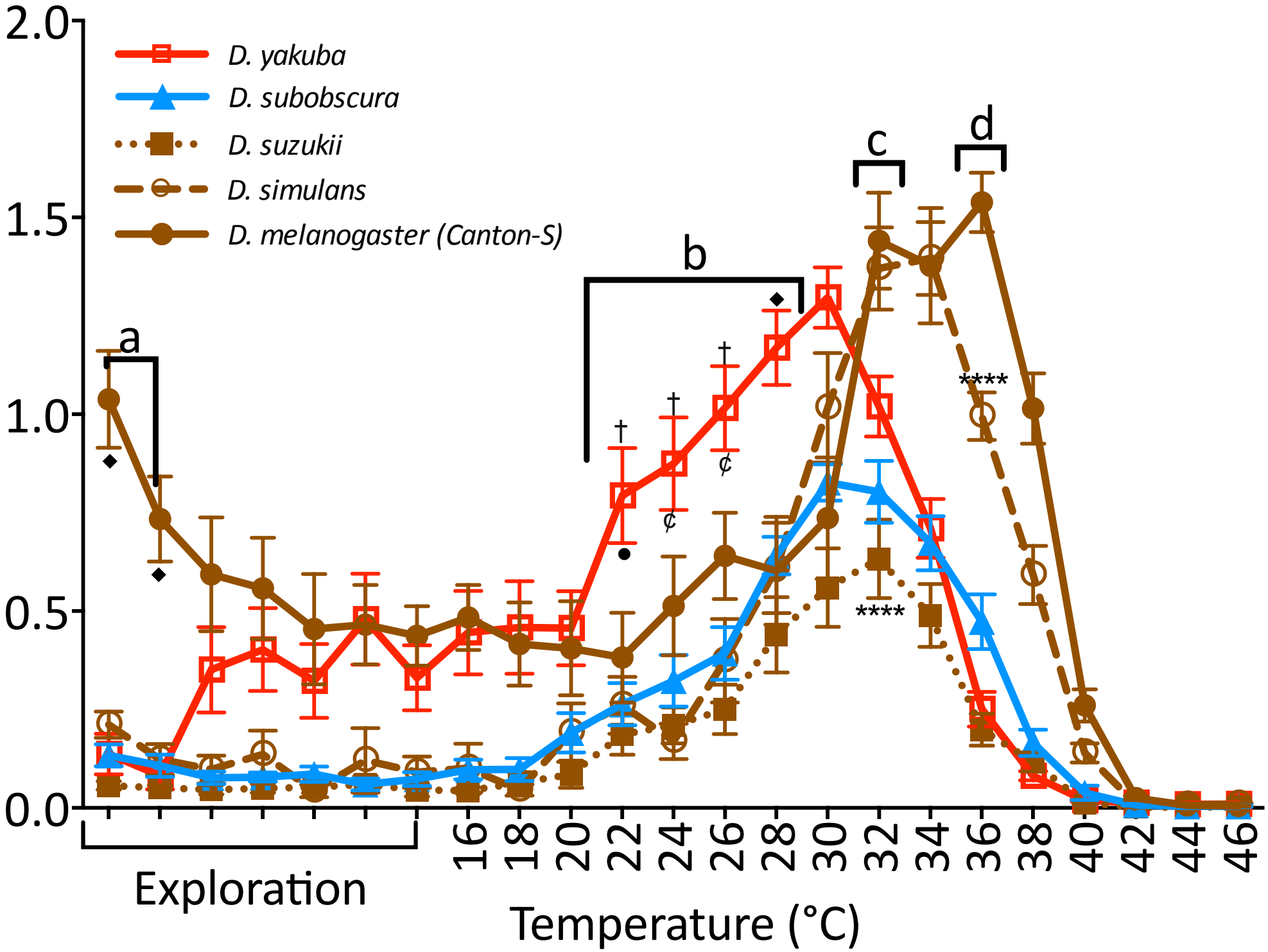

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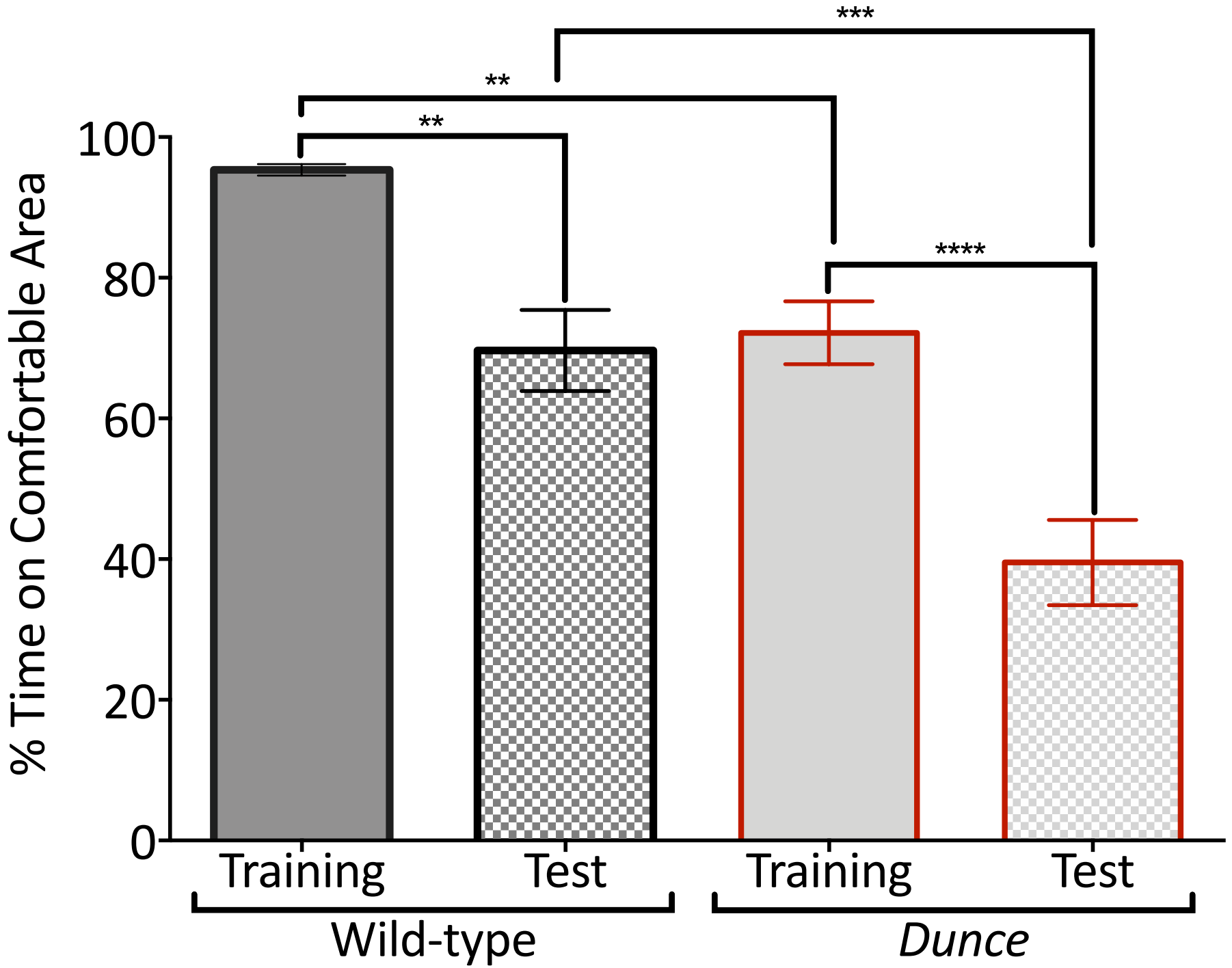

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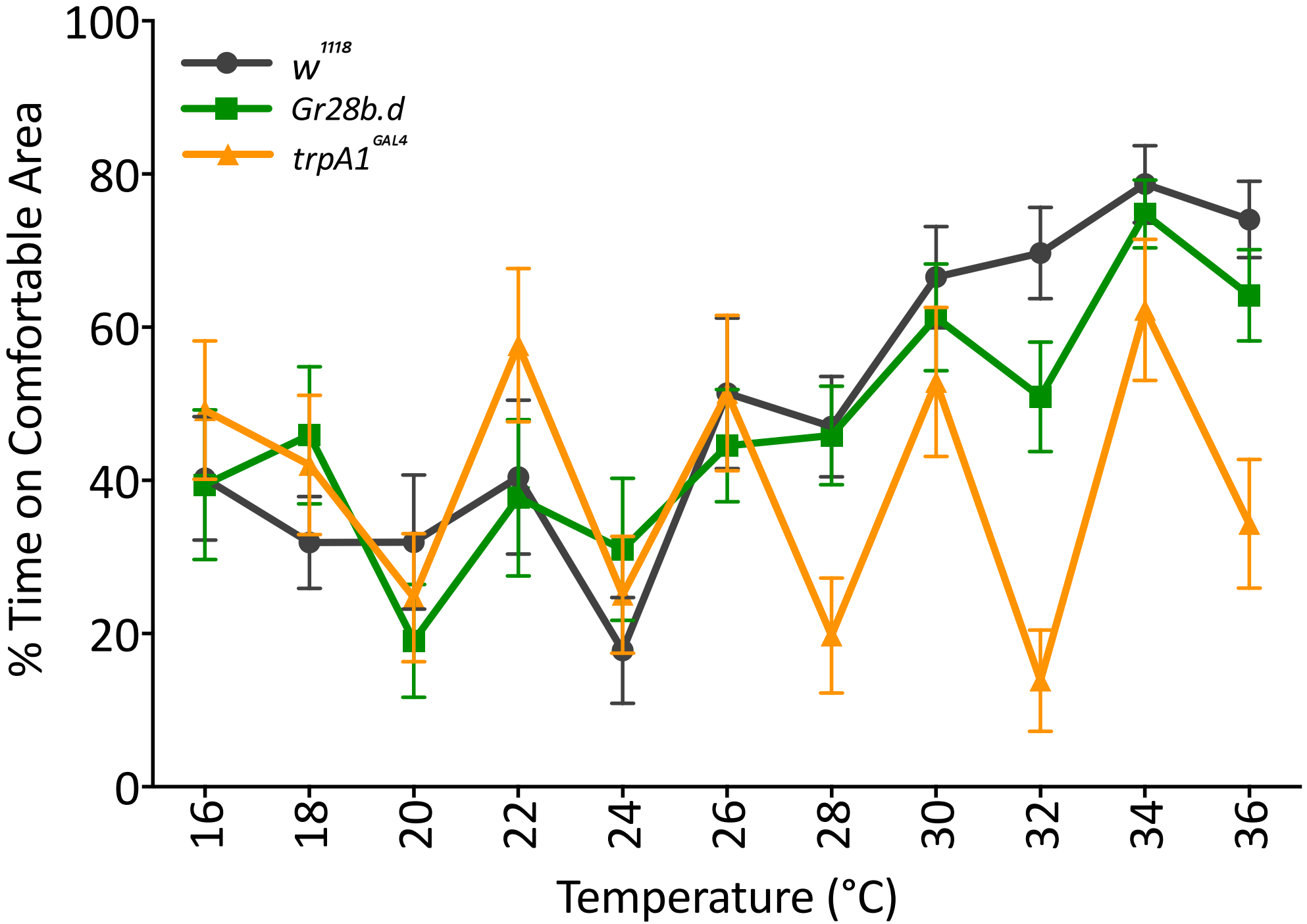

Video 1 describing step 4.3

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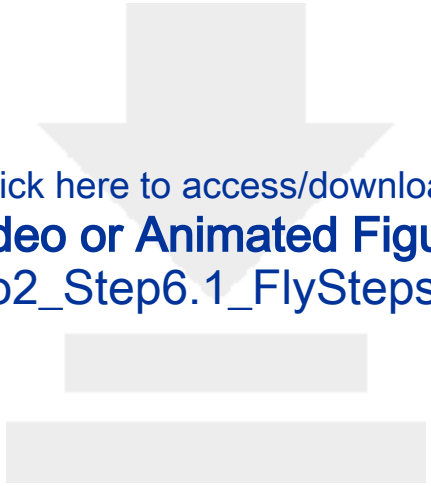
Video or Animated Figure

Video1_Step4.3_TemperaturePhases.mov

Video 2 describing step 6.1



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Video or Animated Figure

Video2_Step6.1_FlySteps.mov

Table of Materials

Click here to download Table of Materials List_materials.xlsx

Name of Material/Equipment	Company
Arduino Due	Arduino
Electronics Board	Ruijsink Dynamic Engineering
Power supply Boost	XP-Power 48. V 65 W
Power supply Tile Heating	XP-Power 15. V 80 W
Power supply Cooling	XP-Power 15. V 130 W
Peltier elements	Marlow Industries
Heat sink	Fisher Technik
Temperature sensors	Measurement Specialties
Copper block/tiles	Ruijsink Dynamic Engineering
Auminum ring	Ruijsink Dynamic Engineering
Tesa 4104 white tape 25 x 66 mm	RS Components
Red LEDs	Lucky Ligt
Warm white LED strip	Ledstripkoning
Switch Power Supply	Generic
Logitech c920	Logitech Europe S.A
QuickTime Player	Apple Computer
Tracking analysis software	R
Tracking analysis software	MATLAB
Thermal Imaging	FLIR T400sc
Graphs and Statisticts Software	Graph Pad Prism
Sigmacote	Sigma-Aldrich
Fly rearing bottles	Flystuff
Flypad	Flystuff
Fly rearing vials	Dominique Dutscher
Incubator	Sanyo
Magnetic hot plate	Heidolph
Agar	Caldic Ingredients B.V.
Glucose	Gezond&wel
Sucrose	Van Gilse
Cornmeal	Flystuff
Wheat germ	Gezond&wel
Soy flour	Flystuff
Molasses	Flystuff
Active dry yeast	Red Star
Tegosept	Flystuff

Catalog Number		Comments/Description
A000062		Software RUG
FF-Main-02-2014		
ECS65US48		Set to 53 Volt
VFT80US15		
ECS130U515		
RC12-4		2 Elements, controlled DC feed
LA 9/150-230V		Decoupled for vibration
MCD_10K3MCD1		Micro Thermistor Probe
FF-CB-01-2014		
FF-RoF-02-2015		
111-2300		White conductive tape
ll-583vc2c-v1-4da		Wavelength between 625 nm, 20 mAmp and 6 V
HQ-3528-SMD		60 LEDs per meter
T-36-12		
PN960-001055		
		Recording program
		Packages: pacman
SL2-100ML		Siliconising agent
32-130		6oz Drosophila stock bottle
59-114		
	789008	Drosophila tubes narrow 25x95 mm
MIR-154		
505-20000-00		MR Hei-Standard
010001.26.0		
	1019155	Dextrose/Druivensuiker
		Granulated sugar
62-100		
	1017683	
62-115		
62-117		
20-258		100%

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Title of Article: An automated method to determine the performance of Drosophila in response to temperature changes in space and time

Author(s): Andrea Soto-Padilla, Rick Ruijsink, Mark Span, Hedderik van Rijn, Jean-Christophe Billeter

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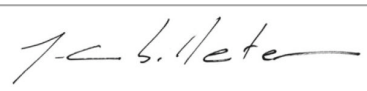
Signature:	 Date: April 25th, 2018

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Groningen June 21, 2018

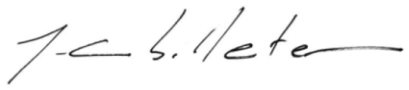
Concerns: Response to Editor: manuscript *JoVE58350*

Dear Editor,

Thank you for your thorough and helpful comments on our manuscript. We have addressed your comments as indicated in your Email of June 15th and the resulting changes are highlighted in the manuscript.

I detail below the action we have taken.

Yours sincerely,



Jean-Christophe Billeter

POINT-BY-POINT RESPONSE TO ISSUES RAISED BY THE EDITOR

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain the same.

- All steps have been formatted following the editors direction.

2. Please address all the specific comments marked in the manuscript.

- All comments have been addressed. We have uploaed a manuscript with tracked changes, and you will find an itemized answer to your comments below.

3. For the protocol section please be specific as to how you performed the experiments and describe the steps involved in these in imperative tense as if directing the reader to do something.

- All steps have been modified accordingly.

4. Please provide Graphical User Interface and button clicks for the software programs and hard experimental steps for the experimental part. We cannot film calculations and scripting steps.

- We have uploaed two Video files (Step 4.3 and 6.1) illustrating how to use the software. There is no particular GUI for either software, as the user must add all specific conditions of the experiment in the main script. The videos illustrate how to use these scripts.

5. Explaining how you do the experiment is important for filming.

- Thank you for asking for clarification on the necessary steps within the manuscript. We have adjusted our directions accordingly.

6. Once all changes are done, please check that highlighted part of the protocol are no more than 2.75 pages including heading and spacings which is the hard cut limit for filming.

- We have verified this.

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- We are not using any figures from previous publications.

Specific comments in the manuscript:

Main document changes and comments

Comment [1] Author 14/06/2018 14:57

Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes. I have done some of it as an example.

- Thank you for pointing this out. All the numbering has been changed and adjusted accordingly.

Also, for the protocol step please write it with respect to a specific protocol which you performed (details are in the result section). So briefly preparation of fly food, rearing of flies, then making the temperature-controlled arena, making the frame of lights, then introduce behavioral experiments- in this case what was the temp, what kind of flies, how do you do everything, then video tracking and data analysis.

- The order of the steps has been changed according to the editor's comment. We have specified temperature settings in the set-up of the temperature controlled arena (Step 4.3.2) and type of fly used in the description of the behavioral experiments (Step 5.3).

Please ensure that we need hard experimental steps for filming. As of now I'm unsure whether the highlighted steps can be filmed. Please provide some graphical user interface for the software being used, button clicks, etc. if these steps need to be filmed. e.g. Note: Use a commercial software for video tracking (Table of Material). 1.1. Open the video by clicking ***Open.*** *Then click* ***....***

- We have added two videos (Step 4.3 and 6.1) that explain the software set-up for the temperature-controlled arena and the usage of the tracking software respectively. We have also changed the steps related to the these two processes to clarify each of the user's actions (e.g clicks). Besides, we have adjusted the highlighted sections to include hard steps in the filming besides the videos we have made.

Comment [2] Author 18/06/2018 11:33

This step can be filmed.

- We have decided not to film the making of the fly food because it is a common process for all *Drosophila* experiments and it detracts from the focus on the usage temperature-controlled arena, which is our main methodological contribution.

Comment [3] Author 14/06/2018 15:31

How much in food is poured in the rearing bottles and collection vials.

- This has been clarified in Step 1.6.

Comment [4] Author 18/06/2018 11:33

Please write the steps in the order of it being performed. So you first place 20 males and 20 females in a rearing bottle, and leave them in the incubator, then after 3-4 days you do you collect the virgins at this stage and transfer the other flies into fresh rearing bottles?

- Thank you for asking for this clarification. We have changed the Steps in section 2 to illustrate each action better.

This step can be filmed.

- We have highlighted the steps that could be filmed from section 2.

Comment [5] Author

For how many days? What is the temp 18 or 25?

- We have specified we used 25°C in Step 2.1.1.

Comment [6] Author

Newly eclosed flies from the bottles prepared in 2.1?

- We have added a note in Step 2.1.1 to explain that new flies will eclose from these bottles to make this point clearer.

Comment [7] Author

So only virgin flies are used for this experiment?

- We have added step 2.2.1 in which it is stated that only virgin flies are used. All figures have this commented too.

Comment [8] Author 18/06/2018 11:33

Will this come before Step 4 since this shows how to make the frame of lights.

- Thank you for pointing this out, this definitely makes our protocol clearer. Accordingly, we have changes the frame of lights to be section 3.

These are hard steps. This can be filmed.

- The frame of lights is built once and then repeatedly used for all experiments; hence, we have decided not to film this process.

Comment [9] Author 14/06/2018 15:48

All these are just verification steps. So doesn't need to be filmed.

If this needs filming please format properly by stating how to do the step.

- Thank you for this clarification. We have removed the highlight from most of these steps (section 4), but we have still selected the turning on of the temperature-controlled arena (Step 4.1) and the usage of the *TemperaturePhases* script that controls it (Step 4.3; Video 1) as important for the filming as we considered they should be clearly illustrated for future users of our protocol.

Comment [10] Author 14/06/2018 14:55

Is this correct. Please refer to the figure where ever possible to bring out clarity.

- We have checked that we are referring to the correct figures (Step 4.1)

Comment [11] Author

How do you verify? Also, what is the temperature sequence in this case?

- We have created a video to illustrate each of the steps (Video 1) and added substeps (4.3.1 to 4.3.4) to explain each of the processes and the specific details used for the experiments.

Comment [12] Author

Please do not use unpublished data.If needed this can be added as a supplemental file. We cannot have links added to the manuscript.

- We have removed the link in Step 4.3 and instead created a video (Video 1) to illustrate each of the steps.

Comment [13] Author

How? By looking at something? Are all these incorporated in the script?
Flies from which step? Any sex specific bias? Please provide every necessary detail.

- We have added steps (Step 4.3.3 and 4.3.4) to explain each of the processes and specify that the materials used. The flies are specified in Step 5.3 under the section Temperature Behavioral Experiments.

Comment [14]

Check how?

- This is now explained in Steps 4.3.1 to 4.3.4.

Comment [15]

How and what is the delay that is set up? Again are all these would just involve filming someone checking the script?

- The delay is illustrated in Video 1. The delay is better explained as an initialization process in Step 4.3.3.

Comment [16]

Manually?

- We have clarified this as 'Using the camera USB cable' (Step 4.4).

Comment [17] Author 14/06/2018 17:00

This can be filmed if formatted properly.

- We have re-formatted and re-written the steps in Section 5 to make them clearer, and highlighted the sections that could be filmed.

Comment [18] Author 14/06/2018 16:35

Flies from which step? Any sex specific bias? Please provide every necessary detail.

- We have specified that flies come from the ones collecting in the rearing vials and exemplified that we use one male fly for results of Figure 3 (Step 5.3). We have also specified that the area in which flies are placed is called Fly Arena (Step 5.1).

Comment [19] Author 14/06/2018 16:37

Please mark this in the Figure.

- We have indicated that the gap is the one left by the experimenter when placing the glass cover on top of the Fly Arena in Step 5.1.3. This gap cannot be illustrated in our Figures, but the reference to step in which it is created should be enough to explain which gap we are referring to.

Comment [20] Author 15/06/2018 10:21

How? Also mark this in the figure.

- We have clarified this in Step 5.4.1 and indicated the placement of the ring of lights in Figure 1C.

Comment [21] Author 15/06/2018 10:23

How?

- Step 5.5 is clearer now by referring to Steps 4.5.2 and 4.3.4, where it is stated how to start the recording process and the experimental process respectively.

Comment [22] Author 14/06/2018 17:08

Stop after how long. What kind of behavior is expected? Add a note stating the same.

- This is clearer now by specifying how to stop the recording (Step 5.6) and how to determine when an experiment is done (Note).

Comment [23] Author

Only be filmed if there are some GUI.

- We have added Video 2 in which the steps for using the *FlySteps* tracker are illustrated. We have also re-written this section to make every step clearer.

Comment [24] Author

This needs institutional login to get connected. Also, we cannot have external links in our manuscripts. Please include this information as a supplemental file instead. Do you have any graphical user interface for the same?
Id this section needs to be filmed please provide graphical user interface, button clicks and hard experimental steps of how to do the procedure.

- The tracker does not have a particular GUI, but instead it provides a script in which the user can specify tracking parameters. We have illustrated each of the steps to use this scrip in Video 2. We have also included all the scripts as "supplementary coding files" accompanied by a Readme.rtf instructive manual explaining the use of each of the coding files.

- The data will be publicly available upon manuscript acceptance within DataverseNL. The current link to the DataverseNL is temporary and meant for reviewers and editors to access the data files if necessary. This link also includes a folder with the raw data from our experiments in the spirit of

OpenScience allowing others to verify our results and/or to use this data as example of how data looks like.

Publicly available Dataverse link:
https://dataverse.nl/privateurl.xhtml?token=c70159ad-4d92-443d-8946-974140d2cb78

Comments [25] and [26] Author

How?

- All steps to use the *FlySteps* tracker have been specified under 6.1 substeps.

Comment [27] Author

We cannot film calculation steps, so highlights are removed.

- Agreed. Thank you for this correction.

Comment [28] Author

Some of the experimental details explaining how to perform the experiment can be moved to the protocol section.

- Thank you for this suggestion. We have moved the explanation about allowing flies to explore the arena to a Note under Step 4.3.2 as this consideration is related to the experimental set-up. We have also modified the first paragraph of this section to simplify the descriptive information.

Comment [29] Author

Please remove commercial term and use generic term instead. Please refer to the commercial term in the table of materials.

- We have removed all commercial terms and ensured that they are added to the Material List.

Comment [30] Author

Please correct this.

- We apologize for the lack of correct symbols appearing in the description of Figure 3. We have used the Insert Symbol function within Word to add the diamond and circle that were missing, hoping that this will avoid changes depending on where the document is opened.

Comment [31] Author

Does this need to be in quotes?

- We agreed that quotes are unnecessary for 'Peltier' as this is acceptable named for the material. Thank you for asking for this change.

Comment [32] Author

Is this open source?

- Yes, it will be open source from DataverseNL upon acceptance of the manuscript. As of today, a temporary link in DataverseNL can be accessed by reviewers and editors and all the necessary files can be downloaded from a .zip document added as supplementary information (see Comment 24).

Comment [33] Author

Please include this as supplemental files.

- We have created a .zip file containing all the necessary scripts to use the *TemperaturePhases* and *FlySteps* scripts. We have added this as supplementary information.

Automated Method to Determine Drosophila response to Temperature Changes

The temperature-controlled arena is related to the following scripts:

1. TemperaturePhases.m

This script allows the user to determine the temperature in each of the three copper tiles. It allows specifying when temperature changes occur and of which magnitude each change should be. It also allows determining when and for how long each of the indicative red LEDs should be on. It is directly related to the *LoB.m* script, which contains the temperature controlling functions and to the *ArduinoControl.ino* which contains the main code for the programmable circuit inside the temperature-controlled arena.

The current set up of the script produces a temperature curve between 16°C and 46°C in which temperature changes 2°C every 60 seconds. All tiles are heated up at the same time.

2. TemperaturePhases_ShiftingComfortableTile.m

This script allows the user to produce a temperature curve between 16°C and 46°C in which temperature changes 2°C every 60 seconds and in which one tile is warm up to only 22°C to provide a comfortable location for the flies to walk on. The comfortable tile changes location on every iteration. It is directly related to the *LoB.m* script, which contains the temperature controlling functions and to the *ArduinoControl.ino* which contains the main code for the programmable circuit inside the temperature-controlled arena.

3. FlySteps_Analysis.r

Script to analyse the speed of individual flies that have been tracked by the *FlySteps* tracker. Multiple files can be analysed together. The output results will be stored in a folder called *results* inside the folder that contains the tracker output files. Result will be presented in a .csv file. Each individual fly's information, including name, will be presented in one column of the .csv file. Results are produced in cm/s.

4. FlySteps_Analysis.m

Script to analyse the speed of individual flies exposed to a ShiftingComfortableTile protocol. The script permits specifying which tile was comfortable on each iteration. Results are produced in cm/s. This script also allows correcting for errors in perspective of a rectangular area due to the camera used for recording.

5. Fly_Tracker

Folder containing the python based code of the *FlySteps* tracker. The use must open *configuration_file.ini* to set up the location of the videos to the tracked, the dimensions and shape of the area to be tracked, the number of subjects, the background contrast, and the location and intensity of the indicative red LEDs. The

other files in this folder are necessary functions for the tracking process that will be called automatically when the *configuration_file.ini* is ran.

Files to save in the same location as *configuration_file.ini*:

- _init_.py
- common.py
- common.pyc
- core.py
- core.pyc
- main.py
- tester.py
- Tom.pyc
- video.py
- video.pyc

We ran this tracker in a Ubuntu 14.04 Linux machine.

6. Examples

This folder contains example files of the *FlySteps* output used to produce Figure 2. Each file name represents a *Drosophila* species (*melanogaster* (CS), *simulans* (Sim), *subobscrua* (Sub), *suzukii* (Suz), *yakuba* (Yac)), the sex of the fly (m=male), and the number of experiments (1-15). The first column of the file contains the frame, the second and third columns the on (1) or off (0) state of the left and right indicative red LEDs respectively, and the fourth and fifth columns contain the *x* and *y* location of the fly respectively.

For further questions on set-up and usage contact the authors.

FlySteps output analysis for R

FlySteps output analysis for Matlab

User file for FlySteps

Script for arduino control of the arena

Matlab script for temperature phases

Matlab user script for controlling the gradual increase in temperature in the temperature arena

Matlab user script for controlling independent temperature in each tile of the arena