

1 **Revisiting thermal gradient experiments: Effects of thermal heterogeneity on salamander**
2 **behavior**

3

4 **Running title:** Salamander behavior in thermal gradients

5 **Article type:** Original research

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15 **Key words:** Thermal behavior, *Plethodon cinereus*, Plethodontidae, Preferred Temperature,
16 Body Temperature, Thermoregulation.

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18

19 **Abstract**

20 Thermal gradient experiments are commonly used in studies of ectothermic organisms for a
21 variety of scientific inquiries. Such gradient experiments, performed in the laboratory, are often
22 used to infer the climatic preferences of animals in the absence of other variables. However, the
23 ability to extrapolate laboratory results to the field is only as good as the accumulation of

24 ecological data for that organism. When the variable quantified is interpreted as thermal
25 “preference” there are some assumptions that come with it, namely that the organism selects a
26 particular preferred temperature by positive thermotaxis. Amphibians, as well as most
27 ectotherms, tend to be thermoconformers, so conclusions from thermal gradient experiments
28 carry different meanings than they do for organisms such as heliothermic ectotherms that
29 maintain a narrow range of body temperatures in the lab and field. We tested whether and how
30 the Eastern Red-backed Salamander (*Plethodon cinereus*) behaves when presented with a
31 heterothermal gradient arena in comparison to a control (homothermal) arena. Salamanders in
32 the control arena unambiguously moved towards either end of the arena, despite no variation in
33 temperature being available. We found that salamanders did respond to a thermal gradient, but
34 that their thermoregulatory behavior was limited to the avoidance of the hottest end of the
35 gradient, and not a positive thermotaxis towards a specific temperature as assumed of a thermal
36 “preference”. Our results encourage a broader consideration of how laboratory-measured
37 behaviors relate to the predicted behaviors of organisms in natural settings, and a re-evaluation
38 of the terminology used to describe movement behaviors in thermal gradients.

40 **(Abstract in Spanish)**

41 **Resumen**

42 Los experimentos con gradientes térmicos se utilizan comúnmente en estudios de organismos
43 ectotérmicos para una variedad de preguntas científicas. Dichos experimentos de gradiente,
44 realizados en laboratorio, se utilizan a menudo para inferir las preferencias climáticas de los
45 animales en la ausencia de otras variables. Sin embargo, la capacidad de extrapolar los resultados
46 de laboratorio al campo depende de la acumulación de datos ecológicos de ese organismo.

47 Cuando la variable cuantificada se interpreta como “preferencia” termica, existen ciertas
48 suposiciones, a saber que, el organismo selecciona una temperatura particular por termotaxia
49 positiva. Los anfibios, así como la mayoría de ectotermos, tienden a ser termoconformistas, por lo
50 que las conclusiones de experimentos con gradiente térmico tienen un significado diferente al
51 que tienen para organismos como los ectotermos heliotérmicos, que tienen un menor rango de
52 temperatura corporal en el laboratorio y en el campo. Evaluamos si y como la salamandra de
53 espalda roja del este (*Plethodon cinereus*) se comporta cuando esta presente en un gradiente
54 térmico de arena en comparación con un control (homotérmico) arena. Las samandras en el
55 control de arena se desplazaron de forma clara hacia uno u otro extremo de la arena.
56 Encontramos que las salamandras respondieron al gradiente térmico, pero su comportamiento
57 termoregulador se limitó a evitar el extremo más caliente del gradiente, y no a una termotaxia
58 positiva hacia una temperatura térmica que podríamos asumir como “preferida”. Nuestros
59 resultados invitan a tomar consideraciones en como las medidas de comportamiento en
60 laboratorio se relacionan con los comportamientos predichos de los organismos en ambientes
61 naturales, y una reevaluación en la terminología utilizada para describir los comportamientos de
62 movimiento en los gradientes térmicos.

63

64 **(Abstract in Portuguese)**

65 **Resumo**

66 Gradientes térmicos são comumente utilizados em estudos envolvendo organismos ectotérmicos
67 para uma variedade de perguntas científicas. Esses experimentos realizados em laboratório são
68 frequentemente usados para estimar a preferência térmica de animais na ausência de outras
69 variáveis. Porém, a capacidade de extrapolar os resultados do laboratório para o campo depende

70 do acúmulo de dados da ecologia do organismo em questão. Quando a variável quantificada é
71 interpretada como “preferência”, há algumas premissas implícitas, incluindo que o organismo
72 seleciona uma temperatura preferencial específica através de termotaxia positiva. Anfíbios, assim
73 como a maioria dos ectotérmicos, tendem a ser termoconformadores. Portanto, conclusões de
74 experimentos em gradientes térmicos carregam significados diferentes do que em organismos
75 que mantêm uma amplitude térmica estreita tanto em laboratório quanto em campo, como
76 ectotérmicos heliotérmicos. Nós testamos se e como a salamandra *Plethodon cinereus* reage a
77 um gradiente térmico em comparação a uma pista controle (sem variação térmica). Os resultados
78 mostraram que as salamandras na pista de controle se moveram em direção a uma das
79 extremidades da pista, apesar de não haver variação na temperatura. Nós observamos que as
80 salamandras de fato exibiram respostas ao gradiente térmico, mas a termoregulação observada se
81 limitou a evitar o lado mais quente do gradiente, e não em termotaxia positiva em direção a uma
82 temperatura específica como seria esperado de uma temperatura dita “preferencial”. Os nossos
83 resultados encorajam uma maior consideração de como comportamentos medidos em laboratório
84 correspondem a previsão de comportamentos em contextos naturais, e uma reavaliação da
85 terminologia usada para descrever comportamentos de movimentação em gradientes térmicos.
86

87 **Introduction**

88 Temperature influences function and homeostasis in ectothermic animals, with impacts on
89 physiological processes and spatial distributions (Angilletta Jr. 2009). However, the degree of
90 thermal dependence varies among species, as many lineages can thermoregulate their body
91 temperature using behavior, with great diversity of strategies and limits, according to species,
92 ecological context, and type of thermal heterogeneity (Muñoz 2022; Dubiner et al. 2024;
93 Giacometti et al. 2024). This assortment of thermoregulation strategies matters because the
94 nature of temperature selection in heterogeneous thermal landscapes influences selective
95 pressures on thermal physiology, in turn affecting evolutionary patterns (Muñoz and
96 Bodensteiner 2019). Thermoregulatory behaviors must be properly assessed, as they are relevant
97 for proposing, interpreting, and informing models for vulnerability under climate change (Huey
98 et al. 2012; Kearney and Porter 2017). This scenario highlights the importance of permanently
99 revisiting the methods used to assess behavioral thermoregulation, often consisting in analyzing
100 the behavior of target specimens in a thermal gradient spanning a certain range of temperatures.
101 The data, in this case, are substrate or body temperatures recorded periodically, and the central
102 tendencies of these data are used to infer behavioral preferences and patterns.

103 Many studies seek to connect laboratory-estimated thermal behavior to that of their free-
104 ranging counterparts. This inference requires discriminating between what animals can do, as
105 tested in a laboratory, and what animals will do in the field (Fry 1947; Navas et al. 2021). Such
106 inference is straightforward when field behavior is well known; small, heliothermic lizards, for
107 example, tend to display strong and consistent thermotaxis in laboratory studies, and this
108 behavioral pattern is often replicated in the field (Huey and Stevenson 1979; Hertz et al. 1993;
109 Gunderson and Leal 2016; Muñoz and Losos 2018). However, behavior must also be studied in

110 species for which little information is available, and no generalization applies to all ectotherms.
111 Here, we use an experimental framework to explicitly test some common assumptions when
112 performing thermal preference experiments in a lungless amphibian, the Eastern red-backed
113 salamander (*Plethodon cinereus*).

114 If the objective of a given laboratory intervention is to learn about behavior in the field, then
115 the correspondence between field and laboratory behavioral patterns becomes crucial. However,
116 this association is intricate, and amphibians offer an excellent example to learn more about these
117 complexities. For example, salamanders in the family Plethodontidae (lungless salamanders)
118 respond non-randomly to laboratory variation in temperature (Feder and Pough 1975; Feder
119 1982), pH (Wyman and Hawksley-Lescault 1987; Sugalski and Claussen 1997), and soil
120 humidity (Sugalski and Claussen 1997), but when pH and humidity covary, salamanders may
121 gravitate more strongly towards higher pHs (Sugalski and Claussen 1997), despite field data
122 showing humidity as a key factor guiding activity patterns and spatial distributions (Heatwole
123 1962; Ficetola et al. 2018; Farallo et al. 2020). Laboratory behavior in response to temperature
124 may vary with relative humidity (Galindo et al. 2018), and the body temperature of field animals
125 may disagree with laboratory thermal selection, even under favorable environmental conditions
126 (Feder 1982). Therefore, deducing field behavior from experimental data is not straightforward,
127 as field distributions may reflect many behavioral drives, interactions, and constraints that are
128 not replicated in most laboratory settings (Navas et al. 2021). Even a compelling corroboration of
129 thermotaxis, using temperature as a single variable, does not necessarily grant reproducibility in
130 field settings. In summary, inferring thermal ecology from whole-animal experiments is a
131 complex analytical process that is enhanced by attention to experimental detail, and information
132 on natural history (Bartholomew 1986).

133 Concerns about inference likely explain persistent cautionary advice in the literature regarding
134 both methods and, perhaps more strongly, interpretations. Concerning critical thermal limits (for
135 ectothermic vertebrates this is typically defined as the lowest and highest temperature at which
136 locomotor capacity ceases, determined by the inability of animals to right themselves when
137 flipped onto their backs), for example, articles directly consider the costs and benefits of
138 different methodological approaches (Ørsted et al. 2022) and the analytical consequences of
139 behavioral versus physiological endpoints (Lutterschmidt and Hutchison 1997a, 1997b; Ribeiro
140 et al. 2012). Parallel caveats extend to vulnerability indices based on critical temperatures,
141 emphasizing the need to make underlying assumptions explicit (Clusella-Trullas et al. 2021)
142 and the consequences of the scale at which the analysis occurs, either in time or space (Garcia et
143 al. 2019). With respect to thermal gradients, the discussion emphasizes the importance of
144 experimental controls (Hutchison et al. 1966), the methods to report variance (Feder 1982), the
145 implications of metaphor (such as “preferred”, “selected” or “eccritic”) in variable naming
146 (Pough and Gans 1982), and the need to restrict hypotheses about thermotaxis to its appropriate
147 inference space (Navas et al. 2021).

148 Here, we designed and applied a thermal gradient method to evaluate temperature selection
149 in small animals. We had the goal of both evaluating the nature of the behavior of salamanders in
150 relation to temperature under an experimental setting, as well as testing the methodological
151 factors that can influence inference. We focused on the Eastern Red-backed Salamander
152 (*Plethodon cinereus*), a lungless, terrestrial amphibian found across eastern North America,
153 northward from North Carolina to southern Ontario, and westward from the Atlantic Coast to
154 Minnesota. This species has been widely studied, is abundant and easy to handle, and performs
155 well under laboratory conditions (Jaeger et al. 2016). Focusing on *P. cinereus*, we asked, “Does

156 *the behavior in an experimental arena change with exposure to a thermal gradient? And, if so,*
157 *how does behavior change? ”. In parallel, we ask three methodological questions: (1) What is the*
158 *usefulness of a control in thermal gradient experiments? (2) To what extent do small changes in*
159 *the setup of an experimental thermal gradient influence results? and (3) What confounding*
160 *variables are likely to affect results and require caution? The last two questions relate to*
161 *variables related to the orientation and location of the experimental setup, as described in the*
162 *Methods, and to changes in the temperature range. Question (1) relates to the need for a control,*
163 *which is inherent to most biological questions to be answered experimentally. Because our*
164 *question concerning salamander behavior uses the term “change”, it requires a comparative*
165 *framework. We use the control arena for this purpose, where salamanders experience the same*
166 *conditions as in the thermal gradient, but under uniform room temperature. We aimed for strong*
167 *inference in our biological question and designed a “crucial experiment” (Platt 1964) focusing on*
168 *patterns of movement. Potential results could range from no movement (no experimental signal)*
169 *to random movement (signal unrelated to temperature, the main variable tested), although we*
170 *expected animals to move, and temperature to have some impact on behavior. Based on this*
171 *expectation, we formulated five alternative hypotheses and associated predictions involving*
172 *random movement and specific non-random behaviors related to a temperature gradient. Our*
173 *hypotheses and predictions appear in Table 1 and are related to the Behavioral Response*
174 *Variables described in “Methods”. Our research also includes inductive components, as we had*
175 *no *a priori* hypotheses regarding how certain variables (for example, Collection Date or Body*
176 *Mass) could affect behavior. Notably, our aim is not to propose and describe an ideal thermal*
177 *gradient experiment, but rather to explore the method with the advantages of a common species*
178 *that responds well in captivity, and that might be generalizable to other thermoconforming*

179 species. Thus, we can enhance information from repeated measures, and the use of individuals as
180 their own controls. The setup we used may be impractical, even undesirable, under several
181 contexts, but our intention is to illustrate what we learned from each variable quantified, so that
182 researchers find inspiration to decide what is important for their context-specific cases and
183 conditions.

184

185 **Methods**

186 *Experimental setup*

187 Apparatus: We built four experimental units, each one consisting of two rectangular cases
188 (110 cm long and 9.5 cm wide) termed *arenas*, one of which was activated with a thermal
189 gradient while the other remained at the room temperature of 15°C (*control*). To keep track of
190 the position of animals within each arena, we split and labelled arenas into 22 segments
191 (hereafter, “positions”), approximately 4.5 cm each, with numbers printed in one detachable
192 label placed on the wall behind the units. For logistic reasons, two controls and two gradients
193 were always paired together (Figure S1).

194 Substrate: We originally tried layer-type substrates (paper towels and gauze), but salamanders
195 occasionally hid under the material, which also had to be washed or replaced after each use to
196 minimize pheromone marking, a characteristic of *Plethodon* salamanders (Jaeger and Forester
197 1993). We switched to a 1 cm depth layer of fine vermiculite, a substrate that could be mixed
198 after each test, homogenizing eventual chemical cues. Individuals occasionally moved their
199 bodies or limbs in what looked to be digging behavior, but no salamander was able to fully hide
200 under the vermiculite.

201 Heating and Cooling: Heat was generated by placing a heating cable under the first half of
202 each gradient; the cable was more densely distributed at the extreme and decreased in density
203 towards the middle, where it exited the gradient. To diminish lateral gradients or sharp changes
204 associated with the exact position of the heating cable we added a copper sheet between the
205 gradient and the heating cable so that the heating cable was secured to the copper sheet with
206 electrical tape. The second half of the gradient was not heated, and the final portion (positions 1
207 to 4 according to the labeling code) rested over crushed ice placed on a Styrofoam cooler
208 (contact area space 21 x 19 cm, depth 16 cm). During experiments we added ice as needed to
209 compensate for melting.

210 Thermal structure of gradients: When building the gradient, we aimed for balance among
211 three factors. First, we deemed it important to retain the behavioral information generated by
212 extreme temperatures and opted for a minimum temperature approaching 5°C (mean \pm SD: 5.47
213 \pm 2.91°C, min: 0.5 °C, max: 13.4 °C) and a maximum temperature of about 33°C (mean \pm SD:
214 33.12 \pm 4.30°C, min: 26.2°C, max: 45.6°C). Second, we wanted to avoid overly long or short
215 gradients because the temperature selected by urodeles in thermal gradients may be influenced
216 by the length of the gradient relative to the body length of specimens (Navas et al. 2021), and so
217 opted for arenas of 110 cm in length. Lastly, and despite the impact of the air flow inside an
218 environmental chamber, we decided to perform experiments in the regulated environment of a
219 walk-in environmental chamber (15°C) instead of a laboratory bench at room temperature. The
220 resulting gradient met target temperature values on the ends, although its middle part (positions
221 10-12) was unavoidably influenced by airflow. The thermal structure of the baseline gradient
222 appears in Fig. 1. The gradient was constant within a given experimental day, but we allowed
223 experiment-wise minor changes in the minimum and maximum temperature to evaluate possible

224 impacts. Also, on each experimental day we recorded the minimum, maximum, and central
225 temperature of each gradient. A thermographic image of gradients and controls is presented in
226 Fig. S2.

227 Variables related to orientation and location of the experimental setup: The walk-in
228 environmental chamber had two side-to-side stands, each one supporting two parallel
229 experimental units (two control arenas and two thermal gradients; Fig. 1). Because air flow,
230 light, and air temperature varied inside the chamber, we took measures thought to reduce, or at
231 least turn quantifiable, the impact of such unplanned sources of variation. Regarding light and air
232 flow, we applied red translucent plastic over the light sources (located in the ceiling in the center
233 of the room above roughly the middle of the gradients) and placed panels that reduced direct air
234 flow on experimental units. Despite these actions, we could not eliminate the impacts of the
235 location and orientation of experimental units and thought desirable to alternate the position and
236 orientation of gradients and controls. Thus, we created a systematic protocol to rotate the
237 position and orientation of gradients and controls.

238 Our protocol involved three orientation categorical variables (See diagram in Fig. 2). Each
239 arena occupied one out of 8 possible locations, and these different locations were subject to
240 different spatial effects of the room. Therefore, we assigned Block, a variable with two states,
241 Right (location 1-4) and Left (location 5-8), referring to the right or left stand inside the chamber.
242 At each such stand (Block, hereafter), because arenas were oriented perpendicular to the
243 observer, either controls or thermal gradients could be at the front (nearer to observer, i.e., the
244 open floor space of the environmental chamber), or back (further from the observer, i.e., nearer
245 the wall of the chamber) and this aspect of location was termed Depth, with two states, Front and
246 Back. For practicality, either cold or hot extremes met in between the two blocks, and we

247 associated these two possible orientations to a third variable, Pole, with two states, Cold and Hot.
248 Therefore, a Cold state was defined when the gradients at the left block ran hot to cold (from left
249 to right), whereas those at the right block ran cold to hot, and consequently both sets of gradients
250 were closest at their colder extremes, between the two stands (Fig. 2). Conversely, a Hot state
251 was the opposite, with both sets of gradients meeting at their hottest extreme. No other practical
252 orientation was viable, as the hot and cold ends could not meet without serious interference with
253 the aimed thermal ranges. We shifted from Cold to Hot state every experimental day, so that
254 gradients would necessarily run in different directions. These variables were used exploratorily.
255 For consistency, the hot end of gradients was always associated with arena segment 22, and
256 control arenas followed the number scheme of neighboring thermal gradients. The specific
257 sequence of changes applied is listed in Figure S3.

258

259 *Animals and data gathering*

260 Animal collection: This study included 24 red-backed salamanders (*Plethodon cinereus*)
261 collected between September and November 2023 in New Haven, CT, mainly at East Rock Park
262 and neighboring areas. These salamanders were collected in three groups of 8 individuals, from
263 September (first group) to November (third group). While in the field, we placed salamanders in
264 individual plastic bags that were filled with moist leaf litter, and salamanders remained there
265 until the next day. Then, we transferred the specimens to individual plastic cases containing
266 humid paper towels, which were maintained in a walk-in environmental chamber set at 15°C. We
267 fed salamanders weekly with flightless fruit flies (*Drosophila*) and applied established husbandry
268 protocols (e.g., Reiter et al. 2014).

269 Temporal distribution of data collection: With few exceptions and pauses associated with the
270 academic calendar, we ran experiments twice a week from September to December 2023. During
271 this time, we tested each of the 24 salamanders five times. On the first experimental day for a
272 given salamander group, we individually placed four salamanders in the control arenas (room
273 temperature set at 15°C) and four in the thermal gradients. On the next day of experiments, the
274 salamanders that were previously assigned to the control were assigned to the thermal gradient,
275 and vice versa. We repeated this procedure over two additional experimental days. Then, on one
276 additional experimental day, we tested four salamanders as control and four as experimental. In
277 total all salamanders were tested twice as control, twice as experimental, and once as either
278 control or experimental. We did not attempt to test all salamanders six times, to guarantee that all
279 24 individuals would be studied during the natural surface activity season (Anthony and
280 Pfungsten 2013; Fisher-Reid et al. 2024). Experiments were concluded by mid-December 2023.

281

282 *Experimental protocol*

283 To start an experiment, we randomly defined the position (1 to 22) in the gradient at which
284 each salamander would be released and placed a wet cotton circle at the designated spot. Next,
285 we carefully placed salamanders under the cotton circles, aiming to reduce movement until the
286 onset of formal observations. Once all salamanders were in place, we defined time zero and
287 activated a stopwatch. We collected behavioral data throughout the experiment (*e.g.*, Number of
288 end-to-end movements and visits to the extreme segments of arenas; see below) and every five
289 minutes (standard variables, *e.g.* numbered position in arena and salamander dorsal temperature;
290 see below). The experiment continued for 105 min and all tests started between 12:00 and 14:00h
291 (EST). On two occasions (27 September and 04 October) experiments were finished at 95 and 80

292 minutes, respectively, to accommodate experimenters' schedules; however, the amount of
293 movement was typically small later in experiments. There were three-four people present in the
294 walk-in environmental chamber during data collection: this team comprised two observers, each
295 watching four of the thermal gradients, one note taker responsible for writing the information,
296 and one person who assisted the observers. *Plethodon cinereus* is primarily nocturnal, but
297 individuals are also surface active during the daytime during favorable weather (Anthony and
298 Pfingsten 2013) and were thus expected to respond to thermal cues during our experiments.
299 Every 10 min, all arenas were sprayed with mineral water to keep the substrate humid and the
300 animals fully hydrated. Although spraying water may momentarily change the temperature of
301 salamanders and gradient, we thought that preferable to dehydration, since hydric stress has been
302 shown to affect salamander selected temperature (Galindo et al. 2018).

303

304 *Variables quantified*

305 Our primary goal concerned how salamander position and movement differed between the
306 control arenas and thermal gradients, and which of the hypotheses concerning salamander
307 behavior summarized in Table 1 were supported. In addition, we recorded several variables
308 related to salamanders and experimental setup to explore their effect on inference.

309 Behavioral Response Variables: These are aspects of behavior quantified in terms of three
310 dominant dimensions: movement, position, and temperature. The variables recorded
311 continuously were: 1) Reaches to 22 (number of independent visits to position 22, which was the
312 warmest in the case of thermal gradients), 2) Reaches to 1 (reaches to position 1, the coldest in
313 thermal gradients), and 3) End-to-end events (events of movement from position 1 to 22, or vice
314 versa). Variables registered every five minutes were 4) Position in gradient, 5) Orientation (the

315 dominant direction of the head of salamanders according to a cardinal plane with four values
316 only), and 6) Salamander dorsal surface temperature (hereafter, body temperature; T_b) measured
317 with an infrared thermometer (General IRT207) aimed at the dorsum on the mid-body of the
318 salamander at a distance of approximately 20 cm from the salamander (distance-to-spot ratio of
319 8:1 inches). Body temperature represents a function of room oscillation in controls, and of
320 salamander position in gradients. From these measurements we also determined 7) Movement
321 (the number of 4.5 cm segments traversed; for example, a salamander at position 8 found five
322 minutes later at position 6 would have a count of “2”), 8) Motionlessness (number of consecutive
323 observations at the same position), 9) Maximum position visited (equivalent to hottest reach in
324 thermal gradients), 10) Minimum position visited (equivalent to coldest reach in activated
325 gradients), 11) Time at last movement (experimental time at which salamanders moved for the
326 last time), and 12) Number of movement events (count of events in which the position was
327 different from the previous observation).

328 Experimental setup variables: Variables that, according to published observations, may
329 influence results. These are: 1) Minimum Gradient Temperature, 2) Maximum Gradient
330 Temperature, 3) Gradient Temperature Range, and 4) Salamander release position (according to
331 labels). In addition, we measured setup variables that were not a part of our conceptual
332 framework but could influence results and require care. These include the already mentioned
333 variables: 5) Block, 6) Depth, 7) Pole, plus 8) Mean control temperature and 9) Mean control
334 variance. The last two mentioned are useful because they reflect unavoidable oscillations of
335 room temperature that changed slightly across experimental days.

336 Salamander variables: Variables related to the collected salamanders that could influence
337 results. These are 10) Capture date, 11) Days to test (between capture and test day), 12) Order of
338 testing, 13) Sex, and 14) Body mass (g).

339

340 *Statistical analyses*

341 To test which of our proposed hypotheses were supported by the data, we ran a series of
342 models, one for each of the response variables listed in Table 1, comparing control arenas and
343 thermal gradients. The statistical model chosen for each variable was selected based on variable
344 type (numerical, ordinal, count) and the evaluation of whether variables conformed to
345 assumptions of models. When applicable, we used salamander identity as a random effect.
346 Specific models used and their hypothesized patterns are listed in Table 2. We report variance
347 explained for the fixed effects and the full model using the marginal and conditional R^2 ,
348 respectively, obtained from function *r.squaredGLMM* implemented in R package ‘[MuMIn](#)’
349 (Nakagawa and Schielzeth 2013).

350 Movement: To investigate salamander movement in our experimental system we took
351 three different strategies: First, we evaluated if treatment (control or thermal gradient) affected
352 total movement of the salamander throughout the trial. We measured movement as “total
353 distance moved” by counting the number of segments of the arena traversed by the salamander
354 every 5 minutes and summing them all for the entirety of the trial (see *Behavioral response*
355 *variables* for details). We used linear mixed effects model (LMM) with treatment (control arena
356 vs. thermal gradient), release position (1-22), salamander mass, and sex as fixed effects, and
357 salamander identity as random effect to account for repeated measurements. We used the R
358 package ‘nlme’ (Pinheiro and Bates 2000) for this and similar analyses. Salamander total

359 movement was log-transformed to conform to the assumption of normality of residuals. Because
360 of one trial in which the salamander had a movement of zero, we added 1 to all values before
361 log-transforming. Running the analysis without log-transforming or removing the 0 value
362 resulted in qualitatively identical results. Sex and mass were not found to significantly affect
363 movement or other response variables, so were excluded throughout.

364 Secondly, we used a Komolgorov-Smirnov test to compare whether movement
365 distributions along time periods were equivalent between control and gradients. Lastly, to test
366 whether there were individual differences in movement between salamanders, we used a
367 repeatability estimation with a Poisson distribution using the function *rpt* implemented in the R
368 package 'rptR' (Stoffel et al. 2017). This analysis tests if the amount of variation within
369 individuals is smaller than variation among individuals, which is evidence for behavioral
370 repeatability. We quantified repeatability using the intraclass correlation coefficient (R) and
371 considered behavior repeatable if the 95% confidence intervals did not overlap zero.

372
373 Salamander Position: To determine if salamander position in thermal gradients differed
374 from controls, we used LMM with mean position as the response variable, treatment as a fixed
375 effect, and salamander ID as a random effect to account for individual differences. We initially
376 also included salamander mass, sex, and release position as covariates, but since they did not
377 have significant effects, we removed them from the final model.

378 Within each of the treatments, we also ran a chi-square analysis to determine if
379 salamanders were randomly distributed across numbered positions in the arenas, or if their
380 observed positions were different than expected by chance. For those analyses we counted the
381 number of salamanders for which the final position corresponded to each numbered position (1-

382 22) in control and thermal gradients separately and then ran a chi-square analysis on each
383 treatment to determine whether salamander position was significantly different from a random
384 (uniform) distribution across positions.

385
386 Salamander body temperature (T_b): We ran a LMM with average temperature for the whole
387 duration of the trial as the response variable and treatment (control or thermal gradient) as a fixed
388 effect with salamander ID as a random effect. We implemented a model in the R package ‘nlme’
389 that accounts for heteroscedasticity since the thermal variance between treatments was different.
390 Running the model without accounting for heteroscedasticity yields qualitatively equivalent
391 results. We ran an additional LMM estimating what control temperatures would be if
392 salamanders were in thermal gradients. We did that by obtaining an average temperature for each
393 of the numbered positions in the gradients (as shown in Figure 1) and then assigning these
394 temperatures to control salamanders based on the positions they were at in each time period. This
395 allowed us to compare actual T_b in thermal gradients with T_b salamanders would have had in
396 control arenas if they were thermally heterogeneous.

397
398 Influence of setup variables on salamander T_b and position: The full list of variables considered
399 can be found in the “Variables quantified” section of the methods. We used linear mixed effects
400 models (LMMs), with salamander ID as a random effect to account for repeated measures, and
401 either T_b or salamander average position (separately) as response variables. We ran separate
402 models depending on the predictor variables as described below.

403 To determine the effect of exploratory variables relating to the gradient structure, (release
404 position, and minimum, maximum, and breadth of temperature in the gradient) we considered

405 only data from thermal gradients. Because thermal breadth was highly correlated with minimum
406 (Pearson's $r=-0.66$, $P < 0.001$) and maximum temperature (Pearson's $r=0.86$, $P < 0.001$), we ran
407 one model that included gradient thermal breadth and release position as predictors and another
408 with minimum and maximum temperature, and release position as predictors. We log-
409 transformed T_b to conform to model assumptions of normality of residuals.

410 To test for the effect of additional setup variables on average T_b we ran linear mixed
411 effects models with salamander ID as a random effect. We first ran one global model including
412 Treatment (control or thermal gradient), Block (Left or Right), and Depth (Front or Back) as
413 fixed effects. We removed Pole (Hot and Cold) from this analysis because this variable isn't
414 meaningful in controls (since it refers to the direction of the cold and hot ends), therefore would
415 yield a spurious interaction with treatment by default. We tested for interactions between
416 predictors and subsequently dropped each interaction effect that was not significant, removing
417 interactions with P -values > 0.05 sequentially, until only significant interactions were left. We
418 repeated this process with each treatment separately, including Pole as a variable in the analysis
419 of thermal gradients. We performed post-hoc tests with the estimated marginal means
420 implemented in the R library 'emmeans' (Lenth 2017).

421

422 **Results**

423 *Salamander movement and position*

424 Although some individuals remained motionless most of the experimental time, both control
425 and treatment groups had non-zero movement, particularly at the beginning of the experiment
426 (Figure 3). Therefore, the hypothesis "No movement" requires no further consideration.
427 Salamanders in both gradients or controls tended to move more often over the first 20 min of

428 experimental time (Figure 3) and displayed a similar distribution of movement over time
429 (Kolmogorov–Smirnov test $D = 0.24$, $p = 0.59$). Salamanders in thermal gradients moved a
430 median of 21.5 segments (mean = 28.62; max = 96; min = 1), and the counterparts in controls
431 moved a median of 20.5 units (mean = 32.02; max = 185; min = 0). Control and gradient
432 salamanders were comparable in the log-transformed total movement throughout the trials
433 (estimate = 0.05, $t\text{-value}_{1,94} = 0.30$, $p = 0.76$; Table 2), and we found a weak but significant effect
434 of initial position on total movement (estimate = 0.03, $t\text{-value}_{1,94} = 2.18$, $p < 0.05$), with
435 salamanders moving slightly more when initially placed on higher positions (warmer side on
436 thermal gradients). Ranges for all variables referenced in Table 1 are reported in Table S1.

437 Despite the equivalent amount of movement between treatments, the thermal gradient
438 clearly influenced the behavior of salamanders in the system, particularly from the perspective of
439 the specific positions occupied. In both treatments, salamanders were distributed non-randomly
440 (control arenas: Chi-squared = 321.33, $df = 21$, $p < 0.001$, thermal gradient: Chi-squared =
441 462.13, $df = 21$, $p < 0.001$) and spent more time at the extremes of the arenas (e.g., around
442 positions 1 and 22). However, the distribution of animals in thermal gradients was unimodal and
443 skewed towards 1 (colder end), whereas that of animals in the control arenas was bimodal,
444 towards both 1 and 22 (Figure 4), which is expressed by salamanders in thermal gradients
445 occupying a lower average position (estimate: -4.24, $t\text{-value}_{1,95} = -3.55$, $p < 0.001$). For the
446 control, records at position 1 or 22 constituted 62.31% of total observations (38.47 % at 1 and
447 23.84% at 22). By contrast, 56.81% of the records for salamanders in thermal gradients were at
448 position 1 (cooler end), and only 2.40% at position 21-22 (warmer end) (Figure 5B).

449

450

451 *Salamander Body Temperature (T_b)*

452 As expected, given the available thermal landscape, control and experimental salamanders
453 differed in average T_b (estimate: -3.40, $t_{1,119} = -4.60$, $p < 0.001$), with control animals reflecting
454 room temperature and its variation in time and space (mean \pm SD = $13.31^\circ\text{C} \pm 0.99$, range =
455 $9.5^\circ\text{C} - 20.8^\circ\text{C}$) and experimental counterparts ranging from 0.1 to 38.7°C (virtually the full
456 range of temperatures available across all tests), with a lower average temperature ($9.91^\circ\text{C} \pm$
457 5.64) (Figure 5A). In the thermal gradients, the average maximum T_b experienced by
458 salamanders throughout a trial was $22.59^\circ\text{C} \pm 7.23$, and the average minimum T_b was $6.04^\circ\text{C} \pm$
459 5.34 .

460 For further analyses, we compared the actual T_b of salamanders in the gradient with the
461 temperatures that controls would have had in a thermal gradient (using the average temperature
462 in each position from gradients as shown in Figure 1). In thermal gradients, salamanders
463 displayed a median T_b of 8.82°C and a mean of $9.91^\circ\text{C} \pm 5.64^\circ\text{C}$, whereas control salamanders
464 would have had substantially higher counterpart values of 11.53°C and $15.20^\circ\text{C} \pm 10.44^\circ\text{C}$
465 (based on average gradient values) (estimate = -5.27, $t\text{-value}_{1,95} = -3.51$, $p < 0.001$). This analysis
466 corroborates the behavioral differences among groups, and the influence of activating a thermal
467 gradient on salamander behavior, providing evidence that salamanders in thermal gradients are
468 thermoregulating.

469

470 *Experimental setup variables*

471 The impact of setup variables is summarized in Tables 3 and 4. The directionality of
472 gradients affected salamander behavior so that the variable Pole had significant effects on both

473 salamander T_b and position (Table 3). Salamander T_b in thermal gradients was higher under a
474 Cold Pole state ($11.96^\circ\text{C} \pm 6.54$) compared to a Hot equivalent ($8.35^\circ\text{C} \pm 4.32$). Simply,
475 salamanders chose warmer temperatures when the hot end of thermal gradients was facing away
476 from the middle position of the environmental chamber. Salamander position tended to be higher
477 on the Cold state of Pole (9.27 ± 7.28) in comparison to the Hot state (5.96 ± 6.30). Since high
478 numbered positions always represented higher temperatures on gradients that means that
479 salamanders tended to move away from the middle of the room.

480 Since salamander Days to Test and Order of Testing were highly correlated (Pearson's $r =$
481 0.93 , $p < 0.001$), we dropped the variable Days to Test from the model and kept only Order of
482 Testing. Because salamander Capture Date consisted of three discrete capture events, we
483 converted that variable to a categorical variable called Salamander Group, with three levels.
484 Results of salamander variables are summarized in Table 4, with no significant effects on either
485 T_b or position. Salamander Group had a marginally significant effect on salamander T_b ($p =$
486 0.06), possibly because minimum gradient temperature tended to be slightly colder through the
487 season (Figure S4).

488 489 *Individual variation and familiarization with the system*

490 Because salamanders in this experiment were each tested five times, we could explore the
491 behavioral repeatability and whether being previously presented with the thermal preference
492 arenas affected salamander behavior. Regarding movement patterns, we observed slight, but
493 significantly repeatable behavior among individual salamanders ($R = 0.197$, $SE = 0.091$, $CI =$
494 $[0.004, 0.362]$, $p < 0.001$), so that some individuals were more active and others more sedentary.
495 This variation was consistent, irrespective of whether salamanders had been presented with a

496 thermal gradient or a control arena. The order of trials (or previous exposure to arenas),
497 putatively related to familiarization with the system, did not influence the amount of movement
498 observed in salamanders (Table 4).

499

500 **Discussion**

501 We tested alternative hypotheses about the thermal behavior of the Eastern red-backed
502 salamander, *Plethodon cinereus*, when exposed to homeothermic (control) and heterothermic
503 (experimental) arenas, with the goal of clarifying their behavioral responses to temperature in the
504 laboratory. This investigation addressed three topics, specifically (1) the benefits of a control
505 arena in thermal preference experiments, (2) the impact of changes in the experimental setup on
506 thermal behavior, and (3) the role of confounding variables on such behavior. From our results,
507 we can discuss the behavior of salamanders in response to a thermal gradient and offer some
508 methodological recommendations for researchers interested in related topics. We hope that this
509 study helps researchers interested in behavioral thermoregulation, behavioral fever, mechanistic
510 niche modeling, and other approaches, and that it provides useful insight related to how small
511 ectothermic animals navigate heterothermal landscapes.

512 *Salamander behavior in control and thermal gradients*

513 We envisioned control tests as null models for salamander behavior in arenas with conditions
514 that approached homothermality. This ‘control’ behavior proved to be neither random
515 (salamanders were not even distributed across the 22 positions) nor equivalent to the
516 experimental counterpart (use of positions varied among treatments). Salamanders in the control
517 arenas consistently moved from the randomly selected starting point towards any of the arena’s
518 ends, followed by a tendency to remain in those extremes. Whereas this pattern was common to

519 both control and thermal gradient arenas, individuals in the latter group that reached the hottest
520 temperatures in the gradient rarely remained there. In controls, salamanders were not at greater
521 risk of thermal stress at one end than the other and so remained at either end. We see two (non-
522 mutually exclusive) explanations for this tendency to remain at the ends of the arena in the
523 control group: salamanders could either be attracted to the end of the gradient, be less inclined to
524 move once ends are reached, or some combination of both. In the absence of refuges
525 (salamanders could not excavate the vermiculite substrate as also reported in Heatwole 1960),
526 individuals could interpret the corner at the end of the arena as a shelter, and so be less inclined
527 to move once this location is reached. Some organisms will move towards and stay near “walls”
528 because they are attracted by touch, a phenomenon known as thigmotaxis and demonstrated for
529 other organisms such as cockroaches (Camhi and Johnson 1999) and rats (Barnett 1963; Treit
530 and Fundytus 1988). This could also influence salamander behavior in our trials, although there
531 is evidence that *P. cinereus* is not driven by thigmotaxis and is more influenced by other factors
532 such as avoidance of light (Test 1946). Avoidance of light might have contributed to the results
533 we observed, because salamanders were more likely to move away from the middle of the room
534 (where the light bulb was located). Irrespective of the motivation, we are positive that a control
535 enhanced the quality of our inference when interpreting salamander thermal behavior, and
536 suggest that controls always be applied in studies with amphibians and other ectotherms, except
537 when thermoregulatory behavior is very well established (Angilletta Jr. 2009; Huey et al. 2012;
538 Muñoz and Bodensteiner 2019).

539 Had we limited our study to consider only behavior in thermal gradients, we might have
540 reasonably concluded that salamanders strongly favor the coldest temperatures. However, this
541 would have been misleading, in the sense that we know, thanks to the control, that other

542 behavioral drives add complexity to the observed responses. Collectively, our results suggest that
543 salamander responses are composite and include 1) exploratory behavior (more pronounced at
544 the beginning of trials), 2) avoidance of the warmest temperatures in thermal gradients, 3) a
545 tendency to remain at the end of arenas, and 4) inclination to remain at cold end of thermal
546 gradients. Furthermore, the positive correlation between salamander T_b and minimum gradient
547 temperature suggests that salamanders in thermal gradients remained in the coldest position
548 available at the end, and did not choose a temperature (the temperature at position 1 was 0.5-
549 13.4°C). Even though we performed experiments during the afternoon and *P. cinereus* is a
550 mostly nocturnal species, we do not think that invalidates our results. Salamanders explored the
551 full range of the gradients, moving particularly often in the first 20 minutes of the experiment,
552 but often throughout the trial. In addition, *P. cinereus* can be surface-active during the day when
553 environmental conditions are ideal (e.g., after rainfall) (Anthony and Pflingsten 2013), and
554 therefore would still be expected to respond to environmental cues during the daytime.

555 Two additional issues to discuss are the concept of ‘preference’ and the potential for these
556 laboratory findings to predict salamander field behavior. Our results suggest preferences for a
557 position in the system, but not necessarily for a target temperature, except avoidance of
558 excessively hot temperatures (Paranjpe et al. 2012; Navas et al. 2021). Heat avoidance seems
559 consistent with field behavior because, when temperatures are too high on the surface, *P.*
560 *cinereus* tend to move underground (Heatwole 1962; Farallo et al. 2020; Waldron et al. 2024).
561 For ectothermic animals — and particularly for amphibians with permeable skin — avoiding
562 extreme heat confers advantages by mitigating eventual stress and minimizing dehydration
563 (Riddell et al. 2024; but see Navas et al. 2022). In these taxa, thermal behavior involves not only

564 evasion of detrimental physiological conditions but also the maintenance of water balance (Feder
565 1982; Feder and Londos 1984; Galindo et al. 2018).

566 Finally, we observed individual variation in salamander behavior. All individuals tested
567 display heat avoidance, but patterns differ, and were repeatable, particularly regarding the
568 configuration of movement. Some individuals had a higher tendency to move and explore the
569 gradient than others, and this behavior was consistent in both gradients and controls. Further
570 studies shall define if these differences are compatible with the idea of animal “personality” (Bell
571 et al. 2009), but at this point our data are consistent with other studies showing repeatability of
572 behaviors such as foraging or risk-taking in *P. cinereus* (Cosentino and Droney 2016; Waldron et
573 al. 2022; Garner et al. 2024). More work is needed to identify how these differences in individual
574 behavior as we observe in the lab translate into fitness and intraspecific variation.

575

576 *Methodological considerations*

577 Logistical and practical constraints will often prevent researchers from applying the full
578 range of controls, repeated measures, induced experimental variation, and location variables that
579 we employed. Therefore, our aim here is not to propose an ideal experimental design for thermal
580 gradients, but rather to discuss the implications of various experimental decisions. This
581 discussion is intended to help researchers prioritize factors relevant to their specific scientific
582 questions, study systems, and experimental contexts. Along these lines, we first examine the
583 influence of setup variables, as there is no ambiguity regarding the impact of the system's
584 position within the walk-in chamber. This observation suggests the potential influence of hidden
585 variables affecting behavior.

586 Our design allowed us to detect the impact of the arena's orientation (Pole) on salamander
587 position and body temperature (T_b), but the measured variables are unlikely to be direct causes
588 of behavioral changes. Plethodontid salamanders are known to detect gradients of humidity
589 (Heatwole 1962; Galindo et al. 2018), light (Heatwole 1960), and soil pH (Vernberg 1955;
590 Wyman 1988), among others. Additionally, they likely detect and react to convection (air
591 movement), which may influence rates of dehydration (Feder 1983). Many uncontrolled or
592 unknown factors may influence salamander behavior, introducing behavioral noise relative to the
593 target variable, which in this case was substrate temperature. Randomizing the position of
594 thermal gradient arenas relative to physical space is a broadly valuable recommendation, as
595 previously suggested by other authors. Because the importance of this variables is context-
596 dependent, we report the numerical impact of such variables, recognizing that magnitudes may
597 be critical for some research questions but acceptable or negligible for others.

598 Despite thermal preferences being commonly reported for ectotherms, our results fail to
599 corroborate this trend in *P. cinereus* and instead suggest avoidance behavior in relation to heat
600 and confounding factors related to the physical structure of the arenas. These results have two
601 main implications. First, they contribute to the understanding of this species, suggesting that its
602 thermal behavior is complex and influenced by broader environmental cues, as indicated by other
603 studies on plethodontid salamanders (Feder and Pough 1975; Feder 1982; Galindo et al. 2018).
604 Second, our findings highlight the critical importance of controls in testing hypotheses regarding
605 thermotaxis (e.g., active temperature selection). Central tendency values derived from thermal
606 gradients are insufficient for a comprehensive analysis of thermal behavior (see review and
607 additional references in Navas et al. 2021). However, we can leverage the inference we can
608 obtain from a common salamander that is easy to work with in a laboratory context to inform

609 studies with rare species or in limited field conditions, in which complex and time-consuming
610 experimentation is prohibitive. Amphibians face significant risks from climate change (Wu et al.
611 2024), and it is therefore important that our inferences from thermal preference experiments are
612 as informative and biologically relevant as possible.

613 Finally, we caution researchers about the reported effects of arena ends, particularly at the
614 cold end of the gradient, given the observed heat-avoidance behavior. One possibility is to
615 provide cover objects throughout the arena (Feder and Pough 1975), or to adopt circular or
616 elliptical thermal gradients that eliminate terminal edges while retaining lateral ones (Touska et
617 al. 2016). While this approach may be helpful if the observed response has a thigmothermic
618 basis, circular designs do not inherently eliminate shading or other illumination-related factors
619 and are challenging to refine. Another possibility is to use arena ends with temperatures beyond
620 the species' tolerance range, assuming they would be avoided; however, such avoidance is not
621 guaranteed, and animals may risk fatal overexposure to heat (Navas et al. 2007). A list of
622 potential recommendations could become exhaustive; thus, our core advice is to conduct pilot
623 experiments in which behavior is carefully observed, both in the presence and absence of
624 induced thermal gradients. Within the limits of feasibility, the positions of arenas should be
625 randomized with respect to light and airflow sources, and controls are invaluable for robust
626 experimental design.

627

628 **Author contributions:**

629 C.A.N and J.L.B developed initial ideas for the work. C.A.N., J.L.B., R.R., A.F.G., A.K.T. and
630 J.C. performed experiments. J.L.B. performed statistical analyses and generated figures with
631 assistance from C.A.N. and R.R.. R.R. compiled references. M.M.M. provided logistical support

632 in the development of the project and contributed with permanent intellectual discussion along
633 the project. M.M.M. and E.A.R. provided funding for the project. C.A.N. and J.L.B. wrote first
634 version of manuscript. All authors contributed in the writing and revisions of the manuscript.

635

636 **Acknowledgements**

637 We thank the Muñoz lab for helpful suggestions throughout the development of this project and
638 for always offering a stimulating and critical intellectual atmosphere. We also acknowledge
639 Shubhi Sharma, Will Rogers, Sina Rometsch, and Brian Waldron for assistance with statistics
640 and helpful comments on the manuscript. Cari Hickerson and Carl Anthony kindly provided
641 comments on the manuscript and offered some insight about *P. cinereus* salamanders. We thank
642 two anonymous reviewers for thoughtful and constructive comments. *All experiments were*
643 *performed in accordance with protocols approved by Yale IACUC 202220297.*

644

645 **Funding**

646 This work was supported by National Science Foundation, grant number DEB-2039476. J.L.B.
647 was supported by a graduate fellowship from the Yale Institute for Biospheric Studies (YIBS).
648 C.A.N. was supported by a YIBS Edward Bass Distinguished Scholar in Environmental Sciences
649 and further supported by FAPESP, the state of São Paulo Science Foundation (2021/14671-0).
650 R.R. was supported by a Fulbright fellowship for a year of study abroad at Yale University.

651

652 **Conflict of interest statement:**

653 The authors declare no competing interests.

654

655 **Data availability:**

656 The data used in the present paper are available upon request from the corresponding author
657 (J.L.B.).

658

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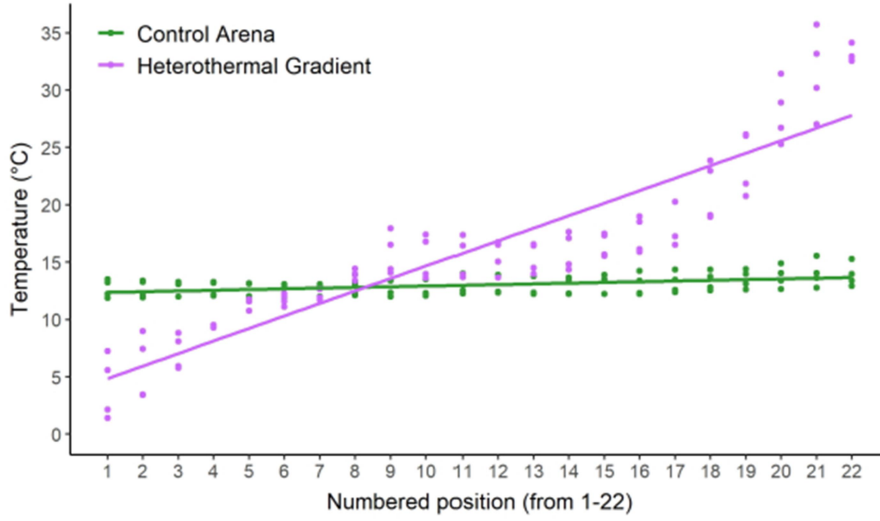
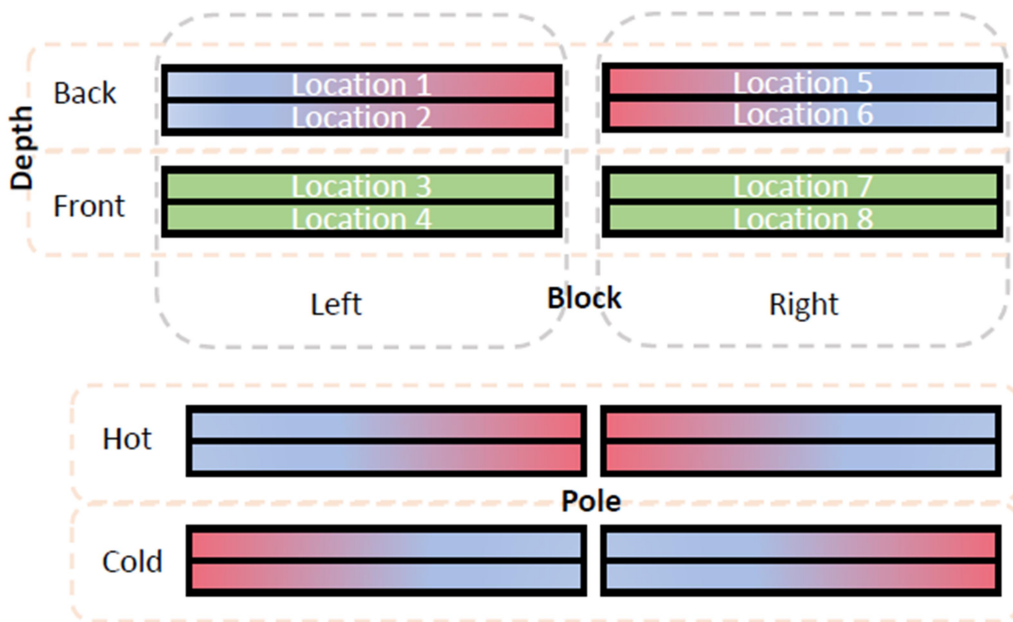


Figure 1. Baseline thermal structure of control and thermal gradient arenas along numbered positions. Gradient and control temperatures were comparable at positions 9-14.

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Figure 2. Illustrative schematic of the setup variables and their states. Block relates to position of arena: Left or Right of the room; Depth represents the position of arenas in relation to observer, Back being further from the observer and Front, closer; Pole refers to the orientation of the hot end of the gradient, which for gradients in both right and left sides faced either towards the middle of the room (Hot) or facing away from each other towards the walls (Cold).

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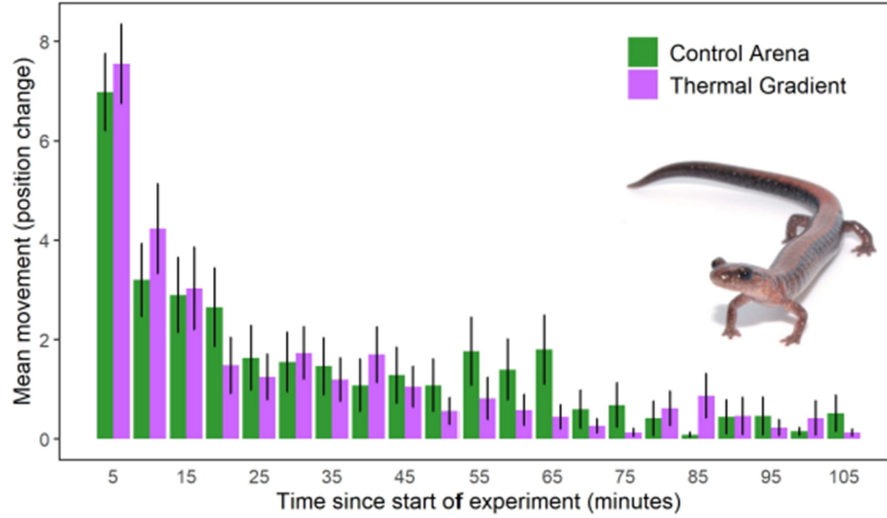


Figure 3. Bar graph showing salamander mean movement through time (minutes since beginning of trial), error bars correspond to standard errors. Movement was defined as the change in position according to numbered sections of arenas. Each numbered section has a width of approximately 5 cm, so each movement unit corresponds roughly to 5 cm.

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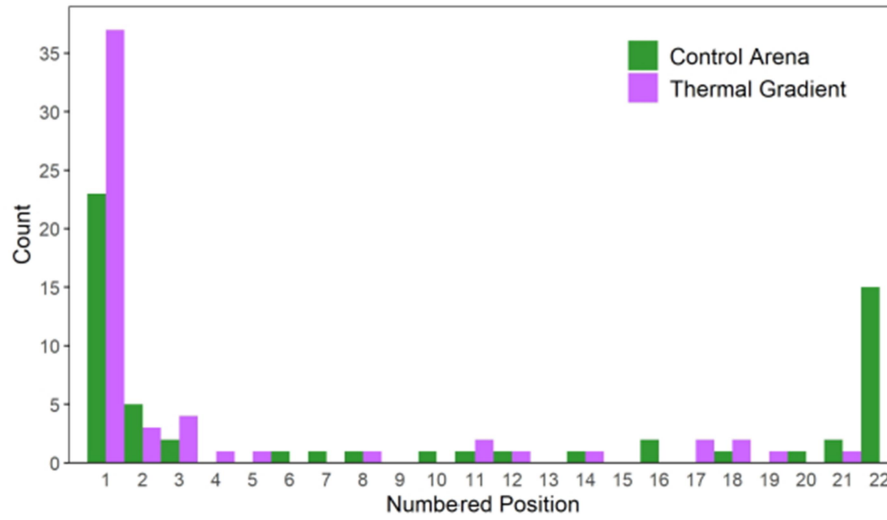


Figure 4. Histogram of salamander median position in arenas (as represented by numbered sections); in thermal gradients lower numbers are always associated with the colder temperatures. Note that in control arenas salamanders tended to remain in both ends of the gradient (positions 1 and 22), while salamanders in thermal gradients remained overwhelmingly more on position 1, which corresponds to the cold end.

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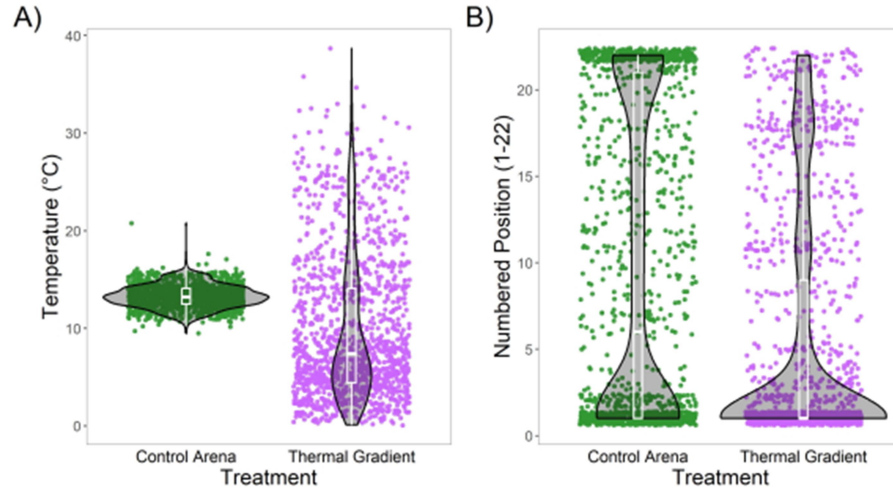


Figure 5. Violin plots showing temperature (A), and numbered position (B) of salamanders in control arenas and thermal gradients. Each point represents one measurement, so each salamander is represented by several points, for as many times it was measured during one trial. Note that salamanders in the thermal gradient exhibited lower temperatures on average than controls (A), this is likely explained by the tendency of salamanders to remain at the ends of the arenas, in both extremes (positions 1 and 22) in control arenas, and only on the cold end (number 1) in thermal gradients.

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The behavior of salamanders in a thermal gradient is:	Unrelated to temperature	Warm avoidance	Cold avoidance	Avoidance of both extremes	Indicative of keen thermoregulation
<i>Reaches to number 22 (warmest)</i>	Similar	Fewer	Similar	Fewer	Fewer
<i>Reaches to number 1 (Coldest)</i>	Similar	Similar	Fewer	Fewer	Fewer
<i>Coast to coast events (1 to 22)</i>	Similar	Fewer	Fewer	Fewer	Fewer
<i>Dominant position in the gradient</i>	Similar	Fewer at high values (>20)	Fewer low values	Fewer extreme values	Fewer in a range to be determined, probably on the colder side.
<i>Total distance moved</i>	Similar	Somewhat lower	Somewhat lower	Lower	Lower
<i>Motionlessness</i>	Similar	Lower at high release number	Lower at small release number	Lower at high and small release numbers	Lower
<i>Maximum number visited</i>	Similar	Lower, less frequent > 20	Similar	Lower, less frequent < 3	Lower
<i>Minimum number visited</i>	Similar	Similar	Fewer or less frequent	Fewer or less frequent	Similar or higher
<i>Time at last movement</i>	Similar	Similar or earlier	Similar or earlier	Possibly earlier	Earlier
<i>Number of movement events</i>	Similar	Somewhat fewer, related to release number.	Somewhat fewer, related to release number.	Fewer as a function of release number	Fewer
<i>Body Temperature (extrapolated in control)</i>	Similar	Lower	Higher	Lower	Lower variance more than anything else.

Table 1. Hypotheses (Column Heads) tested, and predictions based on variables (*italics*, first

column). Comparative statements are Gradient animals relative to Control.

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Table 2. Pattern of behavioral response variables of salamanders in thermal gradient in relation to the controls. Pattern refers to whether the behavioral response value was higher or lower in the thermal gradient relative to the control. GLMM = generalized linear mixed-effects model; LMM = linear mixed-effects model; Ordinal = ordinal regression. When applicable we report the marginal (R^2_m) and conditional (R^2_c) R^2 for the LMMs and GLMMs.

Behavioral Response Variables	Pattern	Mean control (SD)	Mean gradient (SD)	p-value	Model	R^2_m	R^2_c
<i>Reaches to number 22 (warmest)</i>	Lower	1.03 (1.18)	0.70 (1.11)	0.07	GLMM	0.024	0.12
<i>Reaches to number 1 (Coldest)</i>	Higher	1.17 (1.39)	1.65 (1.49)	0.036	GLMM	0.028	0.277
<i>Coast to coast events (1 to 22)</i>	Lower	0.93 (1.56)	0.90 (1.56)	0.28	GLMM	0.012	0.479
<i>Mean position in the gradient</i>	Lower	9.64 (7.80)	5.38 (5.22)	<0.001	GLMM	0.095	0.123
<i>Total movement</i>	Lower	32.02 (37.79)	28.62 (25.08)	0.763	LMM	0.095	0.123
<i>Motionlessness</i>	Lower	16.33 (3.87)	16.18 (3.48)	0.77	LMM	0.001	0.089
<i>Maximum number visited</i>	Lower	17.45 (6.37)	16.12 (5.53)	0.021	Ordinal	NA	NA
<i>Minimum number visited</i>	Lower	3.57 (5.22)	2.65 (3.90)	0.28	Ordinal	NA	NA
<i>Time at last movement</i>	Lower	44.83 (34.76)	41.17 (33.41)	0.56	LMM	0.003	0.003
<i>Number of movement events</i>	Higher	4.20 (3.30)	4.35 (3.20)	0.71	GLMM	0.001	0.104
<i>Body Temperature (extrapolated in control)</i>	Lower	15.20 (10.44)	9.91 (5.64)	<0.001	LMM	0.09	0.135

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Table 3. Effects of setup variables on mean salamander body temperature in thermal gradients, and on mean salamander position in control arenas and thermal gradients.

BODY TEMPERATURE			Fixed effects			
<i>System</i>	Value	Std.Error	DF	t-value	p-value	
Block	-1.44	1.38	31	-1.04	0.3	
Depth	-1.53	1.35	31	-1.13	0.27	
Pole	-3.79	1.5	31	-2.52	<0.05	
Average control temperature	-0.93	1.24	31	-0.75	0.46	
Control temperature range	-0.83	2.38	31	-0.35	0.73	
POSITION			Fixed effects			
<i>System</i>	Value	Std.Error	DF	t-value	p-value	
Type	-4.1	1.19	90	-3.44	<0.01	
Block	-1.1	1.2	90	-0.92	0.36	
Depth	-0.72	1.24	90	-0.58	0.56	
Pole	-3.1	1.32	90	-2.35	<0.05	
Average control temperature	0.22	1.04	90	0.21	0.84	
Control temperature range	0.48	2.14	90	0.22	0.82	

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Table 4. Effects of salamander variables on mean body temperature in thermal gradient salamander (top) and mean position in control and gradient salamanders (top).

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BODY TEMPERATURE	Chisq	DF	p-value
Salamander group	5.59	2	0.06
Order of testing	4.44	4	0.35
Sex	3.68	2	0.16
Body mass	0.03	1	0.87
POSITION	Chisq	DF	p-value
Salamander group	1.67	2	0.43
Order of testing	4.04	4	0.4
Sex	2.68	2	0.26
Body mass	1.22	1	0.27

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