Journal of Visualized Experiments

High Throughput Assays of Critical Thermal Limits in Insects

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61186R1
Full Title:	High Throughput Assays of Critical Thermal Limits in Insects
Section/Category:	JoVE Biology
Keywords:	Thermal limits; Critical thermal minimum; CTmin; Critical thermal maximum; CTmax; Heat knock down time; KDT; Insects; Drosophila melanogaster
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Lexington, Kentucky, USA

TITLE:

High-Throughput Assays of Critical Thermal Limits in Insects

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KEYWORDS:

thermal limits, critical thermal minima, $CT_{min,}$ critical thermal maximum, CT_{max}, heat knock down time, KDT, insects, *Drosophila melanogaster*

SUMMARY:

Thermal limits can predict the environments organisms tolerate, which is valuable information in the face of rapid climate change. Described here are high-throughput protocols to assess critical thermal minima and heat knockdown time in insects. Both protocols maximize the throughput and minimize the cost of the assays.

ABSTRACT:

Upper and lower thermal limits of plants and animals are important predictors of their performance, survival, and geographic distributions, and are essential for predicting responses to climate change. This work describes two high-throughput protocols for measuring insect thermal limits: one for assessing critical thermal minima (CT_{min}), and the other for assessing heat knock down time (KDT) in response to a static heat stressor. In the CT_{min} assay, individuals are placed in an acrylic-jacketed column, subjected to a decreasing temperature ramp, and counted as they fall from their perches using an infrared sensor. In the heat KDT assay, individuals are contained in a 96 well plate, placed in an incubator set to a stressful, hot temperature, and video recorded to determine the time at which they can no longer remain upright and move. These protocols offer advantages over commonly used techniques. Both assays are low cost and can be completed relatively quickly (~2 h). The CT_{min} assay reduces experimenter error and can measure a large number of individuals at once. The heat KDT protocol generates a video record of each assay and thus removes experimenter bias and the need to continuously monitor individuals in real time.

INTRODUCTION:

Thermal limits of insects

Variation in environmental conditions, including temperature, is a major factor influencing the performance, fitness, survival, and geographic distribution of organisms[1,2]. Upper and lower thermal limits determine the theoretical range of environments an organism can tolerate, and, therefore, these limits are important predictors of plant and animal distributions, especially in the face of climate change[3,4]. Thus, protocols to accurately measure thermal limits are important tools for ecologists, physiologists, evolutionary biologists, and conservation biologists, among others.

As they are the most abundant and diverse terrestrial animals, insects have been frequent recipients of thermal limit measurements. Critical thermal maxima (CT_{max}) and critical thermal minima (CT_{min}) are commonly used to assess intra- and interspecific variation in thermal tolerance[5–7]. While CT_{max} and CT_{min} can be measured for multiple phenotypes, including growth, reproductive output, and behavior, they are most commonly applied to locomotor function[5–7]. Thus, CT_{max} (also called heat knockdown temperature) and CT_{min} are often defined as the high and low temperatures at which insects lose motor function and are unable to remain upright[5–11]. CT_{min} coincides with the onset of chill coma, a reversible paralysis brought on by cold temperatures[6]. While paralysis at the thermal limits is often reversible, continued exposure to these temperatures leads to ecological death[5].

Common methods for measuring thermal limits

A variety of apparatuses have been used to measure thermal limits (summarized in Sinclair et al.)[6]. Briefly, insects are heated or cooled in incubators[12,13], containers submerged in fluid baths[11,14–16], aluminum blocks[10,17], or jacketed containers[18], and monitored until locomotion ceases. To monitor insects during the assay, the most common method is direct observation, in which individuals are continuously monitored in real time or retrospectively with recorded video[6,9–11, 15,17]. While direct observation methods have minimal equipment requirements, they are labor-intensive and limit throughput. Alternatively, insects can be observed indirectly by collecting individuals at discrete times as they fall from perches[6,19–21] or using activity monitors[13].

Indirect methods for measuring thermal limits are generally higher-throughput and potentially less error prone than direct observation methods. The most common method for indirect monitoring uses a jacketed temperature-controlled column[6,8,19–21]. Insects are placed inside a column with perches, and the temperature of the inner chamber is controlled by pumping fluid from a temperature-controlled fluid bath through the jacketed lining of the column. Individuals that reach their thermal limit fall from their perch and are collected at discrete temperatures or time intervals. While this method works well for CT_{min}, it has been found unsuitable for CT_{max}, because flies voluntarily walk out of the bottom of the column when the temperature increases. The new method described here circumvents this issue by individually containing flies during automated measurements.

In addition to the method of observation, two types of temperature regimes are commonly used to assess upper thermal limits. Dynamic assays consist of gradually increasing temperature until

motor function is lost; that temperature is the dynamic CT_{max}[7–9,13]. In contrast, static assays consist of a constant stressful temperature until motor function is lost; that time point is the heat knockdown time (heat KDT), also called the static CT_{max} (sCT_{max}) in a recent paper by Jørgensen et al.[7–9,16,22]. Although CT_{max} and heat knockdown assays (heat KD assays) produce metrics with different units, mathematical modeling of the two traits indicates they give comparable information on heat tolerance and are both ecologically relevant[8,9]. Dynamic assays yield a temperature that can be compared to environmental conditions, and they are preferable when there are large differences in heat tolerance, such as comparisons between species with widely different thermal niches. However, due to the high Q10 for heat injury accumulation, a static assay may be preferable for detecting small effect sizes, such as intraspecific variation in heat tolerance[9]. Also, practically speaking, a static assay requires less sophisticated equipment than a dynamic assay.

Objective

The objective of this paper is to formalize methods for CT_{min} and heat KD assays that can be used in future research to assess the thermal limits of motile insects. The protocols are adapted from previously established methodologies and are designed to be high-throughput, automated, and cost-effective. Both assays can be completed in a short amount of time (~2 h), which means that multiple experiments can be conducted in a single day, producing large amounts of data without sacrificing repeatability or accuracy. With this setup, the heat tolerance of 96 flies can be measured simultaneously, while the column for CT_{min} can hold more than 100 flies, provided there is adequate surface area for perching.

The high-throughput method for observing CT_{min} modifies the common jacketed column methodology with the addition of an infrared sensor to automatically count flies. The use of an infrared sensor for counting was first proposed by Shuman et al. in 1996[23] but has not been widely adopted. The addition of the infrared sensor allows for the generation of continuous data rather than collecting data at discrete intervals. This protocol also minimizes experimenter error by eliminating manual data entry and the need to manually switch collection tubes below the jacked column at discrete time points.

The high-throughput method for recording heat KDT is modified from two previous studies of heat tolerance in insects[10,12]. Individual flies are stored in a 96 well plate in a temperature-controlled incubator and video is recorded. This protocol minimizes experimenter bias in determining heat KDT because experiments can be reviewed and verified by playing back the recording. This protocol also provides a set of custom Python scripts that can be used to speed up video analysis. The use of individual wells eliminates interference that can occur when other individuals move around or fall over, which can be a problem when groups of individuals are observed in the same arena[10,17]. Furthermore, the temperature-controlled incubator provides a stable temperature across all 96 wells, unlike the temperature gradient sometimes observed across a temperature-controlled aluminum block[10]. Also note that the 96 well recording method can be adapted to measure dynamic CT_{max} and potentially CT_{min} (see Discussion).

To demonstrate each protocol, the thermal limits of adult *Drosophila melanogaster* females from

select lines of the *Drosophila melanogaster* Genetic Reference Panel (DGRP) were compared[24]. These lines were selected because preliminary experiments indicated significant differences in thermal tolerance. These assays proved to be robust methods for discriminating differences in thermal tolerance. The following two protocols, high-throughput CT_{min} assay (section 1) and high-throughput heat KD assay (section 2), describe the necessary actions to produce CT_{min} and heat KDT data for any motile insect life stage capable of fitting in the apparatuses, such as adult *Drosophila*. For CT_{min} it is also essential that the insect be able to perch. Here, each assay is demonstrated in adult *Drosophila melanogaster*. However, modifications may be required for other taxa or life stages[6]. Minor changes might include using perching material with larger openings to accommodate larger specimens in the CT_{min} assay or using a higher quality camera to discern the subtle KDT of a slow moving insect or life stage in the heat KD assay. This protocol does not describe methods for preparing flies, but it is important to standardize rearing protocols to ensure repeatability[25] (see Garcia and Teets[26] and Teets and Hahn[27]). The protocols provided include information on how to build and set up the apparatuses, how to record measurements, and a brief description of data analysis.

PROTOCOL:

1. High-throughput CT_{min} assay

1.1. Assembling the jacketed column (**Figure 1A**, **Figure 2**)

1.1.1. Cut the widest (7 cm x 6.35 cm x 0.3 cm) and narrowest (5.7 cm x 5.1 cm x 0.3 cm) acrylic tubes to equal lengths (31.5 cm) with a hacksaw (**Figure 2A**). These two tubes will be the outer and innermost walls of the jacketed column.

1.1.2. Cut two rings (2 cm wide) from the midsized (6.35 cm x 5.7 cm x 0.3 cm) acrylic tube with a hacksaw (**Figure 2A**). These two rings will be the spacers between the inner and outermost tubes, creating a space between the two long acrylic tubes for fluid to flow.

1.1.3. Carefully drill two holes in the outer (widest) acrylic tube, one hole at the top and one at the bottom. Ensure that each hole is 3.5 cm from the end of the tube. Drill the holes on opposite sides of the tube (**Figure 2B**).

1.1.4. To reduce cracking, place tape on the tube over the spot of the future hole and drill very slowly on the lowest torque setting of the drill.

1.1.5. Using the threading tap, thread both holes so that the hose adapters can be screwed into the two holes of the outer tube. To reduce cracking, use lubricant, and thread slowly by hand.

1.1.6. Slide the two spacers onto the inner jacket, one at each end (bottom and top). Leave a small space (0.5 cm) between the spacer and the end of the inner jacket (**Figure 2B**).

1.1.7. Weld the spacers into place using acrylic cement.

1.1.8. After the cement on the inner tube and spacers sets, slide this construct into the larger outer tube with the holes. Ensure that the outer and inner tubes are flush on both ends. The spacers will be 0.5 cm from the end, forming small trenches on both ends of the column (**Figure 2C**).

1.1.9. Weld the outer tube to the spacers using acrylic cement, using adjustable steel clamps to hold the apparatus together. Wait for the cement to set.

1.1.10. Thread the hose adapters into the holes of the outer tube now secured to the spacers and inner tube.

NOTE: The adapters should only thread into the outer tube and not into the open space between the inner and outermost tubes. If the hose adapters thread too far in, shorten them to the appropriate length with a hacksaw.

1.1.11. Seal the hose adapters into their threads on the outer tube with silicone sealant.

1.1.12. Fill the two trenches between the inner and outermost tubes at both ends of the jacketed column with silicone sealant.

1.1.13. To test the column, attach 0.6 cm diameter tubing to the hose adapters. Connect the adapter at the bottom of the column to a water source with tubing, and the adapter at the top of the column to a drain with a different piece of tubing.

1.1.14. Run water through the apparatus from the bottom to the top and check for leaks. If there are leaks, identify where they are coming from and seal with silicone.

1.2. Setting up the jacketed column and *Drosophila* Funnel Monitor (DFM)

1.2.1. Secure the jacketed column to a retort stand with a three-prong retort clamp. Align the column vertically with one end open to the ceiling and the other open to the lab bench (**Figure 1B**).

1.2.2. Connect the fluid input and output from a temperature-controlled refrigerated bath to the adapter nozzles of the column with 0.6 cm diameter plastic tubing (**Figure 1B**). Connect the fluid input to the nozzle at the bottom of the column and the fluid output to the nozzle at the top of the column.

1.2.3. Cut two 3 cm thick circular foam insulating plugs (the same circumference as the opening of the innermost compartment of the column). Ensure the plugs fit snugly and seal the innermost column when inserted at both ends (**Figure 1B**).

1.2.4. Pierce a hole through the center of one of the plugs and thread the bare end of a

thermocouple through the hole about 5 cm and secure with tape. Plug the other end of the thermocouple into a thermocouple data logger.

1.2.5. Connect the thermocouple data logger to the computer.

1.2.6. Wedge two pieces of plastic gutter guard (5 cm x 7 cm, with ~0.5 cm diameter openings) inside the column to function as perching material. Place one piece of guard 2/3^{rds} from the top of the column and the other 1/3rd from the top of the column (**Figure 1B**).

1.2.7. Secure the bottom plug (without a thermocouple) and the top plug (with a thermocouple). When the plug is inserted at the top of the column, ensure that the thermocouple does not touch the sides of the column.

1.2.8. Adjust the height of the column on the retort stand so there is a 25 cm distance between the bottom of the column and the bench top.

1.2.9. Secure a retort ring (5 cm diameter) to the retort stand 5 cm below the bottom of the column and rotate the ring off to the side of the column.

1.2.10. Set the DFM directly on the retort ring (**Figure 1B**). Connect all the electronic components: the power supply, power supply interface, and the computer according to the manufacturer's protocol.

1.2.11. Once the components are connected, follow the manufacturer's protocol to finish the setup of the DFM and DFM software.

1.3. CT_{min} assay

1.3.1. Turn the input and output valves of the fluid bath to the open positions.

1.3.2. Push the power button to turn on the temperature-controlled fluid bath and then press the play button to run a program raising and maintaining the temperature of the bath to 25 °C. Give the fluid bath and column 5–10 min to reach and maintain 25 °C.

1.3.3. Remove the plug at the top of the column and replace it with a funnel (5.08 cm diameter; see **Figure 1C**).

1.3.4. Tap flies from their food vial into the column.

1.3.5. Remove the funnel and replace it with the top plug quickly, careful not to let flies escape. Give the flies 5 min to settle, occasionally tapping the bottom plug to encourage the flies to climb.

1.3.6. Press the start button on the fluid bath and begin the CT_{min} ramping program (25 °C for 5 min; 25 °C to 10 °C at 0.5 °C/min; 10 °C for 2 min; then 10 °C to -10 °C at 0.25 °C/min).

NOTE: Other variations of this CT_{min} ramping protocol can be used depending on the research question (e.g., comparisons of the effects of different ramping rates on CT_{min}[28]).

1.3.7. Click open the thermocouple recording software on the computer and then click the **Record** icon to begin recording the temperature inside the column every second for the duration of the assay. Ensure that each temperature recording includes a time stamp specific to the second, so that temperature data can later be merged with data from the DFM.

1.3.8. Add 5 mL of 90% ethanol to a 15 mL conical centrifuge tube and place it in a rack below the column.

1.3.9. Occasionally, tap the bottom plug of the column to entice any flies on the bottom to climb. Most flies will be on a perch or near the top of the column by 15 °C.

1.3.10. At 15 °C, remove the bottom plug and collect any flies still on the bottom plug in the ethanol. Count and note that these flies were collected at 15 °C but their CT_{min} is unknown.

NOTE: The temperature at which the bottom plug is removed should be decided before the assay and based on the predicted CT_{min} of the test species or treatment. For this assay, 15 °C was chosen based on the CT_{min} of these particular DGRP lines found in preliminary assays.

1.3.11. Place a 75 mm outer diameter glass funnel into the DFM. Adjust the retort ring, DFM, and funnel so that they are under the column. Ensure that the lip of the funnel completely seals the bottom of the column (**Figure 1D**).

1.3.12. Insert the bottom of the funnel into the 15 mL collection tube (**Figure 1D**).

1.3.13. Open the DFM software on the computer by clicking the **Software** icon. The software will immediately start recording the time/date at which flies reach their CT_{min}. Flies that reach their CT_{min} lose neuromuscular function and fall from their perches, and thereafter through the DFM.

1.3.14. Monitor whether all the flies have reached their CT_{min} as the temperature decreases by checking the top plug and perches to see if any flies are still perched (i.e., still maintaining neuromuscular function). The trial ends when all the flies have reached their CT_{min}.

1.3.15. At the end of the trial, adjust the DFM and funnel away from the column opening. Some flies may reach their CT_{min} but remain stuck in the column (i.e. wedged in a perch or dangling by a single tarsal hook). Open the top plug and remove these flies. The CT_{min} of these flies is unknown.

1.3.16. Combine the .txt output files from the thermocouple recording software (i.e., temperature, date, and time) and the DFM software (i.e., number of flies through the funnel, date, and time) using the ***Merge*** command in RStudio. Merge the two files based on the shared

date/time variable.

2. High-throughput heat KD assay

2.1. Apparatus assembly and preparation

2.1.1. With an adhesive, fix the steel woven wire mesh (~1.5 mm aperture) to the bottom of a 96 well no-bottom plate.

2.1.2. Attach magnets to the opposite sides of the bottom of a 96 well no-bottom plate with a hot glue gun and hot glue (**Figure 3**).

2.1.3. To craft a custom septum lid with adhesive film designed for 96 well plates, stick two films sticky sides together to form a ridged plastic sheet.

2.1.4. Place the plastic sheets over the 96 well plate and use tape to adhere it to all four sides of the plate. Over the opening to each well on the plate, cut an 'x' in the plastic sheet with a box cutter (i.e., 96 total x's).

2.1.5. Anesthetize flies with CO_2 and load them individually into each well of the modified 96 well no-bottom plate with an aspirator and septum lid. Remove the septum lid from the 96 well plate while the flies are anesthetized with CO_2 and replace it with a tight-fitting clear lid.

2.1.6. Place the 96 well no-bottom plate loaded with flies and with a clear tight-fitting lid on food. Ensure the flies have at least 48 h between CO_2 anesthetization and the start of the assay (steps 2.2.1–2.2.5).

NOTE: The bottom of the modified 96 well no-bottom plates is made of mesh, so flies anaesthetized with CO_2 can be loaded and left on food for at least 48 h before a trial begins. Any plastic container >8.5 cm wide x 13 cm long that is at least 2 cm deep to accommodate a 1 cm deep layer of food can be used.

2.1.7. Fix a webcam to the bottom of the inside of a temperature-controlled incubator with tape. Point the camera directly up (**Figure 4**). Secure an incubator shelf about 10 cm above the camera.

NOTE: The webcam points up and records the 96 well plate from below to ensure as much of the well surface is in view as possible (e.g., not blocked out from view by the well walls of the plate). When the flies reach their KDT they fall to the bottom of the well; in this case, the side closest to the webcam, and are therefore in view regardless of how far their well is from the center of view.

2.1.8. Connect the webcam to a computer.

2.1.9. With tape, attach a white sheet of paper (8.5 cm x 13 cm; the exact area of the bottom of

the 96 well plate) to the bottom of the shelf (**Figure 4**). Ensure that the paper fills the entire frame when viewed through the webcam.

2.1.10. Place a light source in the incubator. Use paper or other materials to dampen the lighting and minimize glare.

NOTE**:** Step 2.1.10 is specific to each incubator because lighting and reflections vary among incubators. The goal is to have sufficient lighting in the incubator to provide a good contrast between the flies in each well and the white sheet of paper behind the plate when viewed with the webcam.

2.2. Performing the heat KD assay

2.2.1. Set the incubator to 37.5 °C and wait about 30 min to give the incubator time to reach and maintain the desired temperature. The exact temperature will depend on the insect being assessed and any other time considerations.

2.2.2. Place the 96 well plate inverted in the incubator, such that the bottom of the plate (mesh side) is against the white paper on the bottom of the tray (**Figure 4**). Take note of the orientation of the wells (column and row names) on the tray and in the frame of the webcam. Colored tape along the sides of the 96 well plate and edges of the white piece of paper can verify the orientation (**Figure 4**).

NOTE: Ensure that the incubator temperature is consistent with the temperature inside the 96 well plate by recording the temperature inside the plate with a thermocouple during a test trial of the heat KD assay. It is also prudent to check that there is negligible variation in temperature between wells of the 96 well plate with multiple thermocouples before conducting the heat KD assay.

2.2.3. Close the incubator door.

2.2.4. Click **Record** on the video recording software.

2.2.5. After 2 h, check the recording to see that all flies have reached their final resting spot and stopped moving. Once all flies have stopped moving, click **Stop** on the video recording software. For the genotypes tested here, reared at 25 °C, most flies reach their KDT by 60 min at 37.5 °C (also see Jørgensen et al.[9]).

2.2.6. Dispose of the flies.

2.2.7. Use the custom Python scripts (**Supplementary Coding Files 1-3**) to approximate the time in the video when flies reach their heat KDT.

NOTE: Step 2.2.7 is optional. To speed up video analysis, a set of custom Python scripts were

developed to measure changes in pixel density over time in each well (see **Supplementary Coding File**). When the flies stop moving, the pixel density is constant, and a plot of these data can be used to locate the approximate time in the video when flies are knocked down. While it may be possible to use this script to automate data analysis, currently slight imperfections in the video lead to minor discrepancies between changes in pixel density and the true KD time.

2.2.8. Click open the video file and record the KDT of each fly in each well. The most consistent measure of heat KDT between trials and observers is recording the time at which a fly reaches its final resting spot.

2.2.9. Track the video in reverse, focusing on a single well, and noting the time at which the fly first moves off its final resting spot. Repeat this process for each well.

REPRESENTATIVE RESULTS:

The thermal limits (i.e., CT_{min} and heat KDT) of females from the *Drosophila melanogaster* Genetic Reference Panel (DGRP) were measured to demonstrate the high-throughput data generated from the two described protocols. CT_{min} was assayed using the DGRP lines 714 (n = 37) and 913 (n = 45). Heat KDT was assayed and compared with the DGRP lines 189 (n = 42) and 461 (n = 42) without the use of the **Supplementary Coding Files**. The total time of the experiments, including watching the video, took <2 h for each protocol.

Females from the DGRP Line 913 had significantly lower mean CT_{min} temperatures than females from the DGRP Line 714 (**Figure 5A**; Wilcoxon rank sum test, W = 1585, P < 0.001). The two lines had clearly distinct distributions of CT_{min}: line 913 had a CT_{min} of 5.00 ± 1.35 °C (mean ± SD) and line 714 had a CT_{min} of 9.60 ± 1.53 °C.

Heat KDT at 37.5 °C differed significantly between females from the DGRP lines 73 and 461 (**Figure 5B**; Wilcoxon rank sum test, W = 1658.5, P < 0.001). Although there was variation in the KDT of both lines, differences in heat KDTs between lines were readily detected. Line 73 had a 14.8 min longer mean KDT than line 461 (Line 73 mean KDT, 55.58 ± 6.92 min; Line 461 mean KDT, 40.78 ± 6.64 min).

FIGURE AND TABLE LEGENDS:

Figure 1: Setting up the jacketed column for the CT_{min} assay. (**A**) Assembled jacketed column. (**B**) Jacketed column with top and bottom plugs sealing the inner chamber. The thermocouple is threaded through a hole in the top plug. The DFM is mounted to a retort ring below the column and moved off to the side. (**C**) Start of a CT_{min} assay. The top plug was removed and flies were poured into the inner chamber via a funnel at the top opening of the column. (**D**) Jacketed column and DFM during a CT_{min} assay. The bottom plug was removed from the column and the DFM and funnel were positioned below the column.

Figure 2: Technical illustration of the jacketed column. (**A**) Each piece of acrylic tubing cut to length: i) two spacer rings cut to 3.5 cm in length (step 1.1.2):ii). the widest acrylic tubing cut to 31.5 cm (step 1.1.1); and iii the narrowest acrylic tubing cut to 31.5 cm (step 1.1.1). (**B**) Two holes

(in grey) drilled into the widest piece of acrylic tubing, 3.5 cm from each end and on opposite sides (i; step 1.1.2). Assembly of the narrowest piece of acrylic tubing with the two spacer rings (ii; steps 1.1.6 and 1.1.7). (**C**) The completed jacketed column after steps 1.1.8–1.1.12. Hose adapters are indicated in grey.

Figure 3: Bottom (left) and top (right) view of the 96 well no-bottom plate. Steel woven mesh is attached to the bottom of a modified 96 well no-bottom plate.

Figure 4: Incubator setup for a heat KD assay. (**A**) Webcam and stage set up at a distance. (**B**) Webcam and stage setup in the incubator before a trial begins. The webcam is fixed to the floor of the incubator and the tray is ~10 cm above the webcam. (**C**) Orientation of the 96 well plate on the white stage above the webcam during a heat KD assay.

Figure 5: Lower and upper thermal limits of select lines from the *Drosophila* Genetic Reference Panel (DGRP). (**A**) CT_{min} values of two DGRP lines. (**B**) Heat KDT of two DGRP lines at 37.5 °C.

Figure 6: Activity output from the video analysis scripts of a test dataset. Each plot represents the activity data from one well of a 96 well plate. A total of 84 samples were tested and are shown. Well ID is labeled on the right of each histogram.

DISCUSSION:

Significance of the CT_{min} and heat KD assays

The two methods detailed above generate high-throughput data of ecologically relevant metrics for upper and lower thermal limits. These protocols build upon previously established methodologies common to research on insect thermal limits (summarized in Sinclair et al.)[6]. Both protocols can be completed in a short amount of time (~2 h each), produce data sets with large sample sizes, do not sacrifice repeatability or accuracy, and minimize experimenter error by eliminating manual data recording and entry (CT_{min} assay), or by creating backup video recordings of each assay (heat KD assay).

Representative results were generated by comparing the thermal limits of adult females from select lines of the DGRP[24]. Both assays showed significant differences in thermal tolerance between lines. The effect size between lines in each of these assays was relatively large, which in turn allowed reliable differentiation of groups with visual and statistical comparisons. The large difference in KDT between the two DGRP lines highlights a potential advantage of a static assay over a dynamic ramping assay; static assays may be better able to detect smaller differences between groups than dynamic assays[9]. The two DGRP lines subjected to the heat KD assay differed in mean KDT by 14.8 min. For reference, using a dynamic ramping protocol, Rolandi et al.[13] showed that the difference of the highest and lowest CT_{max} values of 34 DGRP lines was only 1.42 °C, or <6 min with a 0.25 °C/min ramp.

Relative to other methods, there are several advantages to both the CT_{min} assay and heat KD assay described here. Automated counting in the CT_{min} assay reduces the amount of time an experimenter spends at the apparatus, thus increasing the amount of time that can be spent on

other tasks. The cost to build the acrylic-jacketed column is ~$50, compared to the estimated $400 to purchase a custom-made glass-jacketed column. For the heat KD assay, video recording eliminates the need for direct observations in real time and occupies a small amount of physical space per sample. Other protocols, such as those used by Jørgensen et al.[9], use a large aquarium for viewing individuals submerged in separate vials, but this method requires well-trained investigators to quickly check vials for movement and a large amount of space for the apparatus. Rolandi et al.[13] used infrared sensors to detect movement or lack of movement at CT_{max} in 96 well plates, while this heat KD assay uses an inexpensive webcam (~$70) for detecting motion. This camera can detect subtle movements that might be missed by an infrared activity monitor.

Furthermore, a set of customizable scripts to quickly estimate KDT in the heat KD assay were developed (**Supplementary Coding File 1-3**). These scripts can be used to save time by obtaining a rough approximation of heat KDT in each well before watching the video, and with higher video quality these scripts could potentially automate data recording. Three scripts to process the video have been provided: FirstFrame.py (**Supplementary Coding File 1**), which defines the first image frame of the video; WellDefine.py (**Supplementary Coding File 2**), which defines each individual well of the 96 well plate in the first image frame; and MotionDetect.py (**Supplementary Coding File 3**), which transforms the video file to an activity signal by calculating the change in pixel density between sequential frames. The only input to the program is the video file, and the output includes summary statistics and a time series dataset of activity per well (**Figure 6**). Differences in pixel density between video frames are transformed using a grayscale filter to reduce image dimensions, a Gaussian low pass filter to reduce image noise, and a dilation morphological operation to increase the borders of moving objects. In this case, activity is defined as the absolute difference of pixel values between sequential frames. The heat KDT can then be estimated as the index of the last frame containing an activity value greater than zero. For example, the frame in which activity was last recorded in well g12 of a sample dataset (**Figure 6**) was just after 2,000 s (33.33 min), as indicated by a flat line. An observer can then play back the digital video and quickly find the Heat KDT of well g12 with this time stamp.

With minor modifications and troubleshooting there are additional applications for both assays, most notably with the heat KD assay. The video recording setup could be modified to record static cold knockdown times, chill coma recovery time, or potentially dynamic CT_{max} and CT_{min} values. Chill coma recovery time is the amount of time it takes an individual to resume movement after cold stress[29]. Therefore, chill coma recovery time could be measured with this setup by inducing chill coma in the 96 well plate, then using the video setup to record the recovery time in the incubator. Finally, with careful fine-tuning, dynamic CT_{max} or CT_{min} could be recorded in a programmable ramping incubator. Careful attention to the temperature inside each of the 96 wells would be a concern, because slight variations in temperature in the incubator could be magnified between wells as the temperature changes.

Several considerations should be taken into account when performing either the CT_{min} or heat KD assay. First and foremost, the quality, age, sex, life stage, genetic background, and previous experience of an insect can influence thermal limits[6,13,30,31]. For both assays, test subjects must be motile. Second, only one group can be assayed at a time for each CT_{min} apparatus. Therefore,

variables such as diurnal variation in thermal tolerance[32,33] need to be considered when comparing treatments. One solution to this problem is to conduct CT_{min} assays of multiple treatment conditions with multiple apparatuses at the same time. Third, some species may not be suitable for one or both assays. For example, some species may not readily climb or fly to perches in the CT_{min} assay or may cease activity at high temperatures before their heat KDT is reached, which would make it difficult to discern a knockdown time. Finally, to ensure accurate comparisons in the heat KD assay, it is critical that the criteria for KDT (step 2.2.8) is consistent between replicates, observers, trials, etc. To accommodate different insect species, modifications to either of the test apparatuses may be required. Potential modifications include using different types of perches for the CT_{min} assay, using cell culture plates with fewer wells and more space (48, 24, 12, or 6 wells) instead of the 96 well plate to accommodate larger insects, or adjusting the temperature used for the heat KD assay to ensure a knockdown time that is not too fast or too slow.

ACKNOWLEDGMENTS:

We thank Ellie McCabe for assistance with fly rearing. This work is supported by United States Department of Agriculture National Institute of Food and Agriculture Hatch Project grant 1010996 and National Science Foundation grant OIA-1826689 to N.M.T.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 2

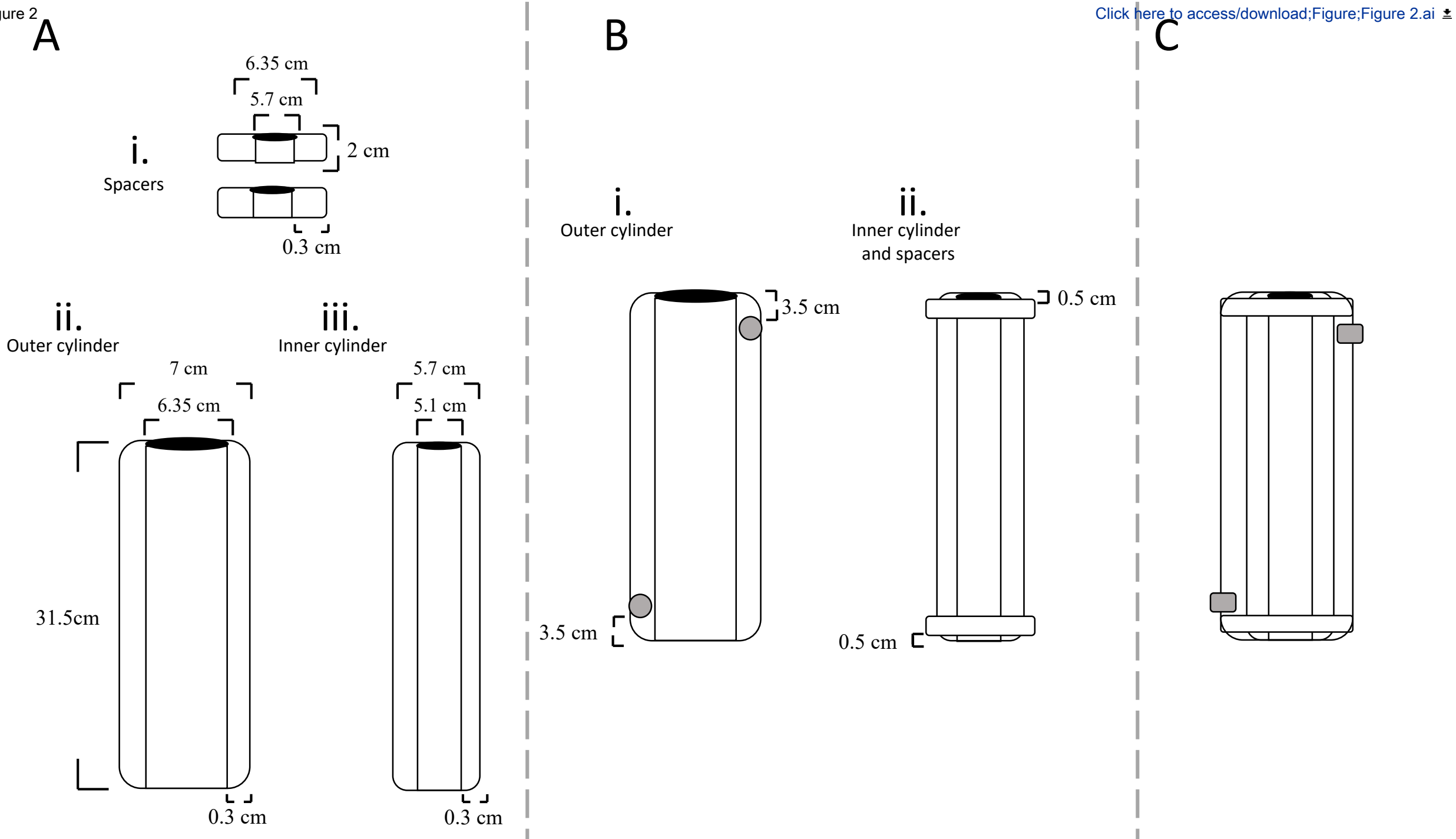


Figure 3

Click here to access/download;Figure;Figure 3.ai

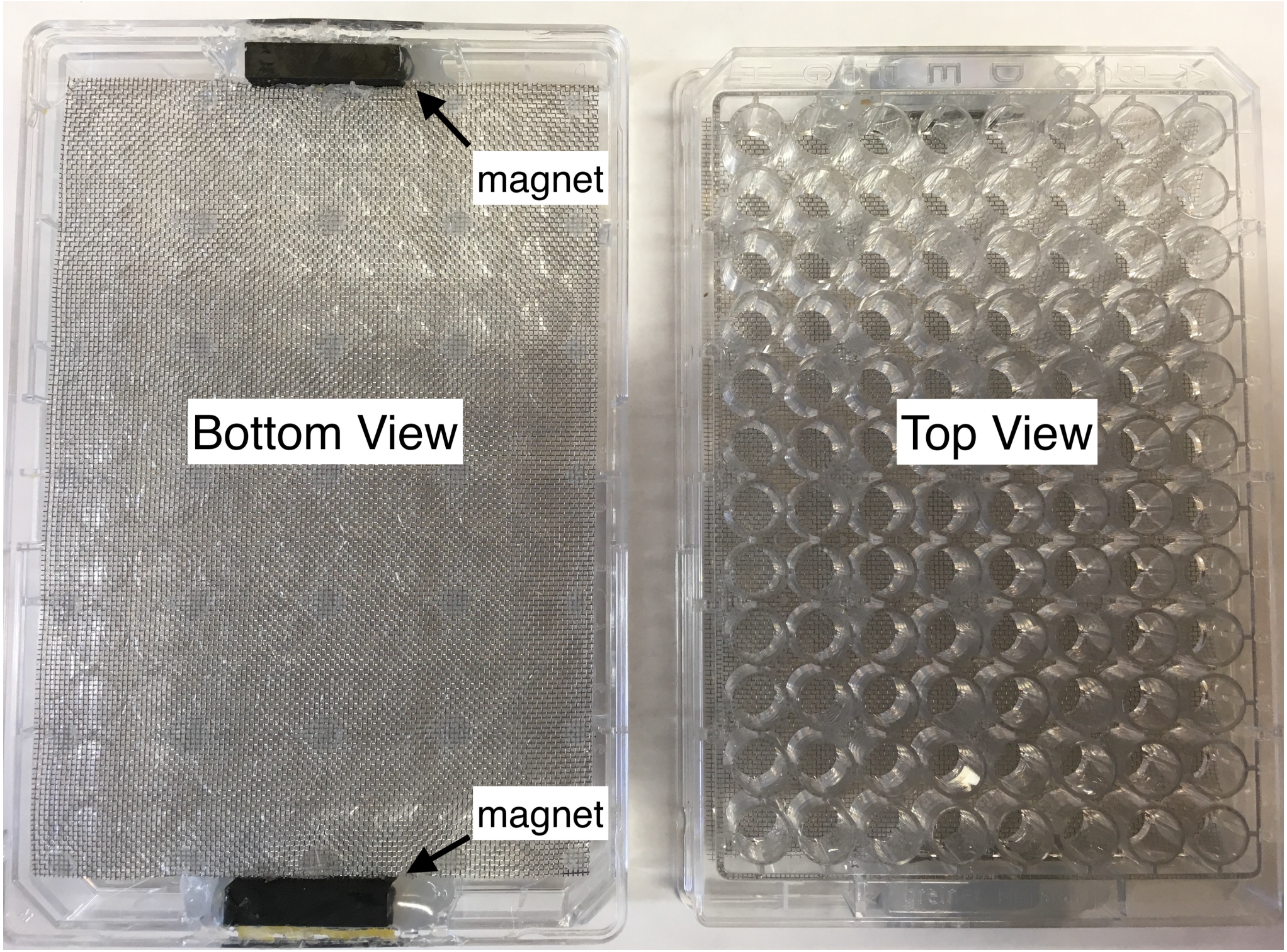
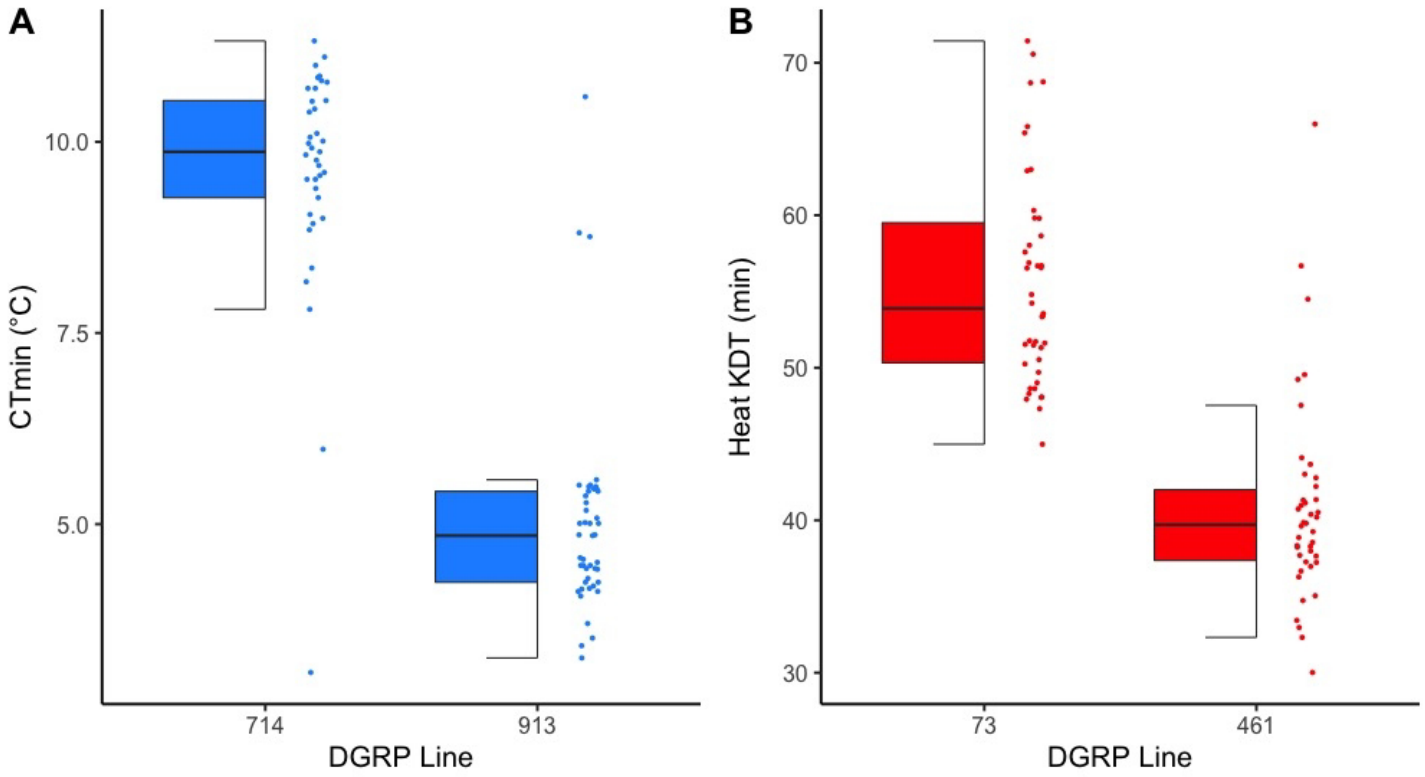


Figure 5

Click here to access/download;Figure;Figure 5.ai



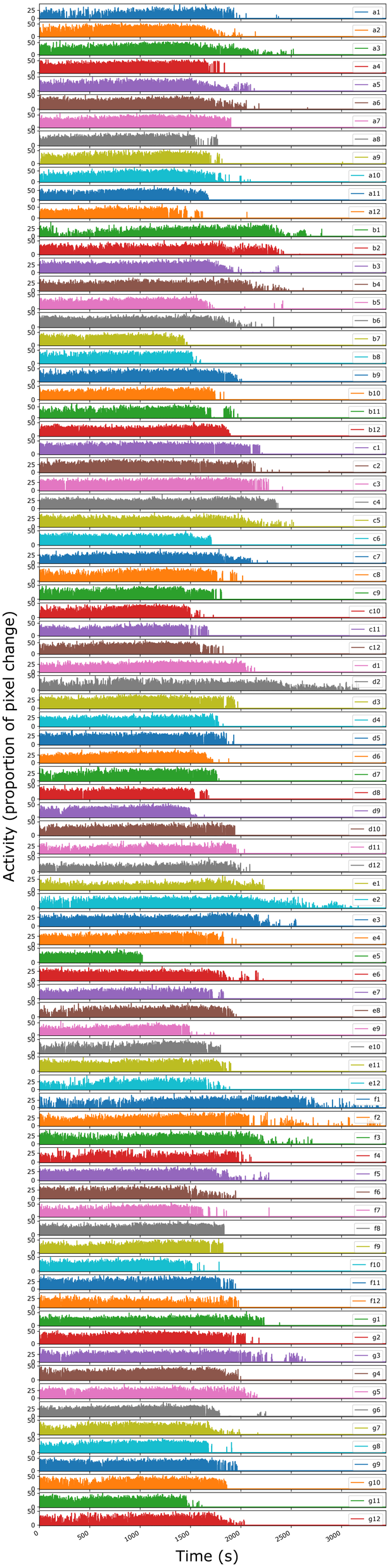


Figure 1

Click here to access/download

Video or Animated Figure

Figure 1.svg

Figure 4

Click here to access/download

Video or Animated Figure

Figure 4.svg

Name of Material/Equipment	Company	Catalog Number
ARCTIC A40 Refrigerated fluid circulator (Programable teperature ramps)	Thermo Scientific; Waltham, MA	153-5401
C922 Pro Stream Webcam	Logitech; Newark, CA	960-001087
Circular adjustable steel clamp – 5.08 cm to 7.62 cm	Any	Any
Clear acrylic tubing – 5.7 cm x 5.1 cm x 0.3 cm	United States Plastic Corp., OH	44036
Clear acrylic tubing – 6.35 cm x 5.7 cm x 0.3 cm	United States Plastic Corp., OH	440515
Clear acrylic tubing – 7 cm x 6.35 cm x 0.3 cm	United States Plastic Corp., OH	44041
Clear silicone sealant	Any	Any
Collection tube (15 ml)	Any	Any
Cordless Drill	Any	Any
Drosophila Funnel Monitor (DFM)	TriKinetics; Waltham, MA	DFM
DAM data collection software	TriKinetics; Waltham, MA	
Fly Storage Lid	FlySorter; Seatle, WA	FS-96LID-5PK
Fly Storage Plate	FlySorter; Seatle, WA	FS-96PLATE-5PK
Fly Food Tray	FlySorter; Seatle, WA	FS-TRAY-5PK
Glass funel	Kimax	28950-75
Gutter gard	Any	Any
Hacksaw	Any	Any
Heratherm Thermo Scientific incubator	Thermo Scientific; Waltham, MA	OMS100
Hose nylon adapters (2) – ¼ MNPT x 3/8	United States Plastic Corp., OH	61135
Hot glue gun and glue	Any	Any
Light Source	Any	Any
Magnets	Any	Any
OMEGA TC-08 Recorder and TC-08 Player Software	OMEGA; Norwalk, CT	
OMEGA thermocouple (Type T)	OMEGA; Norwalk, CT	5LRTC-TT-K-20-36
Plastic funnel	Any	Any
Plastic tubing - 0.6 cm diameter	United States Plastic Corp., OH	62852
Retort ring	Any	Any
Retort stand	Any	Any

Retort three-prong clamp	Any	Any
Rstudio		
Serial port connector (PSIU9)	TriKinetics; Waltham, MA	PSIU9
Styrofoam (2" thick)	Any	Any
Tape	Any	Any
Uninterrupted Power Supply (PS9-1)	TriKinetics; Waltham, MA	PS9-1
Weld-on #4 Acrylic Cement	United States Plastic Corp., OH	45737

Comments/Description

Used to count the number of flies that fall through the funnel at a given time point
Records data input from the DFM
Used to load flies into the storage plate for the sCTmax assay
Used to hold flies during in the sCTmax assay
Used to keep flies on food after loading into the 96-well plate until the sCTmax assay
75mm
~0.5 cm diameter openings

2" diameter

2" diameter

Intermediate connection between the DFM and computer, allows for multiple DFM connections

Power supply for the DFM and PSIU9

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have thoroughly proofread the manuscript and have corrected any spelling or grammatical mistakes.

2. Please define all abbreviations during the first-time use.

All abbreviations have been defined the first time they are used in the text.

3. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: *Styrofoam, DAMSystem3 software, FlySorter Fly Storage Plate, TriKinetics DFM, TriKinetics PSIU9, TriKinetics PS9-1, trikinetics.com, etc*.

We removed all commercial language from the text.

4. 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, alphabets, or dashes. We cannot have non numbered steps or headings in the protocol.

We have fixed the numbering in the protocol. We have also removed the bullet points we used for the notes, and we have included the subheadings with the numbering format.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

We have edited the protocol to describe each step in the imperative tense.

6. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).

All pronouns in the protocol have been removed.

7. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please consider moving lines 140-150 to the introduction section instead.

We have moved this section to the introduction.

8. Please ensure you answer the “how” question, i.e., how is the step performed?

Wherever possible we have made sure to include the specific action required for each step in the protocol.

9. For methods for preparing flies/rearing, please include some citations.

We have included citations to direct readers to information on Drosophila rearing

10. 1: what is being used to perform these steps. Do you use a pair of scissors for cutting the tube? What is the internal diameter of the tubes? Please include all specific details.

We have added the measurements of the tubes to the main text, and included the detail that a hacksaw should be used to cut the tubes. We also include a schematic diagram of the apparatus in Figure 2 to address one of the reviewer's suggestions.

11. 4.9: How do you visually ensure that the flies have reach CTmin?

We have added details to steps 1.39 and 1.39.1 to clarify what "reach their CTmin" means, and how the experimenter can visually ensure that all flies have reached their CTmin.

12. For all the software steps, please include button clicks, knob turns etc.

We have included steps in the protocol to indicate button clicks needed to operate software during the protocol.

13. Please cite the supplementary files wherever it was used in the protocol steps.

We have added a step to the protocol (2.18) to indicate where the supplementary files should be used. We also included a detail in the subsequent note that 2.18 is an optional step that is not required to complete the protocol.

14. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted the steps that we think would be most informative for a researching learning about these protocols.

15. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

We have not use any figures from a previous publication in this manuscript.

16. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

We have highlighted that step 2.19 (recording the KDT of each fly) needs to be consistent across flies, treatments, observers, trials, etc. to ensure that potential biases are not introduced to the data.

b) Any modifications and troubleshooting of the technique

In the procedural comments and potential problems section of the discussion, we have highlighted some troubleshooting steps that may apply when considering different insects or life stages.

c) Any limitations of the technique

We have dedicated a paragraph to limitations and considerations of the techniques in the discussion.

d) The significance with respect to existing methods

This is covered in the discussion (paragraphs 1 and 3).

e) Any future applications of the technique

This is covered in the discussion (paragraph 5).

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Temperature tolerance has attracted a lot of attention as they (thermal limits) are used as proxies to inform scientists about thermal adaptation, sensitivity to climate change etc. As such they methodology has also attracted quite a bit of attention. The current ms described two protocols for improve the throughput of unbiased measurements of CTmin and Time to knock down (static CTmax) in Drosophila melanogaster. Likely, these protocols can be adapted to other taxa successfully. The methodology builds on classic methodology, but improves these by applying high(er) throughput adaptation to these methods. With regards to the methodological discussion, there are several papers pointing to potential biases and problems comparing results and interpreting results e.g. from assays with different temperatures. This does not directly affect this paper, but even of the Jørgensen et al 2019 paper reconciles some of these issues, there are still no clear consensus what signifies an ecological relevant assay and (as it does not really concern this paper) I suggest that authors to keep an methodological approach to their introduction.

We appreciate the word of caution and your suggestion. We have reviewed the introduction and discussion to be sure we have focused our points on the methodology and have not overstated the ecological relevance of measurements used to assess thermal limits.

Major Concerns/Minor Concerns (suggestions and comments):

For the CTmin assay (performed in a "knock-down tube") the authors argue that the device could hold hundreds of flies. I suggest that this might be a bit much (otherwise please provide documentation for this not affecting the outcome) and the authors fail to discuss that only one treatment at the time can be assayed (and this require careful designs to account for diurnal variation in tolerance when comparing groups).

We have tested > 60 flies at once with the set up, but not > 100 flies at once. With sufficient perching material, it shouldn't be an issue to test 100 – 300 flies at once, but we have not directly tested this. Therefore, we changed the wording regarding the number of potential flies (Line 112) .

We agree that careful attention needs to be paid toward experimental design and any variables that may or may not be accounted for. We have made an addition in the "Procedural comments and potential problems" section of the discussion.

Why are CTmin assay description highlighted in yellow?

The highlighted text is a journal requirement to inform the editor and script writers of the steps from the protocol we wish to be used for the video component of this publication.

The "Representative result" are quite limited in extent. In addition, they are compared to published results and not results of the authors own lab. Thus, it is fine to make a qualitative comparison as is done in the ms, but I suggest refraining from a discussion of effect sizes. Different labs have different equipment and likely do things slightly different. More importantly, the units are not similar (degrees versus time), so the comparison of effect sizes are not really a fair or meaningful comparison.

The representative results are limited because the journal specifies to only provide results that were obtained from the described protocol(s). Thus, we were unable to provide a comparison to other methods conducted in our lab, since we did not describe those methods. With this limitation, we do agree that a qualitative comparison of other data sets from other papers is the best approach. We have changed this part of the discussion and provided a qualitative comparison, rather than a discussion comparing effect sizes. Furthermore, as per a suggestion of another reviewer, we have provided a qualitative comparison between the two methods using a common unit (time).

Line 379 "compared" not "compered"?

We have fixed this mistake, thank you for pointing it out.

Line 433 ""…were repeatable…" unclear to what. Among runs in your lab or in comparison to published data on these lines? Please clarify.

We were referring to our own experience handling these lines in the lab. In our hands these results are repeatable between runs in our lab, however we do not show these data, so we have removed this statement from the manuscript.

Line 458 "..three scripts…to(?) process…."?

We have fixed this mistake, thank you for pointing it out.

The 96-well microtitter plate used for time to knock down seems to provide very small space for flies to move (and not at all for anything larger than a D. melanogaster), further, it seems to provide a poor contrast and the deep wells angle, edge and shadow effects that might make detection more difficult. Please elaborate on the experiences and pro's and con's of this vital piece of equipment.

We agree, the 96-well plate was chosen to be suitable for D. melanogaster and other similarly sized insects. The wells provide sufficient space for the flies to move but allow us to maximize the number of measurements that can be taken with a single webcam. We have added text to the discussion to clarify that different sized plates should be considered depending on the size of the test subject.

With respect to the contrast and angles in a 96-well plate, the webcam is situated at the bottom of the incubator and points up at the 96-well plate from below. The plate is suspended upside down (i.e., lid down) from the incubator rack using magnets. This arrangement ensures that no wells are obstructed by the walls of other wells. When a fly reaches its KD time, it falls onto the lid of the plate and reaches its final resting place on the surface closest to the webcam. Therefore, every individual is in view when they reach their heat KDT. We have added a NOTE below step 2.7 to clarify this point.

Finally, given that the DGRP lines are genetically invariable, I am surprised by the seemingly large intra-line variation. Any thoughts on the source of this variation and whether some technical biases are contributing to this variation?

The amount of variation we observed is in line with other studies of the DGRP. Gerken et al. (2018) J. Therm. Biol. found that the survivorship after a rapid cold hardening and a subsequent lethal cold shock treatment varied considerably in some lines (0 – 90% survival) but not others (75% - 85% survival). There are a number of variables that may contribute to this variation, some of which could be accounted for, if necessary. For example, in our experiment, age varied between 5 - 8 days old, which could impact thermal tolerance. That said, we don't think the variation reflects technical artifacts of the thermal tolerance assays themselves. When designing our setup for heat knockdown, we measured temperature across the plate and found minimal well-to-well variation in temperature. We were also concerned that flies were prematurely "slipping" from the column for the CT_{min} measurements, but adding more perching material, and thus decreasing the number of flies clinging directly to the wall, had no effect on CT_{min} estimates (data not shown).

Reviewer #2:

Manuscript Summary:

The authors describe a protocol for measuring static CTmax and CTmin in Drosophila-like insects. While many methods for measuring these traits exist in the literature, the authors have devised a way to do so rapidly with large sample sizes, while supposedly minimizing experimenter effort and error. Briefly, CTmin is performed using the well-known tube jacket method, but has the addition of an infrared sensor

that automatically counts individuals as they fall off their perches due to chill coma. The static CTmax (knock-down temp, KDT) protocol has the addition of a webcam, allowing the researcher to view the video after the experiment to determine the KDT for each individual. The authors suggest the experimental apparatuses for both experiments are relatively adaptable and could be modified to match a researcher's specific insect or question.

Major Concerns:

As a researcher who uses a lot of CTmax and CTmin in my lab, I appreciate a protocol that allows high throughput and minimal error. However, I find this protocol to be quite specific to motile, perching insects, which, while not at all unreasonable, does not do justice to the variety of insects and life stages that are typically of interest to researchers. The authors do mention this as an issue, but I found myself not entirely satisfied with their solutions. Using some non-model insect examples, could the authors suggest ways in which the system could be modified? In addition, with non-model insects, it is sometimes hard to capture or raise adults, but one has access to the sedentary egg, larval, and pupal stages. If the authors suggest a few ways in which the apparatuses could be extended for different life stages, it will increase the general appeal for this paper

Thank you for pointing out this limitation, as we would certainly like for these protocols to be useful for as many systems as possible. To address this concern, we have clarified that the assays might not be suitable for some species or life stages, even with modifications. We have indicated that this assay is ideal motile life stage, i.e., larvae and adults. Our CTmin setup is optimized for perching species, but the video-based knockdown method could be used for CTmin in a cooling incubator, for example for large species like crickets that "slump over" when reaching their thermal limits. However, we do agree that these two assays are unsuitable for sedentary life stages. Testing thermal limits in sedentary life stages would require other methods, such as thermolimit respirometry or assessing survival following a temperature shock, but these approaches are outside the scope of this paper. We have made it clearer that these protocols are designed with subjects similar to adult Drosophila in mind (Lines 141 and 142. Lines 527 and 528, and Lines 531 - 534), and we have provided some possible modifications to the protocols to accommodate other subjects (Lines 144 – 146, and Lines 537 - 541).

Minor Concerns:

1) I was unclear about why the webcam is below the experimental apparatus to record KDT. I couldn't find the explanation in the text, so the authors should make that clearer.

This comment highlights a problem we encountered in earlier setups that was improved in our current method. The webcam points up and films the 96-well plate from below to ensure as much of the well surface is in view as possible, i.e. not obscured from view by the well walls. The plate is suspended upside down, i.e. lid down, using a magnet, so that when flies fall they land on the flat lid of the plate. If the camera were placed above, then portions of wells on the periphery of the plate are obscured by

the well walls. In this situation, flies may reach their final resting place in a portion of the well that can't be viewed by the webcam. We have added a NOTE below step 2.7 to clarify this point.

2) There were highlighted sections of the manuscript that were distracting - were the highlights meant to signify something to the reader?

The highlighted text is a journal requirement to inform the editor and script writers of the steps from the protocol we wish to be used in the video component of this publication.

Reviewer #3:

Manuscript Summary:

The paper presents a nice summary of how to conduct standard measurements of heat/cold knockdown in insects. These methods are already fairly well described in the literature, but the present publication is well focused and contains a number of practical details that will be valuable to newcommers to the field. this includes concrete information on how to build equipment as well as suggestions for automatic video recordings of knockdown. This makes the publication relevant and usefull and I found the paper to be easy to follow. It is also a very well written paper.

Thank you for your positive comments.

Major Concerns:

I have no major concerns - only minor suggestions that the authors can consider (and follow) if they agree.

Minor Concerns:

I think it could be fun and relevant to have a small experiment in which you compare the CTmin/CTmax measured in the knockdown tube, with that measured by individual observation and again to that measured by video - each approach has pros and cons, but a simple comaprasin dat (with data on variance) would be interesting and relevant for this discussion - you could maybe even use it to give some directions as to when the researcher should be cautious. I have seen individual variance from knockdown tubes of > 10C which never occurs when individuals are monitored in individual tubes - this is probably because they fall out of the tube by accident, and I think there are many advantages of more automated systems - but probably more reason to use outlier removal in the automated systems. so my suggestion (to make a good paper better) is to make a small comparison on the same species between the different methods and use this to discuss pros and cons (for heat and cold).

We agree with your first point and certainly see value in such a comparison. Unfortunately the specifications of the journal are to provide representative outcomes following the use of the described protocol(s). This meant that we are unable to provide a comparison to other methods conducted in our lab, since we did not describe those methods. Your suggestion is the logical next step with the tools we have presented here.

I miss the Kelty and Lee paper (2001 as I recall) in the references. this was the first paper I remember that measured CTmin during different ramp rates with an automated system somewhat like yours. but this is maybe just personal preference.

You are correct that the Kelty and Lee (2001) paper does measure CTmin with a similar system to ours. But, it is Kelty and Lee 1999 that investigates the effect of different ramping rates on CT_{min}. We reference the Kelty and Lee (1999) paper when we comment on the possibility of using different ramping rates in the protocol (Reference # 28). Kelty and Lee (2001) is a bit more specific and looks at the effect of rapid cold hardening on a CT_{min} and survival.

I agree that the static assay is preferable when studying small effect sizes - maybe to underline the point say that the dynamic assay is preferable to investigate large effect sizes (i.e. D. melanogaster will knock down in minuttes at 38C, D.mojavensis will continue to be active for days, and it is not usefull to compare large differences in species tolerance in a static assay).

Good point, we agree that a dynamic assay is preferable when there are large differences in heat tolerance. We have added a sentence at the end of the introduction that makes this point (Line 98).

The suggestion in line 130 to measure dynamic CTmax in the video assay is good and another argument for making the small comparison I suggested in the start.

See our reply to your comment above.

Consider to have technical drawings and only have photos as supplement. the photos have the advantage that it is easy to visualise how the system should look, but the technical drawing is what the university workshop will need.

This is a great idea and we have added a technical figure to the manuscript. Considering the nature of the journal we think it is best to have both photos and technical drawings in the manuscript.

Line 311: You recommend to anesthetize the flies with CO2 before the measurement. I would remove this. you can use CO2 a few days ealier to sex the flies, but ideally the should not be anesthetized just prior to measuement - there are lots of studies to show this is a problem (particularly for cold assessments) - you can just aspirate the flies and then sex them after the test. I know this is a minor detail - but it is important eventhough it gives problems when loading the flies (you could suggest to do this in a cool room to reduce activity).

These are good points about using CO^2 prior to testing thermal limits. For our setup, the flies need to be subdued in some way to remove and replace the septum lid with a clear lid after loading. The clear lid is necessary for video recording. For the CT_{min} assay we need to ensure that all the flies are the same sex since the DFM cannot differentiate sex as the flies fall through. Therefore we have tried to

mitigate the effects of CO^2 by giving the flies 48 h to recover from being anesthetized. We have clarified that flies anesthetized with CO^2 should be tested 48 h later in step 2.6 and the NOTE below step 2.6 in the protocol.

Line 350 - just as in the knockdown tube I think it is important to record temperature in the 96 well setup - maybe I missed this, but make sure to show that you also register temperature precisely (in one of the wells).

We agree with this comment and have added an additional NOTE after step 2.13 in the protocol.

As mentioned I think a few more experiments where you also compare methods of observation would be interesting.

See our reply to the first comment above.

Line 438: I suggest you don't compare % difference between static and dynamic assays. The two methods measure the same physiological dysfunction (See jørgensen where she can model the dynamic CTmax from static data). However the units are different and it is not a fair comparison as the base value should not be degrees 0 on either a kelvin or Celcius scale. I wholeheartedly agree that static assays can be better to investigate smaller differences - but I think you are causing confusion with the % change comment. A more meaningful comparison would be the difference in time - here you compare two treatments that take 40 and 55 min (15 min difference) and relate it to the largest diff in dynamic CTmax across the DGRP (1.5C) (I cant remember the ramp rate used for these DGRP data, but if this was 0.1C per min, then this diff is actually also 15 min). I DO NOT think you can compare these time differences directly (in one instance injury rate is constant in the other it is increasing) - but his is at least a more fair comparison. My suggestion is that you do not comment on these percentage changes as you are comparing apples and oranges.

You have pointed out a similar issue as another reviewer, and we thank you for suggesting an alternative approach. We have changed this part of the discussion and provided a qualitative comparison, rather than a quantitative comparison of effect sizes. Furthermore, as per your suggestion, we have provided a qualitative comparison using the common unit, time.

I will end by congratulating with a nice paper - from a purely methodological point of view the paper is fine as it is - but I believe you could make the paper even more relevant if you measured dynamic and static CTmin and CTmax with different assays (collumn/96 well) and different forms of observation (Automated/researcher) - Such a small comparison of means and variances would be relevant to the choice of method for many.

Thank you again for your positive comments.

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01-FirstFrame-v1.0.py

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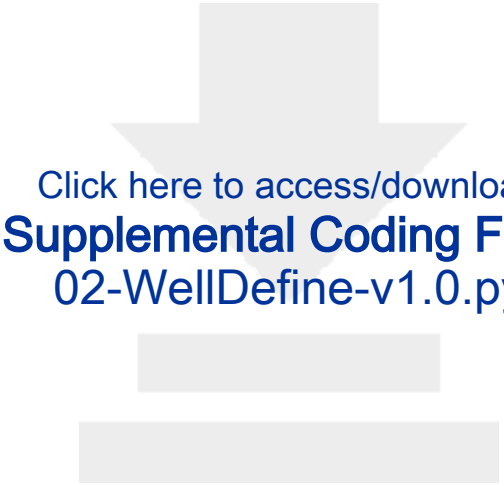

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