1 **TITLE:**
2 An Automated Method to Determine the Performance of *Drosophila* in Response to
3 Temperature Changes in Space and Time
4
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27 **KEYWORDS:**
28 Temperature-controlled arena, locomotor behavior, *Drosophila*, temperature performance,
29 automatic heating mechanism, positional tracking
30
31 **SUMMARY:**
32 Here, we present a protocol to automatically determine the locomotor performance of
33 *Drosophila* at changing temperatures using a programmable temperature-controlled arena
34 that produces fast and accurate temperature changes in time and space.
35
36 **ABSTRACT:**
37 Temperature is a ubiquitous environmental factor that affects how species distribute and
38 behave. Different species of *Drosophila* fruit flies have specific responses to changing
39 temperatures according to their physiological tolerance and adaptability. *Drosophila* flies also
40 possess a temperature sensing system that has become fundamental to understanding the
41 neural basis of temperature processing in ectotherms. We present here a temperature-
42 controlled arena that permits fast and precise temperature changes with temporal and spatial
43 control to explore the response of individual flies to changing temperatures. Individual flies
44 are placed in the arena and exposed to pre-programmed temperature challenges, such as
45 uniform gradual increases in temperature to determine reaction norms or spatially
46 distributed temperatures at the same time to determine preferences. Individuals are
47 automatically tracked, allowing the quantification of speed or location preference. This

48   method can be used to rapidly quantify the response over a large range of temperatures to
49   determine temperature performance curves in *Drosophila* or other insects of similar size. In
50   addition, it can be used for genetic studies to quantify temperature preferences and reactions
51   of mutants or wild-type flies. This method can help uncover the basis of thermal speciation
52   and adaptation, as well as the neural mechanisms behind temperature processing.
53
54   **INTRODUCTION:**
55   Temperature is a constant environmental factor that affects how organisms function and
56   behave[1]. Differences in latitude and altitude lead to differences in the type of climates
57   organism are exposed to, which results in evolutionary selection for their responses to
58   temperature[2,3]. Organisms respond to different temperatures through morphological,
59   physiological, and behavioral adaptations that maximize performance under their particular
60   environments[4]. For instance, in the fruit fly *Drosophila melanogaster*, populations from
61   different regions have different temperature preferences, body sizes, developmental times,
62   longevity, fecundity, and walking performance at different temperatures[2,5–7]. The diversity
63   observed between flies of different origins is explained in part by genetic variation and plastic
64   gene expression[8,9]. Similarly, *Drosophila* species from different areas distribute differently
65   among temperature gradients and show differences in resistance to extreme heat and cold
66   tests[10–12].
67
68   *Drosophila* has also recently become the model of choice to understand the genetic and
69   neural basis of temperature perception[13–17]. Broadly, adult flies perceive temperature
70   through cold and hot peripheral temperature sensors in the antennae and through
71   temperature sensors in the brain[13–20]. The periphery receptors for hot temperatures express
72   *Gr28b.d*[16] or *Pyrexia*[21], while the periphery cold receptors are characterized by *Brivido*[14]. In
73   the brain, temperature is processed by neurons expressing *TrpA1*[15]. Behavioral studies on
74   mutants of these pathways are improving our understanding of how temperature is
75   processed and give insights into mechanisms that vary among populations of *Drosophila* from
76   different regions.
77
78   Here we describe a temperature-controlled arena that produces fast and precise temperature
79   changes. Investigators can pre-program these changes, which allows for standardized and
80   repeatable temperature manipulations without human intervention. Flies are recorded and
81   tracked with specialized software to determine their position and speed at different phases
82   of an experiment. The main measurement presented in this protocol is the walking speed at
83   different temperatures, because it is an ecologically relevant index of physiological
84   performance that can identify individual thermal adaptability[5]. Together with temperature
85   receptor mutants, this technique can help reveal the mechanisms of thermal adaptation at
86   cellular and biochemical levels.
87
88   **PROTOCOL:**
89
90   **1. Preparation of fly food medium**
91
92   1.1. Pour 1 L of tap water into a 2 L glass beaker and add a magnetic stir bar. Put the beaker
93   on a magnetic hot plate at 300 °C until boiling temperature is reached.
94

95   1.2. Stir at 500 rounds/min and add the following: 10 g of agar, 30 g of glucose, 15 g of sucrose,
96   15 g of cornmeal, 10 g of wheat germ, 10 g of soy flour, 30 g of molasses, and 35 g of active
97   dry yeast.
98
99   1.3. When the mix foams vigorously, turn down the hot plate temperature to 120 °C while
100 continuing stirring.
101
102 1.4. Turn the hot plate temperature further down to 30 °C after 10 min and continue stirring
103 until the mix cools to 48 °C. Measure the temperature by inserting a thermometer directly
104 into the food without touching the walls of the beaker.
105
106 1.5. Dissolve 2 g of p-hydroxy-benzoic acid methyl ester into 10 mL of 96% ethanol and add it
107 to the mix, together with 5 mL of 1 M propionic acid. Continue stirring for 3 min.
108
109 1.6. Turn the hot plate off and pour 45 mL of food into the rearing bottles and 6.5 mL of food
110 into the collection vials.
111
112 **2. Preparation of flies**
113
114 2.1. Place 20 male and 20 female flies in the rearing bottles containing 45 mL of fly food
115 medium. Transfer the flies to new bottles after 3 to 4 days by tapping them down and then
116 tapping them into the fresh bottles. Discard the flies after three changes.
117
118 2.1.1. Place the bottles inside the incubator under 12-h light/12-h dark cycles with a constant
119 temperature of 25 °C.
120
121 NOTE: A new generation of flies will eclose after three changes.
122
123 2.2 Anesthetize newly eclosed flies on carbon dioxide pads for a maximum of 4 min and collect
124 them in 2.5 cm x 9.5 cm fly rearing vials with 6.5 mL of fly food medium using a paintbrush.
125
126 2.2.1 Collect only virgin flies and separate them by sex into groups of 20 flies per rearing vial.
127
128 2.2.2 Place the vials inside incubators for 5-7 days, changing the flies to new vials every 2-3
129 days and on the days before experiments.
130
131 **3. Frame of lights**
132
133 3.1. Make a wooden frame of 10 cm length, 4 cm width, 4 cm height, and 0.5 cm thick.
134
135 3.2. On each of the short edges, create a border of 4 cm length, 4 cm height, and 1.5 cm width
136 towards the inside area of the wooden frame. Leave the internal face of the border open.
137
138 3.3. Drill two holes of 0.5 cm diameter at the intersection of one of the long edges of the
139 wooden frame and at each of the borders at the short edges.
140

141   3.4. Place 10 cm of a warm white LED strip inside each of the borders on the short edges. Peel
142   the back of the LED strip to immediately glue it in place.
143
144   NOTE: For experiments in which illumination needs to be eliminated, the warm white LED
145   strip can be substituted for infrared LED strips.
146
147   3.5. Connect one end of the LED strip in one of the borders to the switching power supply and
148   its other end to the LED strip on the opposite border.
149
150   3.6. Turn the switching power supply on to verify that both LED strips turn on.
151
152   3.7. Cover the open side of each border with a white piece of paper.
153
154   3.8. Glue another piece of paper to each of the internal phases of the long edges.
155

4. Temperature-controlled arena

157
158   4.1. Turn on the temperature-controlled arena (**Figure 1A,C**). Ensure that the fan starts
159   running and the aluminum ring starts warming up.
160
161   4.2. Use a USB cable to connect the temperature-controlled arena to the control computer
162   running the *TemperaturePhases* script with the temperature sequences.
163
164   4.3. Open the *TemperaturePhases* script in the control computer and verify that the
165   temperature sequence is properly set up (**Video 1**).
166
167   4.3.1. Check that the duration of each experimental phase is set to 60 s by verifying that
168   "par.StimulusDur" is equal to 60 s.
169
170   4.3.2. Check that the 1) number equal to 23 phases, 2) iterative ON/OFF set-up of the
171   indicative red light emitting diodes (LEDs), 3) 2 °C temperature increase per phase, and 4) 16
172   °C as the starting temperature are all correct under the "Start the experimental block"
173   section.
174
175   NOTE: Allow the flies to acclimate to the Fly Arena for 7 min at 16 °C to avoid an artificial
176   increase of speed during the first experimental phases (**Figure 2**).
177
178   4.3.3 Run the *TemperaturePhases* script. The software will initialize for 5 seconds as
179   determined in "arena.Wait", then stop press the keyboard key to continue.
180
181   4.3.4 Press the spacebar of the keyboard to begin running the experimental phases once a fly
182   has been blown into the Fly Arena (step 5.3).
183
184   NOTE: The *TemperaturePhases* is the current script controlling the box; however, it is possible
185   to create other custom scripts to use this device that adjust to the requirements of different
186   experiments.
187

188 4.4. Connect the camera on top of the arena to the recording computer using the camera's
189 USB cable.
190
191 4.5. Open the video recording program (see **Table of Materials**) in the recording computer by
192 selecting **File | New Movie Recording**. A screen showing the image from the camera will
193 open.
194
195 4.5.1 Ensure that the camera image captures all edges of the arena and the indicative red
196 LEDs.
197
198 4.5.2 Start recording by pressing the red button in the middle of the screen's bottom edge
199 showing the camera image once the frame of lights is set around the arena (step 5.4).
200
201 NOTE: Small changes in lighting can affect accuracy of the tracking. It is recommended to keep
202 the illumination of the temperature-controlled arena constant by fixing the location of the
203 apparatus.
204
205 **5. Temperature behavioral experiments**
206
207 5.1. Prepare the Fly Arena (**Figure 1C**).
208
209 5.1.1 Place a strand of white conductive tape on the top of the copper tiles, ensuring all edges
210 are covered.
211
212 5.1.2. Place the heated aluminum ring around the copper tiles. The edge of the ring fits
213 perfectly around the copper tiles so it is always placed in the same location.
214
215 5.1.3. Clean the glass cover with a clean tissue and place it on the top of the aluminum ring,
216 leaving a gap through which a fly can be blown in.
217
218 NOTE: Before the experiments, coat the glass cover with the siliconizing agent to create a
219 slippery surface. Apply the siliconizing agent for 24 h and rinse it with water before use.
220
221 5.2. Run the *TemperaturePhases* script (step 4.3.3) and open the video recording program
222 (step 4.5).
223
224 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**).
225
226 5.3.1 Take a vial of flies from the incubator, tap it twice to force them to go to the bottom,
227 trap one fly with a mouth aspirator, and close the vial and put it back into the incubator.
228
229 5.3.2. Place the fly in the arena through the gap that has been left between the glass cover
230 and aluminum ring (step 5.1.3).
231
232 5.3.3. Close the gap between the glass cover and aluminum ring by pushing the glass cover
233 until it reaches the edge of the aluminum ring as soon as the fly is introduced to the Fly Arena.
234

235   5.4. <mark>Place the frame of lights around the arena</mark> to ensure symmetric illumination.
236
237   5.4.1. Mark the location (e.g., using a permanent marker) of the frame of lights around the
238   Fly Arena (**Figure 1C**) to ensure that the frame is always placed in the same location.
239
240   5.5. <mark>Start recording with the video recording program</mark> (step 4.5.2) <mark>and press the spacebar on</mark>
241   <mark>the keyboard of the control computer to begin running the experimental phases</mark> (step 4.3.4).
242
243   5.6 After all experimental phases are done, save the video in .mp4 or .avi format and remove
244   the fly from the Fly Arena with the mouth aspirator.
245
246   NOTE: The end of the experimental phases can be determined by both indicative red LEDs
247   being turned off or by the *TemperaturePhases* script stopping.
248
249   5.6.1 Stop the video recording by pressing the stop button in the middle of the screen's
250   bottom edge in the recording program. Press **File | Save us** to save the video.
251
252   <mark>**6. Video tracking and data analysis**</mark>
253
254   6.1. <mark>Use the *FlySteps* tracking software (**Video 2**)</mark> to track the videos.
255
256   6.1.1. <mark>Open the "configuration_file.ini" inside the "FlyTracker" folder.</mark>
257
258   6.1.2. <mark>Set the location of the videos in "video_folder" and the names of the videos in</mark>
259   <mark>"video_files".</mark>
260
261   6.1.3. <mark>Specify the borders of the Fly Arena in "arena_settings" based on (x, y) pixel coordinates</mark>
262   <mark>of multiple points at the edge of the arena.</mark>
263
264   6.1.4 <mark>Specify the location of the indicative red LEDs in "led_settings" based on (x, y) pixel</mark>
265   <mark>coordinates of the location of the center of the LEDs.</mark>
266
267   6.1.5 <mark>Check the location of the borders of the Fly Arena by setting "debug" to "true" in</mark>
268   <mark>"arena_settings", clicking **Save**, and running the script in the terminal. A screen capture of the</mark>
269   <mark>video will appear with a blue square formed by the coordinates inputted in "arena_settings".</mark>
270
271   NOTE: This square surround the area to be tracked.
272
273   6.1.6 <mark>Change "debug" in "arena_settings" to "false", click **Save**, and run the screen in the</mark>
274   <mark>terminal once more.</mark> This will start the tracking process.
275
276   NOTE: Flies can walk out of the tracking area onto the heated aluminum ring. This happens
277   during the first seconds of an experiment, after which flies stop touching the heated ring and
278   remain inside the tracking area. Videos can be tracked with other tracking software according
279   to the experimenter's preferences.
280

281 6.2. Use the (x,y) location of each fly provided by the tracking software to calculate the
282 measure of interest for the temperature performance. Custom scripts (e.g., *FlyStepsAnalysis*
283 in **Supplementary**) can be used.

284

285 6.3. Compare the temperature performance curves of different fly groups using repeated
286 measurements (RM) analysis of variance (ANOVA) and *post-hoc* multiple comparisons using
287 statistical software (see **Table of Materials**).

288

289 **REPRESENTATIVE RESULTS:**
290 The temperature-controlled arena (**Figure 1A**) consists of three copper tiles whose
291 temperature can be individually controlled through a programmable circuit. Each copper tile
292 possesses a temperature sensor that gives feedback to the programmable circuit. The circuit
293 activates a power supply to increase the temperature of each tile. Passive thermoelectric
294 elements act as constant heating elements to maintain the desired temperature, while a heat
295 sink cooled by a fan provides constant cooling. The magnitude of temperature change
296 determines the speed of the process in a non-linear manner. An increase of 2 °C requires only
297 0.1 s, and an increase of 18 °C requires 4 s. A screen connected to the programmable circuit
298 (**Figure 1C**) informs the user of the temperature measured by the temperature sensors in
299 each of the tiles. The copper tiles are surrounded by an aluminum ring constantly heated to
300 50 °C (**Figure 1B,C**) by semiconductors around the periphery. This ring forms the edges of the
301 Fly Arena (**Figure 1C**), the area in which flies are to be placed. The Fly Arena is covered by a
302 siliconized glass cover (**Figure 1A,C**), which provides a 3 mm high space which ensures that
303 flies can walk but not fly. Next to the Fly Arena are two red LEDs (**Figure 1C**) that can be
304 programmed to mark different experimental phases. For example, for the results shown in
305 **Figure 2A**, each LED is associated with a different temperature, while in **Figure 2B**, each LED
306 indicates 60 s. The *FlySteps* software can register when each of the indicative LEDs is on, and
307 the researcher can then use this information to automatically determine the experimental
308 phases based on temperature or time.

309

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

320

321 The temperature-controlled arena can also be used to explore the response to conditioning
322 experiments based on temperature. The simplest form of this approach is an operant
323 conditioning paradigm in which flies are trained to prefer one side of the arena over the other,
324 by warming up the side that will be avoided[23-25]. We exposed individual flies to 40 °C in the
325 middle and one of the side tiles, while leaving the other side tile at a comfortable 22 °C (**Figure
326 4**). Wild-type flies quickly stopped moving along the arena and remained in the comfortable
327 location. In contrast, the classic memory mutant *Dunce* kept exploring the arena and spent

328   less time than controls in the comfortable location. The differences between performance of
329   the wild-type flies and *Dunce* mutants became larger when all tiles were set to 22 °C and
330   comparisons were made between the treatment groups. *Dunce* mutants also showed greater
331   differences between training and test phases in comparison to the wild-type flies (**Figure 4**).
332   These results suggest an effect of memory on remaining in the comfortable location.
333
334   Combinations of temperature and location are also useful to understand the function of
335   different temperature receptors during dynamic temperature changes. We exposed
336   individual *D. melanogaster Gr28b.d* and *TrpA1^{GAL4}* mutants to increasing temperatures (2 °C
337   increase every 60 s) while providing a comfortable location at 22 °C (**Figure 5**). The
338   comfortable location shifted from left to right, and vice versa, per iteration. Results show that
339   the periphery temperature receptor *Gr28b.d* mutants behave as the control, as they spend
340   more time in the comfortable location as temperature increases. However, brain
341   temperature receptor *TrpA1^{GAL4}* mutants are not affected by increasing temperatures and do
342   not change their locations in the arena. The increases and decrease in the curve of *TrpA1^{GAL4}*
343   mutants show the effect in flies that were already sitting in the comfortable location before
344   it became comfortable and remained there during that phase. The consistency of peaks and
345   valleys of the curve of *TrpA1^{GAL4}* suggest that these flies remained still for most of the
346   experiment; hence, they were constantly counted when their location was the one
347   considered comfortable. This conclusion was confirmed by visual inspection of the recorded
348   videos. These results support previous physiological reports suggesting that periphery
349   perception of fast and large changes does not depend on *Gr28b.d*[17] and that flies possess a
350   main central mechanism to sense temperature based on *TrpA1*[14,21].
351
352   **FIGURE LEGENDS:**
353   **Figure 1: Diagram of temperature controlled-arena**. (**A**) A lateral view of the temperature-
354   controlled arena. A programmable circuit connects a power supply and temperature sensors
355   to heating elements under copper tiles to control their temperature. Tiles are constantly
356   cooled down through a heat sink connected to a fan. A heated aluminum ring over which a
357   glass cover rests surrounds the tiles. (**B**) Thermal imaging showing the tiles set at 24 °C (top)
358   and side tiles at 24 °C with a middle tile at 30 °C (bottom). (**C**) A top view of the arena. A
359   camera records the copper tiles, aluminum ring, and red LEDs, then automatically determines
360   experimental phases. A screen in the corner of the box, not recorded by the camera, displays
361   the current tile temperature. (**D**) Ring of light: two warm white LED strips inside a wooden
362   box covered in white paper ensure constant and symmetric illumination of the whole arena.
363
364   **Figure 2: Flies must acclimate to the arena before starting the temperature protocol**. (**A**)
365   Single male flies were introduced to the arena and allowed to explore at a constant 16 °C for
366   1 min, after which the temperature started increasing. (**B**) Single flies exposed to 16 °C, 20 °C,
367   or 24 °C (no group differences; two-way ANOVA $F_{(2,570)}$ = 4.156, p = 0.162) have a higher
368   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
369   virgin female flies 5 to 7 days old tested over multiple days. Asterisk indicates significant
370   difference among groups (****p < 0.0001; Tukey's multiple comparison test, p = 0.05).
371
372
373   **Figure 3: Locomotion of 5 *Drosophila* species exposed to gradually increasing temperatures.**
374   Individual male flies from temperate (blue), tropical (red), and cosmopolitan (brown)

375 *Drosophila* species were exposed to an increasing temperature gradient (2 °C every 60 s)
376 between 16 and 46 °C. The first 7 min were constantly at 22 °C to allow flies to explore the
377 arena. Species were significantly different (two-way RM ANOVA $F_{(4,70)} = 28.46$, $p < 0.001$).
378 (**A**) *D. melanogaster* (brown; filled circles) was faster when introduced to the arena. (**B**) *D.*
379 *yakuba* (red; empty squares) was faster as temperature increased. (**C**) *D. suzukii* (brown; filled
380 square) was slower than the other cosmopolitan flies at its maximum performance point. (**D**)
381 *D. simulans* (brown; empty circles) was in decay at the maximum point of *D. melanogaster.*
382 Each point represents the mean (± SEM) of 15 male flies 5 to 7 days old tested over several
383 days. Significance indicated by symbols (♦ = difference from all, $p < 0.0001$; † = difference
384 from all except *D. melanogaster,* $p < 0.0001$; • = difference from *D. melanogaster,* $p < 0.01$;
385 ¢ = difference from *D. melanogaster,* $p < 0.001$; **** = difference between named groups, p
386 < 0.0001; Tukey's multiple comparison test, $p = 0.05$).
387
388 **Figure 4: The temperature-controlled arena can be used for operant conditioning.** *D.*
389 *melanogaster* Canton-S strain (wild-type; black border) and *dnc^1* (*Dunce*; red border) mutants
390 were trained to prefer a lateral tile at 22 °C after warming the middle and opposite lateral
391 tiles to 40 °C for 4 min (training, no pattern). Memory of the heated areas is then tested by
392 setting all tiles to 22 °C (test; grid pattern). Flies were conditioned to prefer tiles on the left in
393 half of the experiments, then tiles on the right in the other half. The percentage of total time
394 inside the tile at 22 °C during training and testing was measured to compare performances.
395 Groups were significantly different (one-way ANOVA $F_{(3,76)} = 23.23$, $p < 0.0001$), with *Dunce*
396 performing worse than wild-type overall. Data are mean (± SEM) of 20 virgin female flies 5 to
397 7 days old tested over several days. Asterisks indicate significance difference among groups
398 (****$p > 0.0001$; ***$p > 0.001$; **$p > 0.01$; Tukey's multiple comparison test, $p = 0.05$)
399
400 **Figure 5: Response of temperature mutants to increasing temperature when a comfortable**
401 **location is provided.** Temperature mutants *Gr28b.d* (green; squares) respond as controls
402 (*w^{1118}*, black; circles) by increasing the percentage of time in the comfortable area as
403 temperature increases (two-way RM ANOVA $F_{(1,38)} = 0.5107$, $p = 0.479$). *TrpA1^{GAL4}* mutants
404 (yellow; triangles) are different from controls (*w^{1118}*, black), as they do not increase the time
405 in the comfortable area as temperature increases (two-way RM ANOVA $F_{(1,38)} = 1.670$, $p =$
406 0.019). Data are mean (± SEM) of 20 male flies 5 to 7 days old tested over several days.
407 *TrpA1^{GAL4}* is significantly different from *Gr28b.d* and the control ($p < 0.05$; Tukey's multiple
408 comparison test, $p = 0.05$).
409
410 **DISCUSSION:**
411 Here we have presented an automated temperature-controlled arena (**Figure 1**) that
412 produces precise temperature changes in time and space. This method allows exposure of
413 individual *Drosophila* not only to pre-programmed gradual increases of temperature (**Figure**
414 **2** and **Figure 3**), but also to dynamic temperature challenges in which each tile of the fly arena
415 was heated independently to a different temperature (**Figure 4** and **Figure 5**).
416
417 The temperature-controlled arena uses an innovative approach to the heating process.
418 Instead of producing temperature changes in the tiles through thermoelectric Peltier heating
419 elements used in traditional methods, the temperature-controlled arena uses current to
420 warm up a copper mass with the copper tiles, and flies are placed at the top. The copper mass
421 is constantly cooled down by a heat sink block connected to a fan. Peltier-like elements are

422 used to maintain the desired temperature of the copper mass once it has been warmed up.
423 Because these elements are not the main temperature generators, they suffer less stress,
424 which extends their life span and permits faster temperature changes. A programmable
425 circuit that receives feedback from temperature sensors under each of the copper tile, which
426 can also activate the low voltage power supply, coordinates the heating mechanism.
427 Researchers can specify when and where temperature changes occur and determine the
428 intensity and direction of such changes. Furthermore, coupling the method with specialized
429 tracking software, such as *FlySteps*, permits analysis of all aspects relating to *Drosophila's*
430 movement, such as the overall speed at certain temperatures or time spent in certain
431 locations (**Figures 2-5**). Nevertheless, all results must consider characteristics inherent to fly
432 behavior that might affect their locomotion. For example, if flies are not allowed to explore
433 the arena and settle before changing the temperature, speed measurements might be
434 artificially high (**Figure 2**). Flies can also leave odorants that affect subsequent flies; hence,
435 the glass cover must be cleaned, and tape covering the tiles must be changed between
436 subjects. Given that locomotion declines as flies age[26], it is important that flies are
437 standardized for age to avoid variation in results. In our arena, flies have also shown
438 centrophobism, preferring edges over the middle area. Experimenters must control for this
439 by changing the location of comfortable areas to prevent overestimating site preference.
440
441 The current characteristics of the arena and requirements of the tracking process could limit
442 some experimental procedures. For example, the close environment of the arena does not
443 include access points through which odours could be introduced, which prevents studies in
444 which this stimulus is important. Similarly, the *FlyStepts* tracker necessitates videos with
445 uniform backgrounds, which limits the possibility of adding food or other items to the fly's
446 environment. The arena could be adapted to include a connection to a gas valve, and software
447 developments exist that may allow for more objects to be present. Future projects may take
448 advantage of these possibilities to adapt the temperature-controlled arena to specific
449 experimental needs.
450
451 Finally, we have shown in the results that different species of *Drosophila* perform differently
452 as temperature increases (**Figure 3**) and that temperature mutants do not respond in the
453 same way as controls (**Figure 5**). This shows that this new method may be used to explore
454 *Drosophila's* thermal behavior and how it is affected by natural selection and functional
455 characteristics. Finally, it illustrates that our method may help further understanding of
456 thermal adaptation and speciation as well as the interactions of temperature receptors with
457 other stimuli in future studies.
458
459 **DISCLOSURES:**
460 The authors declare that they have no competing financial interests.
461

469

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

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