A simple and dynamic thermal gradient device for measuring thermal performance in small ectotherms

Marshall W. Ritchie*, Jeff W. Dawson, Heath A. MacMillan

Department of Biology, Carleton University, Ottawa, Canada, K1S 5B6

*Author for correspondence: MarshallRitchie@cmail.carleton.ca

Keywords: thermal gradient; thermal tolerance; thermal performance; temperature; ectotherms

Abstract

The body temperature of ectothermic animals is heavily dependent on environmental temperature, impacting fitness. Laboratory exposure to favorable and unfavorable temperatures is used to understand these effects, as well as the physiological, biochemical, and molecular underpinnings of variation in thermal performance. Although small ectotherms, like insects, can often be easily reared in large numbers, it can be challenging and expensive to simultaneously create and manipulate several thermal environments in a laboratory setting. Here, we describe the creation and use of a thermal gradient device that can produce a wide range of constant or varying temperatures concurrently. This device is composed of a solid aluminum plate and copper piping, combined with a pair of programmable refrigerated circulators. As a simple proof-of-concept, we completed single experimental runs to produce a low-temperature survival curve for flies (*Drosophila melanogaster*) and explore the effects of daily thermal cycles of varying amplitude on growth rates of crickets (*Gryllodes sigillatus*). This approach avoids the use of multiple heating/cooling water or glycol baths or incubators for large-scale assessments of organismal thermal performance. It makes static or dynamic thermal experiments (e.g., creating a thermal performance or survival curves, quantifying responses to fluctuating thermal environments, or monitoring animal behaviour across a range of temperatures) easier, faster, and less costly.

Introduction

The thermal environment can directly impact organismal survival and fitness, and small ectotherms can respond to changing thermal environments by altering behaviour or physiology within the lifetime of an individual or over evolutionary time [1,2]. Our understanding of how animals respond to their thermal environment has come largely from studies focused on small ectothermic animals with short generations times that can be reared rapidly and inexpensively in the laboratory. As insects are easily manipulated and maintained in the lab, model insect species are widely used for studies of the genetics, molecular biology, environmental physiology, behaviour, and evolution of thermal performance traits [3–7]. Laboratory studies of *Drosophila* thermal tolerance, for example, have recently allowed for investigation of the molecular or physiological processes that limit their biogeography, and how limits to thermal performance may evolve following changes in abiotic conditions [4,8–10].

A common measure of thermal tolerance is survival following high or low-temperature exposure. Survival experiments are typically assessed using one of two different experimental designs. The first is prolonged exposure to a single lethal temperature [11,12]. This approach is limited to the selected exposure temperature, which may or may not be the ideal temperature to measure the thermal tolerance of the chosen species, or discriminate variation in thermal tolerance among treatment groups. An alternative approach is to use a range of exposure temperatures [13–16]. This approach is used to generate thermal tolerance or performance curves by examining, for example, the effects of temperature on survival [17], growth rates [18], reproductive capacity [8], motor performance [19] or behaviour [20]. In these experiments, different individuals are exposed to different static temperatures to examine underlying

49 temperature effects on life history and to understand better how prior thermal experiences might
50 influence these relationships [19,21,22].

51 In nature, animals do not experience a drastic increase or decrease in temperature but
52 rather experience gradual temperature increases or decreases as well as non-linear and stochastic
53 temperature profiles [2,23]. These predictable or unpredictable changes in temperature affect
54 insects differently than would a sudden increase or decrease in temperature applied in a typical
55 lab setting [23]. Experiments involving ramping temperatures are now common [24–26], and
56 there is concern over the effects of the rate of temperature change on the experimental outcome.
57 In light of this concern, some authors have directly measured the effects of different ramping
58 rates on thermal performance traits [27–29], choosing a handful of ramping rates to test.
59 Ramping temperatures are just one example that demonstrates a growing appreciation for
60 thermal performance and evolution studies that embrace complexity of natural thermal
61 environments as well as the complexity of physiological responses to those environments [30].
62 Experimental design, however, can strongly influence research outcomes, and several factors
63 limit our ability to test a wide range of conditions at once.

64 When performing an experiment that requires multiple constant temperatures or
65 programmed temperature changes over time, it can be challenging to maximize the number of
66 different treatments available because of limited equipment and keep unintended sources of
67 variation among treatments to a minimum. Variation in the resulting data from such experiments
68 can come from either using multiple heating/cooling devices all running at different temperatures
69 or having to use individual specimens from multiple successive generations or of varying age
70 because of limited capacity or versatility of the equipment or time. While accounting for these
71 variables statistically is sometimes an option, controlling for them is preferred. In addition to

72 potentially impacting data quality, equipment limitations can significantly increase the resources
73 and time required to perform a thermal experiment, thereby restricting both data quality and
74 quantity.

75 Here, we describe the manufacturing and testing of a thermal gradient plate and
76 associated thermal bath setup that can streamline thermal experiments. We believe that our
77 design allows for the rapid and accurate quantification of a variety of organismal thermal
78 tolerance or performance metrics. By connecting two heated and/or refrigerated circulating baths
79 to either end of a custom aluminum plate, stable and predictable thermal gradients can be formed
80 (documented here and previously with various designs, see: [31,32]). If one or both of those
81 circulating baths can also be programmed, however, a single setup can become a powerful tool
82 for examining the impacts of both static and dynamic thermal conditions on organismal
83 performance and fitness. We describe the utility of this system from our perspective as insect
84 thermal biologists but small organisms or samples such as other terrestrial or aquatic
85 invertebrates, plants, unicellular eukaryotes, bacteria, cell cultures, or even enzymes could be
86 studied with this approach. Labor-intensive experiments can be easily and rapidly accomplished
87 using the described system in a single run. As a proof-of-concept, we generated low-temperature
88 survival curves of male and female *D. melanogaster* (typically produced using multiple cooling
89 baths) and examined how the amplitude of diurnal temperature cycles influenced the growth
90 rates of tropical house crickets (*Gryllodes sigillatus*).

Materials and Methods

Building the plate

The thermal plate was created using parts (Table 1) that were assembled in the laboratory. These pieces are readily available from a hardware store and/or publicly accessible metal supplier or machine shop. The gradient device was built using a 91.4 cm (36″) x 45.7 cm (18″) x 2.5 cm (1″) solid aluminum plate (Figure 1A). We milled channels on each side and at both ends of the plate (12 channels in total; Figure 1B). Given the scale of the plate, this milling was the most technically demanding part of the build but could be easily completed by a local machine shop if necessary. Each channel had dimensions of 0.95 cm (3/8″) x 0.95 cm (3/8″) and sets of 3 channels were milled 0.95 cm (3/8”) apart. Copper tubing (0.95 cm thick (3/8” OD) x 49 cm (c. 19.25”) long) was gently hammered into the channels, being careful not to damage the tubing in any way that would block fluid flow or cause a leak. Hammering was done until the copper tubing was flat with the surface of the plate (Figure 1A). We left 1.65 cm (c. 0.5”) of tubing overhang on each side of the plate for plumbing connections (Figure 1C).

After several attempts, we could not bend our copper tubing into a ‘U’ shaped configuration without damaging it and therefore opted to construct simple manifolds to complete the plumbing circuit (Figure 1D). Aluminum blocks (3.8 cm (1.5″) x 1.3 cm (1.5″) x 1.3 cm (1/2″)) were used to create the custom manifolds. Two 0.95 cm (3/8″) holes were drilled adjacent to each other in the long face of the block (Figure 1D). The spacing of the holes aligned with the spacing of the adjacent channels in the aluminum block. Another hole was drilled in one end face deep enough into the block so as to create a cavity connecting the first two holes (Figure 1D). This hole was drilled with a 5/16” drill bit to allow the hole to be tapped to accommodate a 3/8”-16 threaded brass plug. Two shorter lengths (c. 2 cm) of 0.95 cm (3/8” OD) copper tubing were

press-fit into the adjacent holes and a 3/8”-16 brass plug was installed in the threaded end-face hole using sealant to prevent leaking when fluid was under pressure. The manifolds were then connected to the ends of the copper tubing in the aluminum plate with 2.5 cm (c. 1”) lengths of 0.95 cm (3/8” ID) clear vinyl tubing (Figure 1C). We additionally secured the vinyl tubing connections with steel wire.

A benefit of using the manifolds attached to the main plate via short lengths of tubing was to compensate for slight variations in spacing of the channels and tubing, and to avoid the possibility of the seals breaking with thermal expansion of the plate.

The remaining two ends of the copper tubing at each end of the plate were attached to their respective cooling baths. By connecting the manifolds to the plate in a specific arrangement fluid was directed through all 6 of the lengths of tubing at each end of the plate (Figure 1F). Extruded polystyrene foam board (rigid foam-board insulation) was then cut to fit all sides of the plate to leave an approximately 7.5 cm (3″) airspace between the top of the aluminum plate and the bottom of the lid. The air gap allowed space for the placement of samples or metal tins containing samples (described below).

System testing

The plate was tested to ensure that a stable and reproducible temperature gradient could be formed on the surface of the aluminum plate. First, eight type-K thermocouples were equally spaced along the length of the plate at the mid-line of the plate width (Figure 2A). The positioning of each probe was pseudo-randomized during each replicate to minimize bias from using the same probe in the same location. Three different thermal gradients were independently tested to examine the functional range of the device. Three gradients 0°C to +10°C, 0°C to +25°C, and 0°C to +40°C) were each tested three times, for 2 h (each replicate). We also

138 measured temperatures across the width of the plate at the same locations across the length of the
139 plate.

140 We tested the plate using three different dynamic programs that were selected based on
141 their utility to insect thermal biologists. The first was two ramping tests from +20°C to +40°C
142 over 1 h and 2 h at one end of the plate while the other was held constant at +20°C (simulating a
143 ramp to acute heat stress). The second dynamic test was five cycles of +10°C to +30°C at one
144 end while the other end was held constant at +20°C. To simulate rapid thermal cycles, this
145 cycling was left to repeat five times, and each cycle lasted a total of two hours from +10 to
146 +30°C and vice versa, but diurnal cycles as used for our cricket experiment (below) are
147 commonly longer. The third dynamic test was a fluctuating thermal regime (FTR). The FTR was
148 done by forming a 0 to +25°C gradient during a cool period, followed by an identical warm
149 period across the plate at +25°C. FTR programs are commonly used and studied in the context of
150 long-term insect storage [33–35]. Each of these programs was tested and recorded once.

151 We next turned our attention to the practical use of the plate. We tested whether small
152 animal containers (glass *Drosophila* vials), could be placed directly on the plate, or if an
153 intermediate containment vessel was needed to achieve stable temperatures inside the containers.
154 We expected issues to be most pronounced with temperature gradients across a sample container
155 that deviated far from room temperature, so these tests were conducted at low temperatures. With
156 a gradient of -10 to +7°C formed across the plate, glass vials (25 mm diameter x 95 mm height)
157 were placed at the -10°C end of the plate. We used type-K thermocouples to measure the air in
158 the vial, and the bottom of the glass along with the plate. Since this experiment indicated that
159 contact with the plate alone was not sufficient to establish stable homogeneous temperatures
160 inside sample vials, we devised an alternative approach. We opted to use narrow troughs

161 containing a mixture of ethylene glycol and water. We created the troughs (39.37 x 7.62 x 7.62
162 cm) using sheet metal (0.9 mm thick), rivets, and silicone caulking. The troughs were placed on
163 the plate with the vials which were then placed in glycol in the troughs. This approach produced
164 stable and homogenous temperatures throughout the glass surface of the vial as well as the air
165 inside the vial.
166
167 ***Proof of concept 1: Drosophila cold tolerance***
168 The population of *Drosophila melanogaster* used [36] were reared in 200 mL plastic
169 bottles containing 50 mL of a banana-based diet (containing primarily banana, active yeast, corn
170 syrup, and barley malt). Flies were kept in an incubator at +25°C in a 12 h:12 h light/dark cycle.
171 Flies were allowed to lay fresh eggs by transferring ~800 adult flies into a population cage
172 containing food in a petri dish and were left for 24 h before being removed. This process resulted
173 in roughly 1200 eggs laid in each cage. The food containing the eggs was divided up and placed
174 into fresh glass vials (approx. 100 eggs per vial).
175 All flies were sorted by sex on the day of adult emergence under light CO_2. Flies were
176 not exposed to CO_2 for more than 10 min to ensure no long-term physiological effects [37,38].
177 Females were transferred in groups of 10 into glass vials with 7 mL of fresh banana food (mainly
178 made up of bananas, corn syrup, agar, yeast).
179 Our sheet metal troughs (described above) were laid across the width of the plate at nine
180 positions across the length of the plate. Each tin was filled with 1 L of a mix of ethylene glycol
181 and water and was placed on the plate once a stable -6°C to -1°C gradient had formed. Nine
182 metal tins were placed on the plate from one end to the other, which resulted in each vial of flies
183 being exposed to one of nine temperature points, depending on location. Type-K thermocouples

were placed at the bottom of two vials in each tin to record temperatures experienced by the flies. Adult flies were transferred to empty glass vials and restricted to the bottom 25% of the vial with a foam stopper. All of the vials were then placed into the metal tins. After 4 h in the cold, flies were removed and transferred to vials with banana food and left to recover at +25°C. At 2 h, 24 h, and 48 h (after removal from the cold), survival was checked and scored based on whether the fly was able to stand upright at room temperature. Using the survival scores from 2 h after the cold stress, we tested for an effect of temperature on the ability of flies to survive at different temperatures using a generalized linear model with temperature and sex as factors. This model had a binomial error distribution and a logit-link. We then extracted data from flies exposed to -2.21°C and used a mixed effects model (with sex and recovery time as factors and vial as a random effect) to examine how rates of survival changed over time after removal from the cold.

***Proof of concept 2: Gryllodes sigillatus* cycling growth**

The *Gryllodes sigillatus* eggs used for the experiment came from Entomo Farms, in Norwood, ON, Canada. The eggs were laid at the farm and were transferred to a +25°C incubator at day one of egg development. Hatchlings that all emerged within a 24 h period were transferred to a plastic container (24.5 cm x 12.5 cm x 19.5 cm) where they were kept at +33°C with food, shelter and water and were maintained for two weeks. Each individual was then weighed to the nearest µg using a microbalance (Sartorius ME5 model) and transferred to a 30 mm petri dish. Each petri dish contained a 200 µL PCR tube lid filled with cricket diet, a 200 µL microcentrifuge tube with water and stoppered with cotton, and a folded piece of paper for shelter. Crickets in their respective Petri dishes were placed directly on the plate at 10 different locations across the length of the plate in replicates of 10, resulting in a total sample size of 100

crickets. The treatment groups (defined by location on the plate) were divided by Styrofoam barriers to minimize airflow across the plate surface. A light strip was attached to the inside of the Styrofoam lid and was set at a 12:12 h day and night cycle (lights on at 8 AM).

One side of the plate was held at a constant 30°C while the other side was cycled daily between 20°C and 40°C. The ramp rate was set to increase or decrease by 10°C over 6 h, and a full cycle was completed every 24 h. Temperatures experienced were measured by placing two type-K thermocouples into empty cricket containers at the far ends of each treatment group. This created 10 treatment levels of daily thermal variability experienced by the crickets with those closest to the cycling end experiencing the widest range of temperatures (+38°C to +22°C). Water for the crickets was replaced on days 2 and 4 of the 5-day cycling period to ensure the animals were not water stressed. Once 5 cycles had been completed, the crickets were removed from the plate, and the body mass of each individual was measured. The effect of thermal cycle amplitude and initial mass on final mass and the effect of thermal cycle amplitude on proportion of mass gained over the five-day period were tested using general linear models.

Results and Discussion

Simple thermal gradients

Based on our design, we predicted that we could achieve linear thermal gradients across the plate that were stable (Figure 2A). Indeed, stable thermal gradients formed on the gradient plate when one refrigerated circulator was set to 0°C and the other was set to +10°C, +25°C or +40°C (Figure 2B). The relationship between distance (across the plate) and temperature always closely fit simple linear models (R^2 between 0.988 and 0.999; Figure 2C). The intercepts of each of the gradients were close to the value of the highest set temperature (Figure 2C).

In a separate run using circulator settings of constant 0°C and 40°C, we examined the reliability of temperatures across the width of the plate. The temperature across the width of the plate was measured to ensure consistent temperatures would be experienced by an organism at the same position across the length of the plate (Figure 2D). The temperature measured across the width of the plate was consistent at each location across the length (Figure 2D). The standard deviation of temperature at each of the positions across the width of the plate varied between 0.3 and 0.6°C (Figure 2D), which is well within the expected error rate of type-K thermocouples (~1.1°C or 0.4%). Thus, like previously described thermal gradient plates [32], our system can produce linear temperature gradients across the length of the plate, and these temperatures are stable over time (Figure 2).

Dynamic thermal gradients

The first dynamic gradient we attempted was an upward temperature ramp with one bath while the other was held at a constant temperature (Figure 3A). This dynamic gradient was repeated twice with a 2 h (0.17°C min^{-1} rate) and 1 h (0.33°C min^{-1} rate) ramp being completed by the bath. Both of these experiments resulted in a range of ramping rates across the plate that varied linearly with position (Figure 3D, G). Thus, this system can easily be used to study the effects of temperature ramp rates on organismal performance and fitness. Since we were not observing animals during this experiment, we used an opaque Styrofoam lid over the plate, but a clear plastic or glass lid could be used and would allow for experiments requiring continuous observation during a thermal ramp.

To create cycling temperatures, we set one circulator to cycle between high and low temperatures while the other was held at a constant temperature in the middle of cycle amplitude

(Figure 3B). As expected, this set of programs produced predictable temperature cycles of varying amplitudes across the length of the plate (Figure 3E). The maxima and minima of each cycle changed linearly across the length of the plate (maxima $R^2 = 0.995$, minima $R^2 = 0.982$; Figure 3H), and thus the amplitude of the temperature variation varied linearly across the plate ($R^2 = 0.982$; Figure 3I). Thus, our system can be used to study the effects of thermal fluctuations on survival, growth, reproduction, or any other trait of interest. By testing the system setup while trialing sample placement, a carefully crafted range of variations in cycle amplitude can be created and used.

As a third and final test of the ability of our system to create dynamic thermal environments, we simulated a fluctuating thermal regime (FTR). FTR is characterized by a period of cool temperatures followed by a warm break and is often used to store insects for long periods for commercial purposes while slowing physiological aging and avoiding long term effects of low-temperature exposure (Figure 3C). To accomplish this, we held one bath at a constant temperature (+25°C) while the other bath completed cycles of 0°C with periodic warming to +25°C. As predicted (Figure 3C), this arrangement produced FTR cycles across the plate with a consistent temperature for the warm break (+25°C), while the cool "storage" temperatures varied linearly across the length of the plate (Figure 3F, J; $R^2=0.994$). Thus, our design allows for careful manipulation of sample conditions following stepwise changes in the thermal program. In the case of FTR cycles, the sample storage or warm "break" temperature can be independently manipulated to study the effects of these treatments on insect survival or fitness. This specific application of our design would allow for high-throughput optimization of commercial or governmental insect storage programs.

276 ***Proof of concept 1: Drosophila cold tolerance***

277 We generated a low-temperature survival curve for *Drosophila* from a single use of the
278 plate (Figure 4A; survival 2 h post-cold stress shown). We noted a sharp decrease in survival in
279 males and females at approximately -2.2°C, and while the effect of sex was small (Fig. 4A), the
280 large sample size contributed to both temperature ($F_{1,1578}$=1945.8, P<0.001) and sex
281 ($F_{1,1577}$=84.6, P<0.001) having statistically significant effects on survival. This sex effect was
282 driven by the fact that males were more tolerant of chilling at just one temperature point in the
283 assay (-2.2°C; Figure 4A). At this temperature, nearly all males were alive 2 h after the cold
284 stress while c. 70% of females were dead (Fig 4A). Survival was again measured at 24 h, and 48
285 h following removal from the cold exposure, so we tested whether survival outcomes changed
286 between males and females in the 48 h following after exposure to -2.2°C (Figure 4B).
287 Specifically, while males were far more likely to be scored as alive 2 h following the cold stress,
288 they were far more likely to die in the ensuing 48 h (Figure 4B; mixed effects model recovery
289 time x sex interaction: $F_{1,45}$=7.34, *P*=0.002). While females never recovered from chill coma,
290 males recovered the ability to stand, but then died. These results are similar to our recent report
291 of latent chilling injury effects in virgin female flies following exposure to 0°C [39], which
292 suggests that conditions leading to latent injury may be temperature-, sex-, and/or reproductive
293 status-specific. Recording the same data with smaller sample size or fewer temperatures, over
294 multiple rounds because of equipment limitations, or not recording survival over multiple time
295 points could have missed these important differences between the sexes.

296 ***Proof of concept 2: Gryllodes sigillatus growth during daily thermal cycles***

297 To test the effects of thermal fluctuations on insect growth, we generated a 24-hour
298 cycling regime from +40°C to +20°C (Figure 4C) that allowed us to create 10 different cycling
299 regimes across the plate that varied in the amplitude of thermal fluctuation. We used this
300 approach to record the growth rates of two-week old crickets over five days (Figure 4D). The
301 starting weight of each cricket was measured before the start of the cycling regime and at the end
302 (Figure 4E). We expected thermal variability to negatively impact growth rates. The initial mass
303 of the cricket strongly predicted the final mass ($F_{1,116}$=61.6, P<0.001), but surprisingly the
304 position of the cricket on the plate (diurnal temperature range) did not affect final mass ($F_{9,107}$ =
305 0.7, P = 0.743), or interact with initial mass to influence final mass ($F_{9,98}$ = 0.4, P = 0.909). Most
306 crickets approximately doubled in mass over the five-day period (Figure 4F). This implies that
307 none of the cycling regimes created (ranging from 1.6 to 16.6°C of thermal range) resulted in any
308 effect on growth rates.

309

310 ***Conclusions***

311 The thermal gradient system described and demonstrated here represents a powerful new
312 approach to characterizing thermal performance in small organisms. This system can be built
313 from readily available parts and with limited technical knowledge and can be used on a wide
314 variety of sample types to measure nearly any thermal performance trait. The system produces
315 stable static and/or predictable dynamic thermal gradients over a large surface area, permitting
316 high throughput investigations. We are optimistic that this system as described or with creative
317 improvements can enhance and accelerate research on the ecology, evolution, physiology, and

318 molecular biology of ectothermic organisms, while also making these studies more economical
319 for researchers with a limited budget.

320

321 Acknowledgments

322 The authors wish to thank Charlie Reid and Hannah Davis for helpful discussion throughout this
323 project, the staff at Entomo Farms for providing the crickets, and Matt Muzzatti for useful advice
324 on caring for the crickets.

325

326 Competing Interests

327 The authors declare no competing interests.

328

329 Funding

330 This work was supported by a Natural Sciences and Engineering Research Council (NSERC)
331 Discovery Grant (RGPIN-2018-05322) and infrastructure funding to H.A.M. from the Canadian
332 Foundation for Innovation and Ontario Research Fund Small Infrastructure Fund.

333

334 Data Availability

335 All data described in this manuscript is provided as supplementary material.

336

References

[1] M. Schiffer, S. Hangartner, A.A. Hoffmann, Assessing the relative importance of environmental effects, carry-over effects and species differences in thermal stress resistance: A comparison of Drosophilids across field and laboratory generations, J. Exp. Biol. 216 (2013) 3790–3798. DOI: 10.1242/jeb.085126

[2] P. Kern, R.L. Cramp, C.E. Franklin, Physiological responses of ectotherms to daily temperature variation, J. Exp. Biol. 218 (2015) 3068–3076. DOI: 10.1242/jeb.123166

[3] K.L. Hollis, Ants and antlions: The impact of ecology, coevolution and learning on an insect predator-prey relationship, Behav. Processes. 139 (2017) 4–11. https://doi.org/10.1016/j.beproc.2016.12.002

[4] H.A. MacMillan, J.L. Andersen, V. Loeschcke, J. Overgaard, Sodium distribution predicts the chill tolerance of *Drosophila melanogaster* raised in different thermal conditions, Am. J. Physiol. Regul. Integr. Comp. Physiol. 308 (2015) 823–831. DOI: 10.1152/ajpregu.00465.2014

[5] M.R. Frazier, R.B. Huey, D. Berrigan, Thermodynamics constrains the evolution of insect population growth rates: "Warmer is better ", Am. Nat. 168 (2006) 512–520. DOI: 10.1086/506977

[6] N. Tüzün, L. Op de Beeck, K.I. Brans, L. Janssens, R. Stoks, Microgeographic differentiation in thermal performance curves between rural and urban populations of an aquatic insect, Evol. Appl. 10 (2017) 1067–1075. DOI: 10.1111/eva.12512

[7] D. Santos, J. Vanden Broeck, N. Wynant, Systemic RNA interference in locusts: Reverse genetics and possibilities for locust pest control, Curr. Opin. Insect. Sci. 6 (2014) 9–14. DOI: 10.1016/j.cois.2014.09.013

[8] H.J. MacLean, J.G. Sørensen, T.N. Kristensen, V. Loeschcke, K. Beedholm, V. Kellermann, J. Overgaard, 2019. Evolution and plasticity of thermal performance: an analysis of variation in thermal tolerance and fitness in 22 *Drosophila* species, Phil. Trans. R. Soc. B. 374, 20180548. DOI: 10.1098/rstb.2018.0548

[9] J. Overgaard, M.R. Kearney, A.A. Hoffmann, Sensitivity to thermal extremes in Australian *Drosophila* implies similar impacts of climate change on the distribution of widespread and tropical species, Glob. Chang. Biol. 20 (2014) 1738–1750. DOI: 10.1111/gcb.12521

[10] J.E. Pool, D.T. Braun, J.B. Lack, Parallel evolution of cold tolerance within *Drosophila melanogaster*, Mol. Biol. Evol. 34 (2017) 349–360. DOI: 10.1093/molbev/msw232

[11] J.T. Lennon, V.H. Smith, K. Williams, Influence of temperature on exotic *Daphnia lumholtzi* and implications for invasion success, J. Plankton. Res. 23 (2001) 425–433. DOI: 10.1093/plankt/23.4.425

[12] L. Boardman, J.S. Terblanche, B.J. Sinclair, Transmembrane ion distribution during recovery from freezing in the woolly bear caterpillar *Pyrrharctia isabella* (Lepidoptera: Arctiidae), J. Insect. Physiol. 57 (2011) 1154–1162. DOI: 10.1016/j.jinsphys.2011.04.022

[13] H. Colinet, C. Pineau, E. Com, Large scale phosphoprotein profiling to explore *Drosophila*

cold acclimation regulatory mechanisms, 2017. Sci. Rep. 7, 1713. DOI: 10.1038/s41598-017-01974-z

[14] R.L. Kobey K.L. Montooth, Mortality from desiccation contributes to a genotype–temperature interaction for cold survival *in Drosophila melanogaster*, J. Exp. Biol. 216 (2013) 1174 – 1182. DOI: 10.1242/jeb.076539

[15] J.L. Andersen, T. Manenti, J.G. Sørensen, H.A. MacMillan, V. Loeschcke, J. Overgaard, How to assess *Drosophila* cold tolerance: chill coma temperature and lower lethal temperature are the best predictors of cold distribution limits, Funct. Ecol. 29 (2015) 55–65. DOI: 10.1111/1365-2435.12310

[16] B.J. Sinclair, L.E. Coello Alvarado, L.V. Ferguson, An invitation to measure insect cold tolerance: Methods, approaches, and workflow, Journal of Thermal Biology. 53 (2015) 180–197. DOI: 10.1016/j.jtherbio.2015.11.003

[17] C. Christiansen-Jucht, P.E. Parham, A. Saddler, J.C. Koella, M.G. Basáñez, 2014. Temperature during larval development and adult maintenance influences the survival of *Anopheles gambiae* s.s., Parasites and Vectors. 7, 489. DOI: 10.1186/s13071-014-0489-3

[18] J. Chopelet, P.U. Blier, F. Dufresne, Plasticity of growth rate and metabolism in *Daphnia magna* populations from different thermal habitats, J. Exp. Zool. A. Ecol. Genet. Physiol. 309 (2008) 553–562. DOI: 10.1002/jez.488

[19] M.W. Lachenicht, S. Clusella-Trullas, L. Boardman, C. Le Roux, J.S. Terblanche, Effects of acclimation temperature on thermal tolerance, locomotion performance and respiratory metabolism in *Acheta domesticus* L. (Orthoptera: Gryllidae), J. Insect. Physiol. 56 (2010) 822–830. DOI: 10.1016/j.jinsphys.2010.02.010

[20] J.X. Jiang, J.H. Yang, X.Y. Ji, H. Zhang, N.F. Wan, Experimental temperature elevation promotes the cooperative ability of two natural enemies in the control of insect herbivores, Biol. Control. 117 (2018) 52–62. DOI: 10.1016/j.biocontrol.2017.09.001

[21] I.J. McGaw, N. M. Whiteley, Effects of acclimation and acute temperature change on specific dynamic action and gastric processing in the green shore crab, *Carcinus maenas*, J. Therm. Biol. 37 (2012) 570–578. DOI: 10.1016/j.jtherbio.2012.07.003

[22] W.D. Crill, R.B. Huey, G.W. Gilchrist, Within- and between-Generation effects of temperature on the morphology and physiology of *Drosophila melanogaster*, Evolution. 50 (1996) 1205–1218. DOI: 10.1111/j.1558-5646.1996.tb02361.x

[23] H. Colinet, B.J. Sinclair, P. Vernon, D. Renault, Insects in fluctuating thermal environments, Annu. Rev. Entomol. 60 (2015) 123–140. DOI: 10.1146/annurev-ento-010814-021017

[24] C. Rolandi, J.R.B. Lighton, G.J. de la Vega, P.E. Schilman, J. Mensch, Genetic variation for tolerance to high temperatures in a population of *Drosophila melanogaster*, Ecol. Evol. 8 (2018) 10374–10383. DOI: 10.1002/ece3.4409

[25] J.S. Terblanche, J.A. Deere, S. Clusella-Trullas, C. Janion, S.L. Chown, Critical thermal limits depend on methodological context, Proc. R. Soc. B Biol. Sci. 274 (2007) 2935–2943. DOI: 10.1098/rspb.2007.0985

[26] K.A. Mitchell A.A. Hoffmann, Thermal ramping rate influences evolutionary potential and species differences for upper thermal limits in *Drosophila*, Funct. Ecol. 24 (2010) 694–700. DOI: 10.1111/j.1365-2435.2009.01666.x

[27] C. Nguyen, M.H. Bahar, G. Baker, N.R. Andrew, 2014. Thermal tolerance limits of diamondback moth in ramping and plunging assays, PLoS One. 9, e87535. DOI: 10.1371/journal.pone.0087535

[28] J. Overgaard, T.N. Kristensen, J.G. Sørensen, 2012. Validity of thermal ramping assays used to assess thermal tolerance in arthropods, PLoS One. 7, e32758. DOI: 10.1371/journal.pone.0032758

[29] L.B. Jørgensen, H. Malte, J. Overgaard, How to assess *Drosophila* heat tolerance: Unifying static and dynamic tolerance assays to predict heat distribution limits, Funct. Ecol. 33, (2019) 629–642. DOI: 10.1111/1365-2435.13279

[30] V. Kellermann, A.A. Hoffmann, T.N. Kristensen, N.N. Moghadam, V. Loeschcke, Experimental evolution under fluctuating thermal conditions does not reproduce patterns of adaptive clinal differentiation in *Drosophila melanogaster*, Am. Nat. (2015) 582-593. DOI: 10.1086/683252

[31] O. Sayeed, S. Benzer, Behavioral genetics of thermosensation and hygrosensation in *Drosophila*, Proc. Natl. Acad. Sci. 93 (1996) 6079–6084. DOI: 10.1073/pnas.93.12.6079

[32] P.A. Siver, A new thermal gradient device for culturing algae, Br. Phycol. J. 18 (1983) 159–164. DOI: 10.1080/00071618300650201

[33] J.P. Rinehart, G.D. Yocum, M. West, W.P. Kemp, A fluctuating thermal regime improves survival of cold-mediated delayed emergence in developing *Megachile rotundata* (Hymenoptera: Megachilidae), J. Econ. Entomol. 104 (2011) 1162–1166. DOI: 10.1603/ec11062

[34] H. Colinet, T.T.A. Nguyen, C. Cloutier, D. Michaud, T. Hance, Proteomic profiling of a parasitic wasp exposed to constant and fluctuating cold exposure, Insect. Biochem. Mol. Biol. 37 (2007) 1177–1188. DOI: 10.1016/j.ibmb.2007.07.004

[35] V. Koštál, D. Renault, A. Mehrabianová, J. Bastl, Insect cold tolerance and repair of chill-injury at fluctuating thermal regimes: Role of ion homeostasis, Comp. Biochem. Physiol. A Mol. Integr. Physiol. 147 (2007) 231–238. DOI: 10.1016/j.cbpa.2006.12.033

[36] K.E. Marshall, B.J. Sinclair, Repeated stress exposure results in a survival–reproduction trade-off in *Drosophila melanogaster,* Proc. R. Soc. B. 277 (2010) 963–969. DOI: 10.1098/rspb.2009.1807

[37] N.R. Bartholomew, J M. Burdett, J.M. Vandenbrooks, M.C. Quinlan, G.B. Call, 2015. Impaired climbing and flight behaviour in *Drosophila melanogaster* following carbon dioxide anaesthesia, Sci. Rep. 5, 15298. DOI: 10.1038/srep15298

[38] T.L. Nilson, B.J. Sinclair, S.P. Roberts, The effects of carbon dioxide anesthesia and anoxia on rapid cold-hardening and chill coma recovery in *Drosophila melanogaster*, J. Insect. Physiol. 52 (2006) 1027–1033. DOI: 10.1016/j.jinsphys.2006.07.001

[39] M.I. El-Saadi, M.W. Ritchie, H.E. Davis, H.A. MacMillan, 2020. Warm periods in repeated

454 cold stresses protect *Drosophila* against ionoregulatory collapse, chilling injury, and
455 reproductive deficits, J. Insect. Physiol. 123, 104055. DOI: 10.1016/j.jinsphys.2020.104055

456

Tables

Table 1: List of material to assemble the plate with the dimensions of each item as well as the number required. In addition to the other materials, two refrigerated circulating baths were used. These can be programmable or non-programmable depending on the experimental design. All programs shown herein were conducted with one programmable and one non-programmable bath (capable of static temperatures only).

Material	Source	Dimensions	Quantity
Aluminum plate (solid)	Metal Supermarket (local source of stock metal)	91.4 cm x 45.7 cm x 2.5 cm (36” x 18” x 1”)	1
Copper tubing – Utility grade	Hardware store	0.95cm (3/8”) outer diameter and 0.6 mm (0.03”) wall thickness 49 cm (19.25”) lengths	12 pcs
Copper tubing – Utility grade	Hardware store	0.95cm (3/8”) outer diameter and 0.6 mm (0.03”) wall thickness 2 cm lengths	20 pcs
Clear vinyl tubing	Hardware store Scientific supply store	0.95 cm (3/8”) inner diameter 2.54 cm (1”) length	20 pcs
Aluminum blocks (for manifolds)	Metal Supermarket (local source of stock metal)	3.8 cm x 1.3 cm x 1.3 cm (1.5” x 0.5” x 0.5”)	10 pcs
Styrofoam (Rigid foam board insulation)	Hardware Store	91.4 cm x 11.4 cm 45.7 cm x 11.4 cm 111.8 cm x 55.9 cm 91.4 cm x 45.7 cm (all pcs 1.3 cm (0.5”) thick)	7 pcs 4 pcs 2 pcs 2 pcs
Brass plug	Hardware Store	0.95 cm (3/8”-16 thread) (standard plumbing item)	10
Marine grade silicone sealant	Hardware Store	small tube	1

Figures

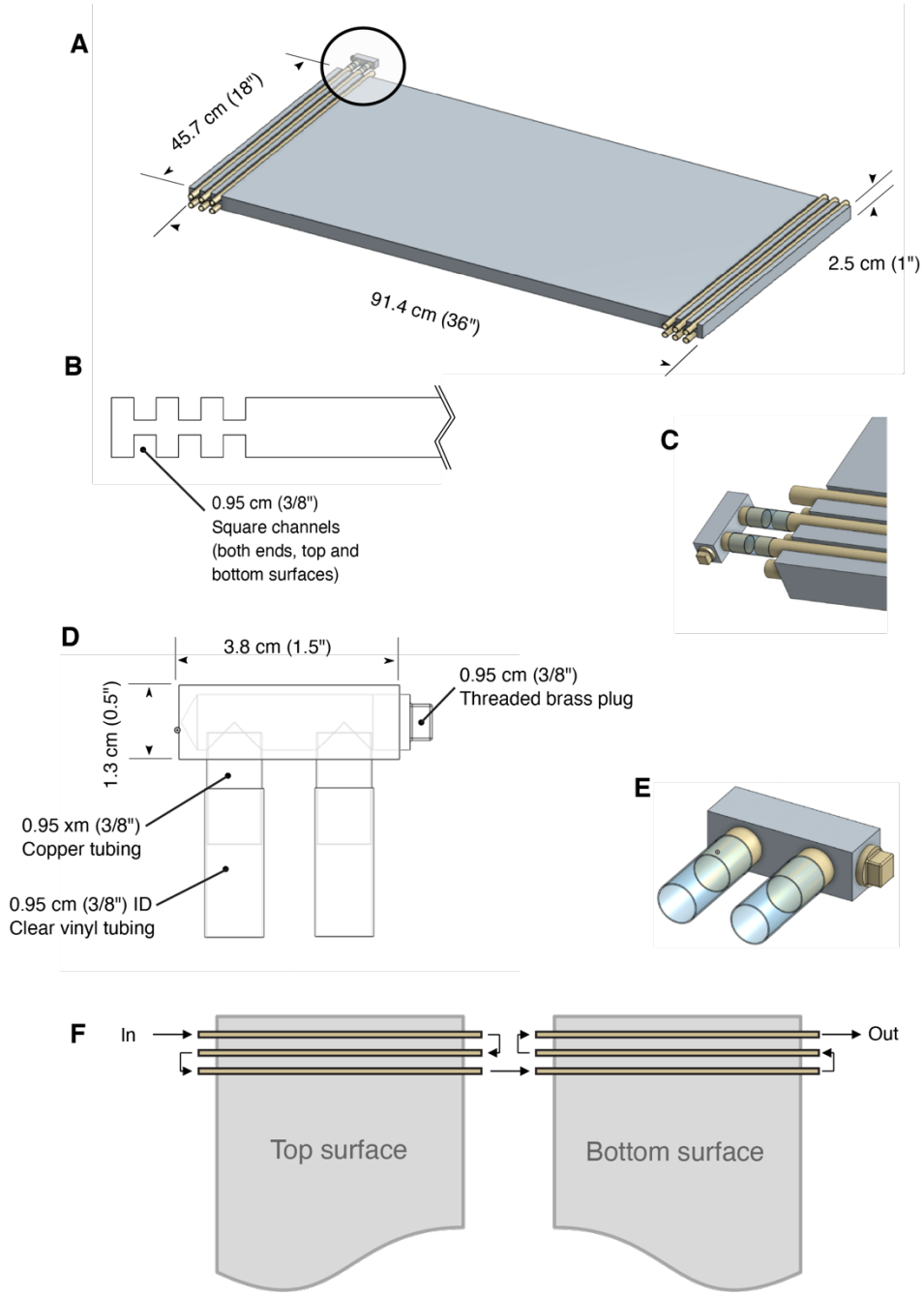


Figure 1: Design and dimensions of the thermal gradient plate. A) Assembled plate and copper tubing with example manifold shown in place (circled region). B) Side view of milled square channels in aluminum plate. C) Manifold connection to exposed copper pipe ends using clear vinyl tubing. D) Manifold design from aluminum block, copper pipe, brass plug, and tubing. E) Close-up of example manifold. F) Direction of fluid flow through the system once all manifolds are connected (manifolds not shown for clarity).

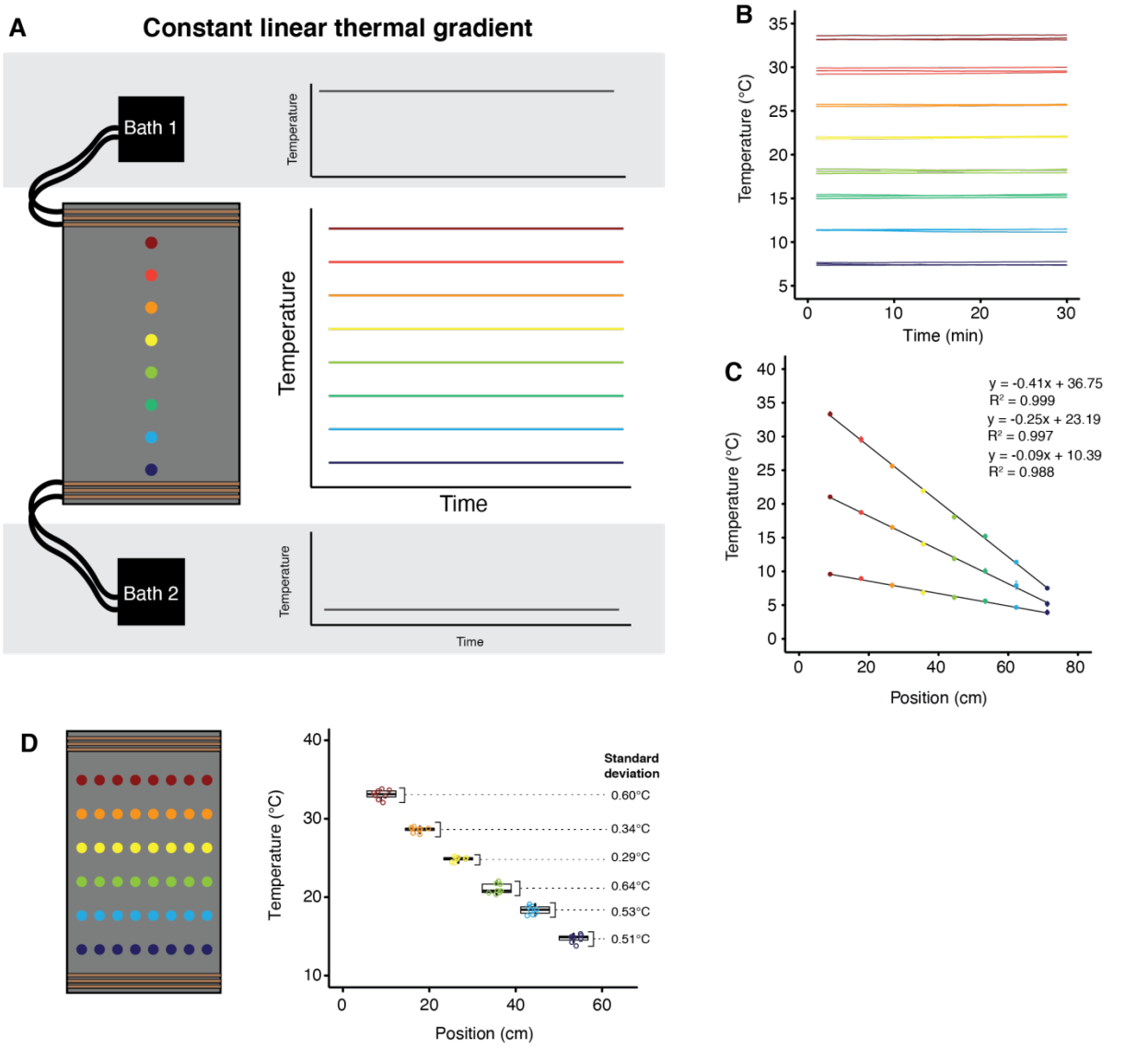
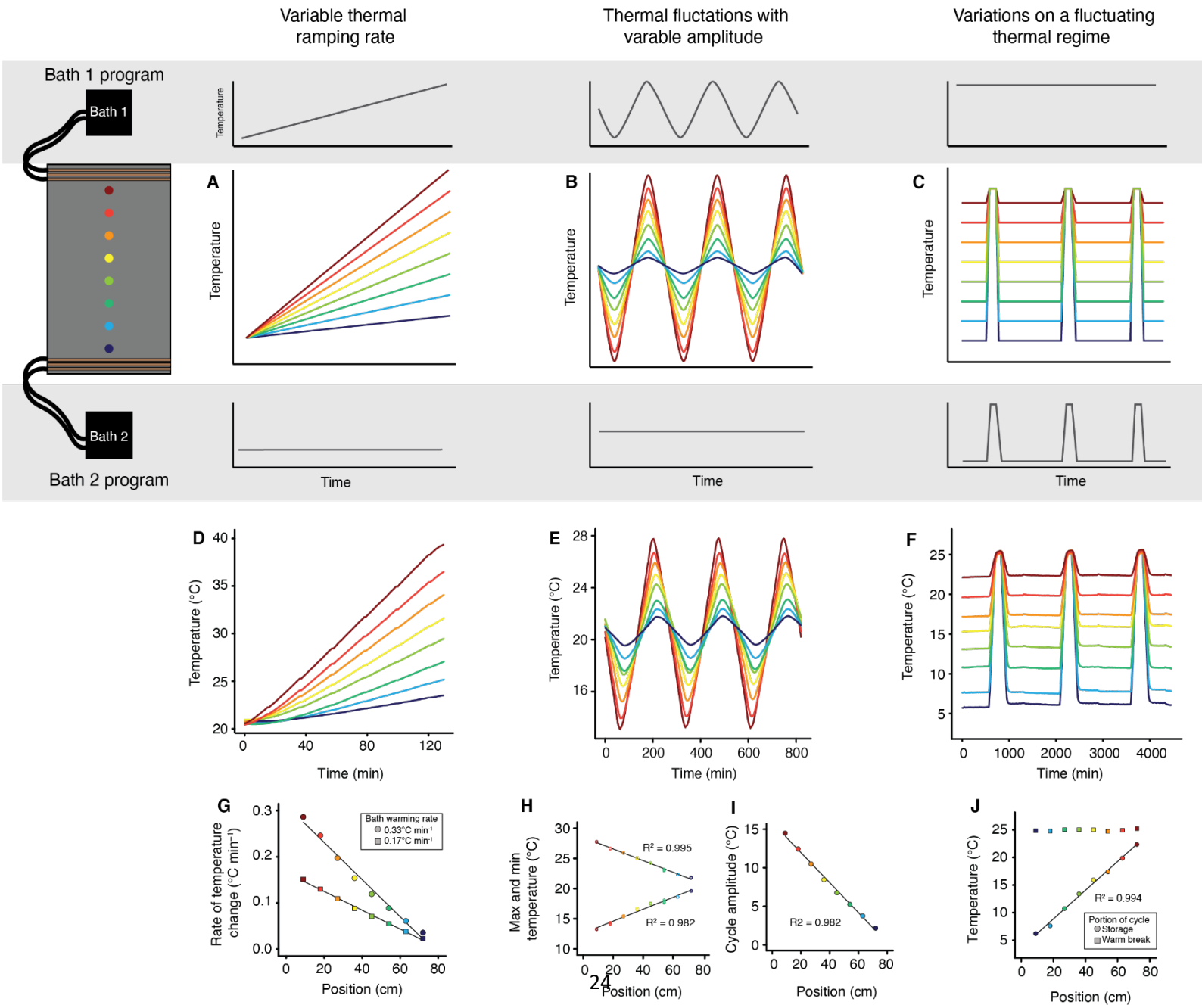
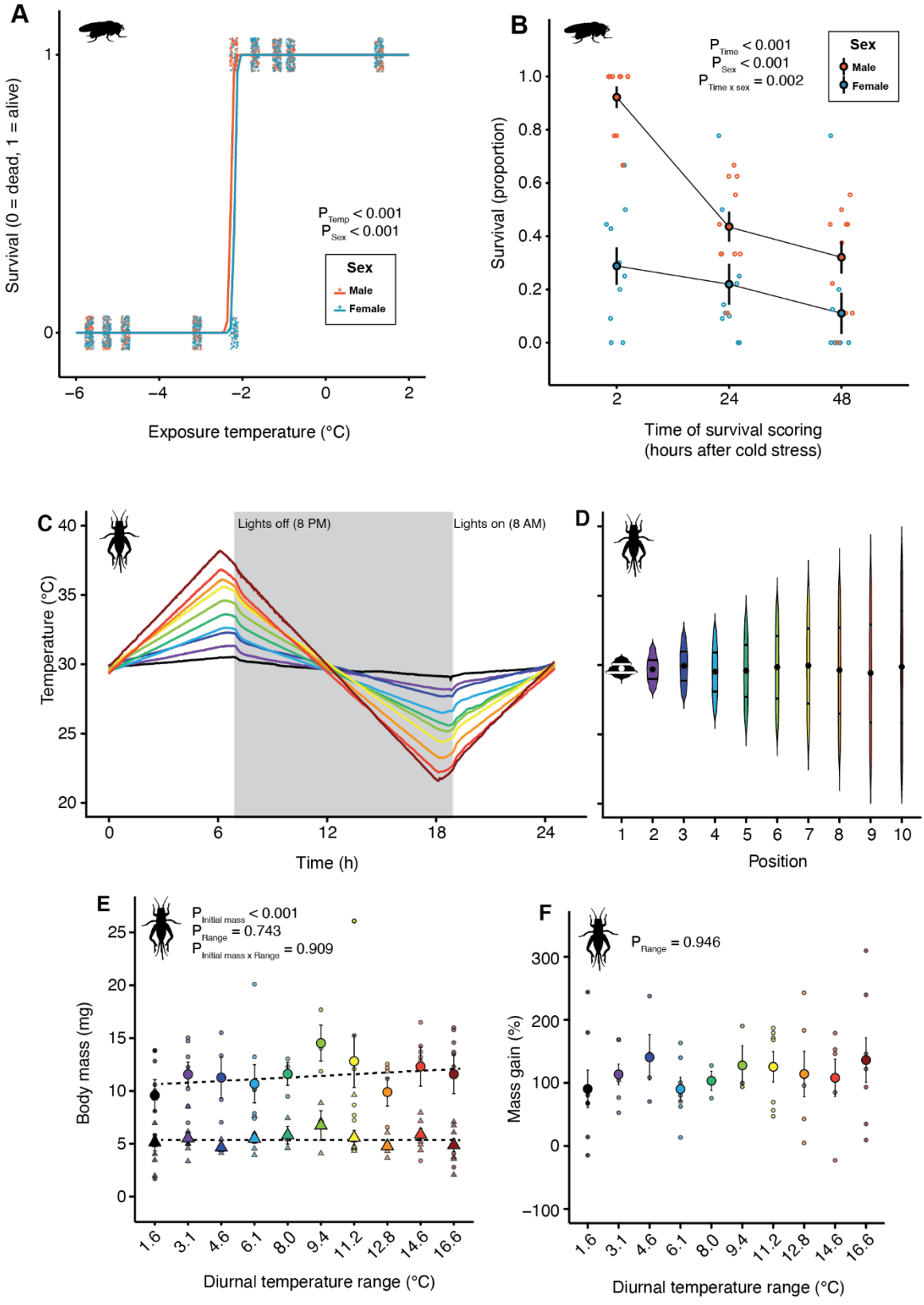


Figure 2: Production of stable linear gradients along the length, but not the width of the gradient plate. A) Probe positions and expected outcomes from establishing constant temperatures at either end of the gradient plate. B) Actual data recorded at eight locations in the position of the plate with an established linear gradient. Circulating baths connected to the plate were set to +40°C (bath 1) and 0°C (bath 2). Each line represents a single type-K thermocouple recording over a 30 min period (n=3 per position). C) Temperatures recorded at eight locations along the length of the plate while maintaining a stable gradient. Solid circles represent the mean of three replicant measurements at each location (open circles, barely visible because of low variance). Note that the R^2 of the temperature-position relationship is close to 1 (0.988 to 0.999). D) Measurements of temperature along the width of the plate at several points in the length of the plate with a thermal gradient of 0 to +40°C. Colors denote position along the length of the plate, and eight temperature recordings across the width of the plate were made for each position along its length. Boxplots show the variation of temperature across the plate width.


Variable thermal ramping rate
Thermal fluctations with varable amplitude
Variations on a fluctuating thermal regime
Bath 1 program
Bath 1
Bath 2
Bath 2 program
Temperature
Time
A
B
C
D
E
F
Temperature (°C)
Time (min)
G
H
I
J
Rate of temperature change (°C min⁻¹)
Bath warming rate
0.33°C min⁻¹
0.17°C min⁻¹
Position (cm)
Max and min temperature (°C)
R² = 0.995
R² = 0.982
Cycle amplitude (°C)
R2 = 0.982
R² = 0.994
Portion of cycle
Storage
Warm break

487

(previous page) Figure 3: Dynamic thermal gradients allow for complex experiments using programmable refrigerated circulators. Programs shown produce predictable variations in warming rate, thermal cycle amplitude, or a chosen aspect of a fluctuating thermal regime over time. A-C) Expected relationships between time (x-axis) and temperature (y-axis) along the length of the gradient plate (color represents position across the length of the plate) when baths 1 and 2 are programmed as shown (grey background). D-F) Actual outcomes of bath programming experiments (temperature recordings over time). Each line represents the temperature of measurements at each position using type-K thermocouples. G) Rates of temperature change at each position during two runs using different warming rates of bath 2. This approach creates a linear relationship between position and thermal ramp rate. H-I) Maximum and minimum temperatures and cycle amplitude recorded using a cycling program. Cycling temperature of one bath (bath 1) while holding another constant (bath 2) produces repeatable cycle maxima and minima that vary by position across the plate and linearly alters cycle amplitude relative to the position. J) Relationship between temperature and position for cold storage temperature and warm break period in a fluctuating thermal regime simulation. A single aspect of the program can be altered predictably across the length of the plate (e.g., storage temp) while holding another aspect (warm break temperature) constant.



505

(previous page) Figure 4: Proof of concept experiments to demonstrate the utility of the dynamic thermal gradient system. A) Survival of adult male (red) and female (blue) *D. melanogaster* following constant exposure to different temperatures for 4 h on the thermal plate. Each solid circle represents an individual fly. 1 = survived, 0 = dead. We noted that males were much more cold-tolerant when measured specifically at -2.21°C. B) Survival following exposure to -2.21°C for 4 h in the same flies across multiple rounds of survival assessment (2 h, 24 h, and 48 h). Although males appear more tolerant of chilling at first, they are more likely to suffer latent mortality. Each open circle represents an independent vial (containing ~10 flies); solid circles represent the mean (± sem) survival. C) One day of the diurnal cycling program experienced by developing crickets (*Gryllodes sigillatus*). Different colours denote 10 different locations across the length of the plate with temperature ranges from 1.6°C (black) to 16.6°C (dark red). Small deviations in temperature can be seen when the lights switch on and off. D) Violin plots showing temperatures experienced by the crickets each day. Width denotes the amount of time spent at a given temperature. Solid circles denote the mean temperature experienced across the entire day (constant +30°C across the plate). Horizontal lines denote the 25% and 75% quartiles. E) Initial (triangles) and final (circles) mass of crickets at each location across the plate. Small symbols denote individual crickets and large symbols denote the mean ± sem. Variance in final body size was strongly related to initial size, but not significantly impacted by thermal variation. F) The same is true when adjusting for initial body size by expressing growth as a percentage of initial mass; thermal variability did not affect growth rates of *G. sigillatus*.