

1 Thermal boldness: Volunteer exploration of extreme temperatures in *Drosophila*
2 *melanogaster*

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17

18 **Abstract**

19 A dominant perception is that small and motile ectothermic animals must use
 20 behavior to avoid exposure to critical or sub-critical temperatures impairing
 21 physiological performance. Concomitantly, volunteer exploration of extreme
 22 environments by some individuals may promote physiological adjustments and enhance
 23 ecological opportunity. Here we introduce to the literature a Thermal Decision System
 24 (TDS) which is fully modular, thermally stable, versatile, and adaptable to study
 25 navigation through thermal landscapes in insects and other small motile animals. We
 26 used a specific setting of the TDS to investigate volunteer navigation through critical
 27 cold and hot temperatures in *Drosophila melanogaster*. We demonstrate that a
 28 thermally bold behavior (volunteer crossings through a Critical Temperature Zone,
 29 CTZ) characterized a fraction of flies in a sample, and that such a fraction was higher in
 30 an outbred population relative to isofemale lines. As set, the TDS generated a thermal
 31 gradient within the cold and hot CTZs, and the exploration of this gradient by flies did
 32 not relate simply with a tendency to be thermally bold. Mild fasting affected thermal
 33 exploration and boldness in complex manners, but thermal boldness was evident in both
 34 fasted and fed flies. Also, thermal boldness was not associated with individual critical
 35 temperatures. Finally, some flies showed consistent thermal boldness, as flies that
 36 performed an extreme thermal cross were more likely to perform a second cross
 37 compared with untested flies. We hypothesize that a simple “avoidance principle” is not
 38 the only behavioral drive for *D. melanogaster* facing extreme temperatures over space,
 39 and that this pattern may characterize other small motile ectothermic animals with
 40 analogous natural history. The physiological correlates, genetic architecture, and
 41 interspecific variation of thermal boldness deserve further consideration.

42

43 **Keywords:** Behavior, Critical Temperatures, *Drosophila*, Orientation, Thermal Biology.

44

45 **1. Introduction**

46 The study of thermal adaptation in ectothermic animals incorporates principles from
 47 physiology, behavior, and ecology (Cowles and Bogert, 1944; Dawson and Templeton,
 48 1963; Messenger, 1959). One early and broad-spectrum principle is the thermal
 49 dependence of many biological functions, an important factor in evolution. Thus, one
 50 approach to the study of thermal adaptation includes analyzing the relationship between
 51 biological functions and body temperature (thermal performance curves, TPCs), and the
 52 processes under which these curves evolve. In a typical case, a TPC depicts the thermal
 53 sensitivity of a given fitness-related trait over a range of body temperature and zero-
 54 performance values define the critical temperatures for activity (Huey and Slatkin,
 55 1976). Then, the role of behavior in interpreting these curves is paramount because
 56 body temperature may constrain behavior and in turn, behavior has potential to affect or
 57 even modulate body temperature (Angilletta et al., 2006). Accordingly, the body
 58 temperature of ectothermic animals relates in a complex manner with environmental
 59 temperature given the interconnected influences of morphology, physiology, and
 60 behavior. For motile forms, and in manners conditioned by morphology and physiology,
 61 body temperature would be affected by patterns of navigation across thermal landscapes
 62 (Sears et al., 2016). Thus, if animals are granted self-producing (i.e., autopoietic)
 63 qualities and a cognitive domain (Thompson, 2007), their motility would include
 64 cognitive navigation through a thermal environment, and their orientation would follow
 65 decision rules to limit, avoid or even promote exposure to environmentally induced
 66 shifts in body temperature. These cognitive processes would also outline subtleties
 67 regarding thermal niche, a term used rather loosely in the biological literature (Gvoždík,
 68 2018).

69 Orientation in thermal landscapes is influenced by the dynamics of temperature
 70 distribution across space and time (Sears et al., 2016), by social aspects of behavior
 71 including potential for aggregation (Auberson et al., 2019) or heterospecific interactions
 72 (Winterová and Gvoždík, 2018), and by the feedback of behavioral decisions on
 73 physiology (Hutchison and Maness, 1979). This complexity highlights the general role
 74 of behavior as a driver for evolutionary shifts (Mayr, 1959, 1963), and the specific
 75 considerations of this postulate matter in the context of thermal adaption (Huey et al.,
 76 2003). However, incorporating the multifaceted nature of behavior into studies of
 77 thermal adaptation poses many challenges, and those linked to critical temperatures
 78 seem, so far, neglected. By definition, critical temperatures (minimum or CT_{min} , and

79 maximum or CT_{max}) set thermal limits for field activity, and exceeding these
 80 temperatures can lead to behavioral impairment and lethality (Cowles and Bogert, 1944;
 81 Gunderson and Leal, 2016). A derived rationale, then, is that avoiding critical
 82 temperatures would be adaptive, as exposure to critical temperatures implies thermal
 83 risks (Andrew et al., 2013; Sunday et al., 2014). This is a compelling, well supported,
 84 and widely accepted postulate. Furthermore, the avoidance of critical temperatures is
 85 not only pervasive in motile animals but is perhaps the most ancestral type of
 86 thermoregulatory behavior (Nelson et al., 1984). However, Hutchison and Manes
 87 (1979) bring to debate a postulate of utmost importance: thermal landscapes, when
 88 explored behaviorally, create a dynamic physio-behavioral frame in which physiological
 89 adjustments such as hardening become possible. Therefore, navigation through thermal
 90 landscapes is both cause and consequence of individual thermal physiology.

91 It is possible that a simple “avoidance principle” may not be the only driver of
 92 evolution regarding navigation around critical temperatures in thermal landscapes. Even
 93 if the individual risk of approaching lethal temperatures seems ultimate, gains could
 94 exist in terms of cumulative hardening and enhanced ecological opportunity (e.g., short-
 95 term survival, shifting thermal niche, expanding animal distribution, finding resources,
 96 etc.) for some species (Hoffmann et al., 2007; Lee and Denlinger, 2010). Therefore,
 97 thermally risky behaviors may persist in a population, even if only at low densities.
 98 Such a possibility can occur in small ectothermic animals like many insects, whose
 99 motile forms can explore thermal landscapes, equilibrate rapidly with environmental
 100 temperatures, and reproduce in large numbers. With these considerations in mind, we
 101 set two primary goals with this study. First, we designed and tested a system to study
 102 exploration of extreme thermal landscapes in small insects and other motile ectothermic
 103 animals, which we introduce to the literature and describe in detail. Second, we take
 104 advantage of the versatility of the system to explore whether thermally risky behaviors,
 105 defined as the tendency to voluntarily enter and cross zones of critical temperatures (a
 106 behavior hereafter termed “thermal boldness”), exists in fruit flies of the species
 107 *Drosophila melanogaster*. We chose *Drosophila* because they meet the natural history
 108 criteria stated above, are experimentally versatile, and constitute a traditional model.
 109 Besides, previous findings encourage research on this topic. *Drosophila* lineages
 110 respond differently to experimental thermal regimes (Loeschcke et al., 1999), and in *D.*
 111 *melanogaster* laboratory fluctuating environments designed to mimic nature fail to
 112 reproduce wild adaptive patterns (Alton et al., 2017; Kellermann et al., 2015). This may

113 be so because, compared to natural counterparts, experimental thermal environments
114 depress spatial variance and reduce the relevance of navigation rules relative to other
115 traits of thermal biology.

116 The scientific goal of this project is to obtain empirical evidence confirming that
117 thermal boldness is a possible behavioral trait in small and motile ectothermic animals,
118 using *D. melanogaster* as a model. At this point we do not engage into any hypothetic-
119 deductive examination on the evolution of thermal boldness, a development we would
120 consider premature for a foundational study. Specifically, we ask three related
121 questions: i) Do flies voluntarily expose themselves to cold and hot critical
122 temperatures? ii) If yes, does mild fasting enhance such thermally risky behaviors? and
123 iii) Do fly lineages of different genetic makeup differ regarding inclination to approach
124 critical temperatures? We anticipated that, if present, thermal boldness would
125 characterize only a fraction of flies in a sample, and that such a fraction could be
126 enhanced by mild fasting. Also, we supposed that potential differences in thermal
127 boldness among fly lineages would validate future studies on its underlying genetic
128 bases. Additionally, we explored –with no *a priori* hypotheses– a possible coupling
129 between thermal physiology (temperature tolerance) and thermal boldness, and the
130 possible consistency of this type of behavior for given individuals. Finally, we
131 considered the possibility that, given the inequality of TPCs (Martin and Huey, 2008)
132 and the less lethal nature of low critical temperatures, navigation towards or into
133 extreme cold temperatures would be more common relative to navigation around or
134 across extreme hot temperatures. We found that thermal boldness did characterize a
135 fraction of individuals in both an outbred population and isofemale lines of *D.*
136 *melanogaster*, with variation, nuances and unexpected patterns that are discussed in
137 detail.

138

139 **2. Material and Methods**

140 *2.1. Description of the system*

141 *2.1.1. Overall design*

142 The apparatus is depicted in Fig. 1, A-C and references to the parts cited in bold (Fig.
143 1A). Detailed measures of the system are provided in Fig. S1. Overall, the system has
144 six compartments called “**Thermal Decision Systems**”, abbreviated “TDSs”, one of
145 which was used to collect temperature data. The basic parts of each TDS are: 1) a **Home**
146 **Bottle** at the base (which may or may not contain food), being a typical transparent

147 stock bottle for *Drosophila* maintenance; 2) an assembly hereafter called **T-System**
 148 (Fig. 1A) given its “T” shape. The T-System connects to the home bottle through an
 149 ascending tube, and on top split into two symmetrical tubular structures of the same
 150 material and light diameter. Once the TDS was thermally stable, a 3) **Thermal**
 151 **Gradient** was conformed along the horizontal part of the T-System, and we use that
 152 term (or just “gradient”) to name this zone between the onset of CTZs. The thermal
 153 gradient is marked by the two black-rings placed 3.5 cm in both sides of the T-System
 154 (Fig. 1A). 4) Two **Temperature Coils**, located at each branch of the horizontal part of
 155 the T-System and externally delimited by black rubber rings, were responsible for
 156 creating both the thermal gradient and 5) the **Critical Temperature Zones** (CTZs),
 157 where cold and hot temperatures were most extreme (Fig. 1C). After the CTZs, and
 158 connected to each distal extreme of the horizontal part of the T-System, we placed 6)
 159 the **Feeding Bottles**, of the same type than the home bottle but always containing food.
 160 Finally, 7) a **Removable Stopper**, a tiny foam circle, was installed to temporally bar
 161 access from the home bottle to the T-System. A nylon thread was attached to the
 162 stopper and left the home bottle via a tiny perforation (Fig. 1A). This configuration
 163 served the purpose of easily allowing fly access by simply pulling the nylon thread,
 164 while causing virtually no disturbance to flies during removal (Video S1). The
 165 connections of the T-System to home and feeding bottles relied on perforated foam
 166 stoppers of the type used to lid *Drosophila* stock bottles (Fig. S1).

167 2.1.2. Temperature control

169 Cooling and heating were provided simultaneously by using two independent
 170 laboratory thermal baths (NESLAB, RTE-300D for cooling; HETO, CBN 8-30 and
 171 HMT 200RS for heating) fit with a water-circulation system, creating the average
 172 thermal landscape depicted in Figure 1C. A silicon hose of 0.7 mm of internal diameter
 173 was fit to the outlet vent of each bath and then connected to the first TDS, as follows:
 174 each TDS was fit with a silicon coil of five loops at each side of the horizontal part of
 175 the T-System (Temperature Coils), placed 3.5 cm after the opening of the ascending
 176 tube, next to the black-ring working as a visual indicator (Fig. 1A, B, additional details
 177 in Fig. S1). The silicon coils connected TDSs in sequence via plastic tubes (1 cm). This
 178 set-up conferred both independent temperature control of cold and hot sides in each
 179 TDS and minimal temperature differences between TDS (conditions granted by a vast
 180 number of preliminary assays, see *Pilot studies* in Supplementary Material). Five TDSs

were used simultaneously and one, always the front most as to reduce interference with observations, was fit with both temperature data loggers and a TC-08 Thermocouple Data Logger (Pico Technology) as to have online temperature records of the inner part of the tube surrounded by temperature coils. The central part of the portion of the inner tube bordered by the silicon hoses displayed the peak thermal barriers (see data table in Fig. 1C). Calibration tests allowed to estimate the inner temperature of the T-System under the influence of the silicone coils, with stable values at the target temperatures (Fig. S2). The system also created a non-planned gradient of relative humidity (Fig. 1C).

2.2. Flies maintenance and handling

Unless otherwise indicated, the data here reported refers to the fly lineage called Odd2010, which was derived from a wild population of *D. melanogaster* collected in October 2010 at Odder, South to Aarhus, Denmark (<https://goo.gl/maps/yEG54oASEU5CynMB7>). This lineage has been kept at 19°C since initial collection, with a generational time of 18 days. Additional tests were performed with isofemale lines of *D. melanogaster* from the Bloomington Drosophila Stock Center (ID number and genotype), which we chose randomly among those available in the laboratory as examples of highly inbred lines: 28240, **ISO40**, DGRP-812/RAL-812; 55014, **ISO14**, DRGP-31/RAL-31; 28213, **ISO13**, DRGP-589/RAL-589 (MacKay et al., 2012), hereafter abbreviated as lines 13, 14 and 40. All flies used were 3-4 days old, except when specifically noted. Flies were kept in 8 oz. (ca. 236.6 mL) stock bottles with 70 mL standard medium (5000 mL water, 200 g sugar, 150 g oatmeal, 80 g agar and 300 g yeast) at 25°C.

For each fly lineage, samples were composed by newly emerged flies of the same generation (i.e., breeding bottle). Briefly, we counted a low number of parents (ca. 30 pairs per breeding bottle) and applied routine transfers as to maintain comparable density among breeding bottles. For final fly selection, we first transferred flies from the breeding bottle to a fresh stock bottle (with food) using a systematic procedure leading to a presumably similar, yet uncounted, number of flies. From this new stock bottle, we aspirated about 50 flies using a silicone tubing with a glass point and a filter at one end, and then transferred them into a 7 mL empty vial (transfer vial) for later transfer. Then, we allowed 30 of these so aspirated flies to move upwards from the transfer vial to a home bottle to be installed in a TDS according to protocols. Flies to be fasted were

215 transferred to home bottles with agar but no food 10 h before the onset of behavioral
216 observations, always at daytime, whereas non-fasted flies were transferred to home
217 bottles with food 60-90 min before data collection.

218 To achieve the final count of 30 flies passing from the transfer vial to home bottles,
219 we used a small funnel fit to a silicon hose split by an aquarium air-valve that could be
220 closed after the target fly number was attained. Thus, we controlled the number of flies
221 for each sample, but not sex ratios. We opted for such a counting procedure because
222 pilots showed that flies sorted under CO₂ anesthesia 3-4 days after emergence moved
223 less in the TDS than non-anesthetized flies (see *Preliminary tests* in Supplementary
224 Material). Once the target fly number was attained, home bottles were transferred
225 randomly to the TDSs. Occasionally up to two flies escaped or were squashed by the
226 stopper in the setting of home bottles to T-Systems. These differences were ignored.

227

228 *2.3. Behavioral observations and data collection*

229 *2.3.1. Overall approach*

230 The study was carried out at the fly lab of the Department of Biology - Genetics,
231 Ecology and Evolution, Aarhus University. We turned the thermal baths on 70 min
232 before the onset of data collection to let target temperatures stabilize in the system (see
233 Supplementary Material for further details). Our overall thermal landscape (Fig. 1C)
234 involved maintaining home bottles and ascending tubes at a room temperature of
235 26±1°C throughout the duration of the test. We started the test by removing the stoppers
236 of all TDSs (< 5 sec) and granting flies access to T-Systems (Video S1). Flies reaching
237 the horizontal part of T-Systems found an average thermal gradient ranging from ca.
238 15°C to 41°C, and CTZs with most extreme temperatures measured at ca. 10°C and
239 47°C. These values were established empirically and correspond to the most extreme
240 temperatures at which we reported attempted crosses in preliminary tests (Fig. S2).

241 We observed fly behavior every 10 min and scored the behavioral variables
242 described in the next section, all of them associated to activity and tendency to approach
243 or enter the CTZs. Thus, 10 min after stoppers were removed, we collected behavioral
244 data the first time (i.e., at time zero or t_0) and kept collecting data at 10-min intervals.
245 Given that the CTZs were extreme and could act as thermal barriers, we did not
246 maintain a constant thermal setting. Rather, after ending a data collection cycle every 10
247 min, we increased (cold side) or decreased (hot side) settings by 0.5°C, so that the
248 thermal configuration of the system slowly advanced from critical to subcritical. The

option for a progressive change in temperature (as opposed to a fix setting) is part of the exploratory nature of this study. We anticipated that a temperature progression would lead to higher exploration and crossings for evaluation, and to enhanced analytical options (for example, regarding the temperatures at which a given fraction of the tested flies would cross). However, it turned out that most crosses were performed early in tests, as we discuss in the *Results*.

Cycles of data collection and thermal control were repeated up to A) two hours, or until B) at least 5% of flies (2 flies) in a sample had made a cold-cross *and* at least another 5% (2 flies) had made a warm-cross, or until C) at least one cross per side had occurred in each one of the TDSs (it turned out that only one test out of 8 was limited by time). Under protocol B, data collection on a given side, either cold or hot, was terminated when target crosses were reached. Then, we fixed the temperature at this first crossing side and maintained the temperature shift protocol only at the counterpart, until reaching target crosses or time. Cold and warm sides of TDSs were alternated among days in terms of left or right, relative to the system longitudinal axis, as placed on the working bench.

2.3.2. Behavioral variables

At t_0 , and at 10-min intervals hereafter, we scored the number of flies i) exploring (i.e., moving) the COLD side of the T-system before the inner black-ring (*COLDEXP*); ii) exploring the WARM side of the T-system before the inner black-ring (*WARMEXP*); iii) touching the inner black-ring of the COLD side or inside the COLD-coiled area (*COLDCONTACT*); iv) touching the inner black-ring of the WARM side or inside the WARM-coiled area (*WARMCONTACT*); v) in the COLD-coiled area in atypical position and not moving (*CBI*); vi) in WARM coiled area in atypical position and not moving (*HEATCOM*); vii) in the feeding bottle after COLD coils (*COLDCROSS*); viii) in the feeding bottle after WARM coils (*WARMCROSS*). *CBI* stands for Cold-Induced Behavioral Impairment, a set of behavioral responses we observed in some flies attempting to cold-cross and that were reverted when temperature in the cold CTZ increase during our changing-temperature protocol (see *Results*). We avoid the term “chill coma” for it may suggest a physiological collapse to some readers, and this was not supported by further observations. We observed very few cases of *HEATCOM*, probably because of how flies crossed the hot CTZ (see *Results on Behavior after hot CTZ*), therefore, we did not analyze these variables formally but report anecdotally.

283 Also, we observed few back-crossings from any feeding bottle to the thermal gradient
284 (e.g., Video S2), but could not operationalize or analyze these occasional events.

285

286 2.3.3. *Consistency of behavior*

287 To determine whether flies crossing CTZs could be generally more prone to thermal
288 boldness as an individual trait, we performed a second test (next day) using cold-
289 crossing or hot-crossing flies, according to a previous and first test. Here the protocol
290 was modified slightly. First, we obtained crossing flies using 6 samples $\times$ 40 flies each,
291 and set the CTZs at 44°C and 14°C (cold temperature elevated relative to original
292 design as to avoid Cold-induced Behavioral Impairment or *CBI*, see *Behavior* in
293 Results). With this thermal landscape configuration we obtained 40 Hot-crossing flies
294 and 40 Cold-crossing flies (given the need of previous test, these flies were 4 days old).
295 A third group of 40 non-previously tested flies (3 days old) was used as control. This
296 procedure was repeated twice, so that we obtained 2 samples $\times$ 40 flies for each
297 treatment (First Cold-crossing, First Hot-crossing, and Control). For final analyses we
298 used total values (the sum of both tests) and compared the three groups of flies so
299 treated in a test performed under identical conditions.

300

301 2.3.4. *Assumptions*

302 The protocol here reported was based on these assumptions: i) despite the many
303 stimuli that may coexist in the system, the number of flies leaving home bottles to
304 circulate by (or even remain stationary at) any side of the T-System (cold or hot) relates
305 to their behavioral inclination to explore the thermal gradient; ii) the number of flies
306 inside CTZs or in feeding bottles after a CTZ cross relates to their tendency to explore
307 critical or subcritical temperatures (cold or hot), i.e., is an indicator of thermal boldness;
308 iii) eventual divergence in thermal exploration and boldness between fasted and not-
309 fasted flies would result from enhanced motivation in fasted flies for exploring and
310 crossing thermal barriers to get food (fasting enhanced activity in pilot tests, see
311 *Preliminary tests* in Supplementary Material); iv) departure from symmetry in cold-
312 crossers and hot-crossers indicates different inclination to explore cold and hot critical
313 temperatures. In addition, some inferential statements in the Discussion assume that v)
314 the navigation rules used by flies in the system somehow relate to those leading thermal
315 exploration in nature. Also, we suppose that, as preliminary insights, vi) differences
316 across lineages (e.g., outbred population vs. isofemale lines) in the inclination to

317 navigate critical temperatures suggest a genetic basis for thermal boldness; and vii)
318 consistent inter-individual variation in exploration of critical temperatures within
319 lineages has basis on individual traits (e.g., genetic makeup, thermal history).

320

321 *2.4. Measure of thermal tolerances*

322 To explore possible associations between the tendency to perform an extreme
323 temperature cross and physiological thermal tolerances, we planned a specific test based
324 on 6 samples $\times$ 20 flies each (disregarding sex). Each of these six samples was
325 associated to previous behavior in the testing system, as to have two samples of 20 hot-
326 crossers, 20 cold-crossers and 20 non-crossers (i.e., flies remaining in the T-System at
327 the end of a test). Flies composing these samples were obtained from behavioral
328 experiments over two days, so that these thermal tolerances were measured on five-day
329 old flies. We used one sample per treatment to test for the critical thermal minimum
330 (CT_{min}), the other for the critical thermal maximum (CT_{max}). For testing, individual flies
331 were distributed in small glass vials tightly sealed with plastic caps. We tested 60 flies
332 (20 for each treatment) at once for a given critical temperature by immersing vials in a
333 glass-made water bath (aquarium) allowing a clear view of each glass. To measure
334 CT_{max} , onset water temperature was 20°C, and then water temperature increased at a rate
335 of 0.1°C/min. To measure CT_{min} , onset bath temperature and rates of temperature
336 change were the same, but the system contained a mixture of ethylene glycol and water
337 in equal parts to avoid eventual freezing. Four observers collaborated with this measure
338 by reporting end-temperatures for each fly according to typical behavioral observations,
339 consisting mainly of flies falling down to the vial and showing no movement after
340 tapping the vials gently with a metal stick. In that moment a fly was considered in
341 thermal coma, and the temperature at the time was reported as the respective critical
342 temperature.

343

344 *2.5. Dry body mass*

345 We measured dry body mass of individual flies, male and female. The procedure
346 used follows Schou et al. (2015) and consisted in drying flies at 60°C for 24 h and then
347 flash frozen them for later weighting. Flies were split in groups according to their
348 behavior and feeding condition, and then stored in small containers with silica gel, as to
349 avoid water absorption. Individual weight was measured with a Sartorius Laboratory
350 balance (type MC5, Göttingen, Germany).

351

352 2.6.Data analysis

353 Although we report several variables and methods reaching some complexity, the
 354 research here reported is essentially inferential. We basically report keen observations
 355 of fly behavior and propose a biological hypothesis to explain them. We refer to
 356 statistical hypotheses when asking whether data display patterns according to standard
 357 statistical procedures. When pertinent we described temporal patterns of fly behavior
 358 along experiments, but this is exceptional and for most formal analyses the final number
 359 of flies exhibiting a given behavior is sufficient for proper inference. We applied
 360 parametric or non-parametric tests according to the assumption-wise profile of data sets.
 361 Briefly, we used either *t*-tests or Mann-Whitney U tests for comparing behavioral
 362 variables between two groups (e.g., fasted vs. non-fasted flies). For analyzing
 363 behavioral consistency among first crossers (cold or hot) and non-previously tested
 364 flies, we used Repeated Measures ANOVA, in this case to account for the temporal
 365 pattern of crossings within a given TDSs. To explore whether fly lineages differ in
 366 exploratory or bold behaviors we used General Linear Models (GLMs) followed by
 367 Bonferroni Post-hoc Tests (*BPHTs*). Physiological (CT_{min} and CT_{max}) and
 368 morphological (dry body mass) correlates of fly behavior among groups were tested via
 369 either GLMs or *t*-tests when applicable. All statistical analyses were performed in SPSS
 370 v. 22. In the main body of the paper, we provide type of analysis and level of
 371 significance, but placed full statistical details in the Supplementary Material, Table S1.

372

373 3. Results

374 3.1.Behavior

375 3.1.1. Observation on fly behavior in the system

376 Our preliminary tests included numerous behavioral observations at room
 377 temperature that were performed with uncontrolled flies (for sex, density, and age), and
 378 are not suitable for a formal analysis. However, such observations were important to
 379 define our final procedure, and we report main conclusions as Supplementary Material.
 380 Briefly, flies tested at room temperature (no thermal gradient active) displayed diverse
 381 behaviors, with active flies that readily moved into the feeding bottles, more passive
 382 counterparts remaining in the home bottles, and many possible intermediate options. In
 383 formal tests with a thermal gradient, flies retained similar behaviors in the sense that
 384 some typically left the home bottle at the onset of the test, ascended the vertical tube of

the T-System, and found the thermal gradient. Once in it, some flies remained stationary, other explored mainly one side of the gradient, and several explored the full gradient up to its limits, i.e., up to the onset of both CTZs. A fraction of the tested flies voluntarily approached CTZs and attempted either cold- or hot-crosses, and several flies died or were impaired in these attempts (Fig. S3).

Flies approached the hot CTZ by walking, advanced into it, but almost immediately made a sharp U-turn, escaping back into the gradient. Most flies barely surpassed the hot CTZ, but few entered it about 1 cm. At t_0 , i.e., at the highest temperatures, flies that did not U-turn after about 5 mm often switched to flight, sometimes hot-crossing erratically (Video S2), so that virtually all early hot-crosses occurred through this behavior. After t_0 , temperatures were slightly less extreme and flies increased the depth of initial advances into the hot CTZ, eventually reaching about 2 cm before a U-turn, or just not performing a U-turn at all and making a very rapid hot-cross by walking. Although rare observations, very few flies fell in heat coma (*HEATCOM*), few were found dead at the hot side and one fly died while attempting a hot-cross.

When approaching the cold CTZ, flies were exposed to about 15°C and theoretically retained physiological ability to U-turn, but only few flies displayed that behavior. Some approached the cold CTZ by slow walking and progressed into the cold CTZ exposing themselves to progressively lower temperatures. Other flies just stopped by the cold CTZ boundary. Some flies that entered into the cold CTZ adopted atypical positions such as curved bodies, wings opened and legs upwards (Fig. S3), responses we referred to as Cold-induced Behavioral Impairment (*CBI*). Normally at t_0 several flies had attempted a cold-cross already, and at this time some flies under *CBI* accumulated within the cold CTZ (Fig.2, Fig. S3). However, most flies recovered from this condition as we increased temperatures in the cold side (Fig. 2). Finally, we observed two unmistakable cases of back crosses from the feeding bottle to the T-system through the cold CTZ.

3.1.2. Exploration of the thermal gradient

Regarding side selection in the gradient (cold vs. hot) by active fasted (F) or non-fasted (NF) flies, F flies stayed more often at the cold side of the gradient (*U-MW*, $P < 0.01$), while NF flies seemed to explore both sides similarly (*U-MW*, $P = 0.051$; *COLDEXP*, Fig. S4A; *WARMEXP*, Fig. S4B). Pooling all flies, active or stationary, the

pattern was repeated and more flies stayed at the cold side of the gradient, independently of fasting condition (t test, $P < 0.01$). Apparently, the active exploration of the gradient did not display obvious patterns between fasting conditions, at least at the beginning of the experiment (Fig. S4A; Fig. S4B).

Regarding actual approaches to CTZs, F flies were bolder than NF flies when approaching the cold CTZ (*COLDCONTACT*; U -MW, $P = 0.029$; Fig S4C). However, this pattern lasted up to minute 10 (t_{10}) and then diluted (Fig. S4C), partially because cold-crosses occurred or were attempted and led to *CBI* (Fig. 2). Then, the number of flies in condition to cross decreased with experimental time (Fig. 3). Fasted flies also approached the hot CTZ more often than NF flies throughout the test (*WARMCONTACT*; U -MW, $P < 0.01$; Fig. S4D). Overall, only a fraction of approaches to CTZs translated into successful crosses. Most flies exploring the thermal gradient approached CTZs, sometimes insistently, but did not attempt crosses.

3.1.3. CTZ crosses

The mean time to meet protocol option B (5% crosses out of 30 flies in samples, see *Overall approach* in Methods) in cold crosses was shorter than the hot-cross equivalent, regardless fasting condition (cold-crosses, NF flies = 10.5 ± 1.82 min, F flies = 8.8 ± 0.85 min; hot-crosses, NF flies = 45.5 ± 1.35 min, F flies = 47.2 ± 1.32 min; see Table S2 for further details). In terms of cold-crosses, F flies displayed similar values than NF flies despite the former were bolder at exploring critical cold temperatures early in the experiment (*COLDCROSS*; U -MW, $P = 0.966$; Fig. 4A). A partial correlate for this pattern was that F flies displayed more cases of *CBI*, mostly early in the experiment (U -MW, $P = 0.025$; Fig. 2). On the other hand, more F flies appeared to cross through the hot CTZ relative to NF flies, but with considerable higher variation, so that no formal difference could be reported (*WARMCROSS*; U -MW, $P = 0.833$; Fig. 4B). Given that these patterns were heavily influenced by what happened up to t_{10} , for an additional perspective we compared the isolated 10-60 min period of the experiment. During this time, F flies did show higher tendency to perform hot-crosses than NF flies (U -MW, $P = 0.037$), but the number of cold-crosses remained comparable among fasting groups (Fig. 4B).

3.1.4. Consistency of behavior

Sample-wise (we did not track individuals), flies that had performed a first extreme cross, cold or hot, displayed a tendency to accumulate more second crosses along time, relative to flies tested by their first time (Fig. 5A-B). The progression of second cold-crosses with time was clearly elevated in flies that had performed a first cold-cross relative to first hot-crossers and control flies (Repeated Measures ANOVA and *BPHT*, $P < 0.01$; Fig. 5A). A similar pattern was observed for originally hot-crossers performing a second hot-cross (Repeated Measures ANOVA, Crosses $\times$ Time, $P = 0.045$; Fig. 5B), but original cold-crossers also displayed a higher tendency to perform second hot-crosses relative to the control group (*BPHT*, $P < 0.01$).

3.1.5. Lineage-related differences

Overall, isofemale lines seemed behaviorally inhibited in terms of exploration of the thermal gradient, and their thermal boldness was low relative to that of Odd2010. For example, the number flies remaining in home bottles was higher for any isofemale line compared to Odd2010. Flies of the outbred population displayed more crosses through extreme thermal barriers (*TOTAL NUMBER OF CROSSES*; GLM, $P < 0.01$). Among isofemale lines, flies from line 14 performed more crosses through extreme thermal barriers compared to lines 13 and 40, which were more similar to each other (*BPHT*, $P < 0.001$; Fig. 6A, Fig. 6B). This pattern was mostly due to hot-crosses (Fig. 6D), particularly between 14 vs. 40 (*BPHT*, $P = 0.041$), whereas no differences among lines occurred for the very low values of cold-crosses (Fig. 6C). Despite pronounced differences in thermal boldness, flies of all lineages explored the cold CTZ similarly until the first 30 min of the test (GLM, $P = 0.07$), although line 13 exhibited less approaches to the cold CTZ relative to Odd2010 (*BPHT*, $P < 0.01$) but not to other lines (*BPHT*, $P > 0.239$). In contrast, Odd2010 flies approached the hot CTZ more often than any isofemale lines (*WARMCONTACT*; *BPHT*, $P < 0.002$ in all cases), but isofemale lines were also comparable (*BPHT*, $P > 0.205$).

3.2. Thermal physiology and behavior

Cold-crossers were slightly less cold tolerant (i.e., higher CT_{min} , measured only in Odd2010 flies after behavioral experiments) than other groups, yet not significantly (cold-crossers, $N = 20$, $6.6 \pm 0.72^\circ\text{C}$; hot-crossers, $N = 19$, $6.15 \pm 0.37^\circ\text{C}$; non-crossers, $N = 19$, $6.25 \pm 0.56^\circ\text{C}$; GLM, $P = 0.074$). Regarding CT_{max} , hot-crossers were less heat tolerant than cold-crossers, but similarly tolerant than non-crossers (hot-crossers, $N =$

485 20, $39.6 \pm 0.75^\circ\text{C}$; cold-crossers, $N = 20$, $40.2 \pm 0.36^\circ\text{C}$; non-crossers, $N = 20$, $39.9 \pm$
486 0.64°C ; GLM, $P = 0.011$).

487

488 3.3. Morphological correlates

489 We measured dry body mass (BM , in mg) in a subsample of 352 flies, 195 females
490 (139 NF and 56 F) and 157 males (112 NF and 45 F), collected after experiments. Full
491 BM data appears in Table S3. As expected, females were on average 38% larger than
492 males (females, $N=195$, $BM = 0.274 \pm 0.058$ mg; males, $N=157$, $BM = 0.198 \pm 0.034$
493 mg), and post-experimental F flies were about 13% smaller than NF flies ($BM-F =$
494 0.216 ± 0.045 mg; $BM-NF = 0.248 \pm 0.065$ mg; t -test, $P < 0.01$). Cold-crossers and hot-
495 crossers had comparable BM (cold-crossers, 0.256 ± 0.059 mg; hot-crossers, $0.245 \pm$
496 0.061 mg; $BPHT$, $P = 0.207$). Non-crosser flies were smaller than crosser flies (BM ,
497 0.223 ± 0.061 mg; $BPHT$, $P < 0.0001$). A parallel pattern was found when comparing
498 males only, but the trend was weaker among females.

499

500 4. Discussion

501 We present a modular, versatile and adaptable Thermal Decision System (TDS) to
502 study the navigation of small and motile ectothermic animals through laboratory
503 thermal landscapes. The system also allows an investigator to capture and isolate
504 individuals that respond to a given set of decision rules, and so isolated individuals can
505 be used for additional testing. Also, because the system is fully modular, researchers
506 can choose the setup that is best suited to tackle a given research problem. For example,
507 after making a given thermal cross, individuals could find another T-System with
508 further settings, and so on. Finally, the contraption is not particularly expensive, can be
509 set in a small climatic room, and has no special requirements. We provide all technical
510 specifications as Supplementary Material, so that the system can be reproduced,
511 modified, and enhanced.

512 Using one specific configuration of the system we confirmed that individual *D.*
513 *melanogaster* voluntarily enter zones of critical temperatures, and that this is a
514 populational phenomenon requiring the observation of many individuals. Our data show
515 that 1) some flies voluntarily explore temperatures able to impair their behavior or even
516 kill them; 2) this is not a common behavior and extreme temperatures act as barriers for
517 most flies; 3) thermally bold individuals are more prone to engage in additional
518 thermally risky behaviors; and 4) thermal boldness does not relate to thermal tolerance

limits in any obvious manner. Because this is an introductory study, we refrain to incorporate thermal boldness into a theoretical evolutionary framework, a step that will develop as this behavior is further assessed. Also, we ignore how idiosyncratic this study is, for example given that both rearing and acclimation temperature may affect the thermal biology of *Drosophila* (Dillon et al., 2009).

Despite limitations, we infer that for small and motile ectothermic animals that reproduce in large numbers, a small fraction of individuals in a population might be thermally bold. If our results reflect intra-populational diversity (Wolf et al., 2007), as strongly supported by the outbred lineage, thermal boldness could typify the behavioral profile of a small fraction of individuals, along a continuum of risk-taking choices across thermal landscapes (Réale et al., 2007; Wilson et al., 1994, Fig. 7). Regarding underlying mechanisms, thermal boldness could have a genetic basis and be heritable, yet admitting contributions of the developmental environment, maternal effects, social influences, and other individual experiences (Falconer and Mackay, 1996). However, the behavioral differences among tested lineages of *D. melanogaster* favor the hypothesis of a genetic basis for thermal boldness. Although epigenetic sources of variation might be substrate for evolution (Burggren, 2016), differences in shyness and boldness in humans and other animals are mostly genetic and have proved heritable (Wilson et al., 1994 and citations therein), just as some decision-making behaviors of *D. melanogaster* like egg laying substrate selection (Miller et al., 2011). Finally, the notion of thermal boldness, as built upon our data, is compatible with examples of behavioral and physiological diversity within insect populations, including thermal biology, morphology (Forsman, 2000) and larval feeding behavior, as in the rovers vs. sitters *Drosophila* case (which has an autosomal basis, see Debelle and Sokolowski, 1987).

In terms of ecological significance, thermal boldness could be linked to both ecological opportunity and impacts of thermal exposure on physiology (Hutchison and Maness, 1979; Terblanche et al., 2007), but the latter relationship remains to be established. Our data do not corroborate any obvious relationship between thermal boldness and physiological thermal tolerance, although admittedly this behavior could lead to cumulative hardening, perhaps realized in nature, given that some flies were persistent in their volunteer exposure to critical temperatures (e.g., Video S1). Albeit previous studies have suggested little relation between thermal tolerance and some behavioral traits of *D. melanogaster* (e.g., locomotor activity, feeding behavior and place memory) (Bährndorff et al., 2016; Gioia and Zars, 2009), the relationship between

553 thermal physiology and behavior may vary when thermal variation along space is
554 involved (Salachan et al., 2021). However, experimental selection studies have
555 prioritized thermal variations in time, but natural fluctuations involve both time, space,
556 and navigational possibilities. Perhaps this fact explains why attempts to mimic natural
557 thermal fluctuations have failed to replicate the adaptive trends observed in the wild
558 (Kellermann et al., 2015).

559 The onset temperatures used in this study were based on empirical identification of
560 the coldest and warmest temperatures at which crosses occurred (Supplementary
561 Material). So, at least from this perspective, both CTZs were “similarly extreme”.
562 Despite this care, exploration and thermal boldness were asymmetrical regarding cold
563 and hot extremes, a pattern perhaps related to physiological risk. As set in our system,
564 cold temperatures impaired but did not kill flies, contrary to hot temperatures (even if at
565 low frequencies), a finding perhaps capturing one aspect of the asymmetrical nature of
566 TPCs (Martin and Huey, 2008). Even so, fly behavior at and across the hot CTZ suggest
567 that the upper thermal limits of TPCs, as typically measured, do not necessarily relate to
568 the impossibility to explore such thermal zones (Fig. 8). Collectively, the experimental
569 system presented in this paper provides new options to study the relationships between
570 TPC structure and individual thermal physiology and behavior, with specific nuances
571 for cold and hot extremes. Also, in the context here reported, flies immobilized within
572 the cold CTZ did recover when temperature increased. These flies may have entered
573 chill coma but only if understood as a reversible physiological state (Hazell and Bale,
574 2011), though our observations did not support that possibility.

575 Finally, mild fasting enhanced exploration and thermal boldness, particularly at the
576 hot side, with more pronounced effects soon after release into the TDS. Enhanced
577 exploration and boldness were expected given that fasting may elicit more risky
578 behaviors (Moran et al., 2021). In parallel, the behavior of first crossers could have
579 affected other flies via odor clues because fasting also enhances odor-tracking behavior
580 in *Drosophila* (Farhadian et al., 2012). Another caveat is that odor-tracking behaviors
581 may be collaterally affected by temperature, for example through differential dynamics
582 of odor clues at each side of the thermal gradient. Despite these uncertainties, thermal
583 boldness was expressed at both cold and hot sides and was well-defined also in non-
584 fasted flies, even if at lower frequencies. Thus, we propose that thermal boldness may
585 be enhanced by some types of ecological risk, but it is not exclusive to such scenarios.

586 In this sense, our results may not reflect proclivity to forage on substrates above critical
587 temperatures, such as in the ant species *Iridomyrmex purpureus* (Andrew et al., 2013).

588

589 **5. Conclusions**

590 In small and motile ectothermic animals, thermal physiology relates in complex
591 manners with orientation in thermal landscapes. This is evident in the flexibility and
592 limits of TPCs (Angilletta et al., 2002; Navas, 2006), their response to experimental
593 selection (Huey and Kingsolver, 1993), the diversity among measures of performance
594 (Kellermann et al., 2019), and the impact of level of organization (Rezende and
595 Bozinovic, 2019). These analyses assume optimality (Martin and Huey, 2008) and the
596 generalization that critical temperatures, which by definition cause physiological and
597 ecological damage, must be behaviorally avoided by individuals (Andrew et al., 2013;
598 Sunday et al., 2014). Although this perception is supported by a strong theory, the
599 avoidance of critical temperatures, at least as typically measured, may be less pervasive
600 than originally thought. Alternative behaviors, including thermal boldness, may be
601 perpetuated given potential links with physiological adjustment and ecological
602 opportunity. Thermally bold individuals could pioneer the expansion of distribution into
603 some new adaptive zones, as reported for some species during the first stages of
604 invasion (Lindström et al., 2013; Mayr, 1963; Wright et al., 2010). Although it is clear
605 that *D. melanogaster* exhibits thermal boldness, the generality of this behavior needs to
606 be further scrutinized in other species, as well as its consistency, heritability, and
607 evolutionary potential.

608

609 **Authorship contribution statement**

610 **Carlos A. Navas:** Conceptualization, Methodology, Validation, Investigation,
611 Formal analysis, Writing, review & editing, Visualization, Funding acquisition (travel).

612 **Gustavo A. Agudelo-Cantero:** Writing, review & editing, Visualization. **Volker**

613 **Loeschcke:** Conceptualization, Methodology, Writing, review & editing, Project
614 administration, Funding acquisition.

615

616 **Declaration of competing interest**

617 The authors declare that they have no known competing financial interests or personal
618 relationships that could have appeared to influence the work reported in this paper.

619

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630

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760 **Figure Captions**

761

762 **Figure 1.** A) Schematic drawing of the system, showing 4 out of 6 replicates termed
763 ‘Thermal Decision Systems’ (TDS), one of which served as control; B) Photograph of
764 the system as installed; C) Thermographic images of an isolated TDS (upper panel) and
765 each temperature coil (lower panel). The data shows average values of temperature and
766 relative humidity for some key spots on the thermal gradient conformed in the upper
767 part of the TDS. t=0’ refers to ‘time zero’, first event of data collection, and t=60’ refers
768 to conditions one hour after. The numbers in red are estimates based in the temperature
769 of the home bottle.

770

771 **Figure 2.** Average number of flies in Cold-induced Behavioral Impairment (*CBI*) as a
772 function of time and temperature (right axis, dotted curve during tests. Values are non-
773 cumulative over time. Green boxes for fasted (F) flies and blue boxes for non-fasted
774 flies (NF). Boxes depict the first, second (median) and third quartiles containing 50% of
775 data, and whiskers show the maximum and minimum values, except outliers ($\geq 1.5 \times$
776 IQR [the inter-quartile range] from the median) and extremes ($\geq 3 \times$ IQR from the
777 median).

778

779 **Figure 3.** Number of flies approaching the onset of Critical Temperature Zones (CTZs,
780 marked by black rubber rings in the T-System), i.e., summing up cold
781 (*COLDCONTACT*) and hot (*WARMCONTACT*) approaches. Given a progressive
782 reduction in exploratory drive and number of flies (due to both successful crosses and
783 *CBI*), the total number of approaches declined after about 30 min (vertical dashed line).

784

785 **Figure 4.** Cumulative number of crosses through extreme thermal barriers as a function
786 of time. A) Successful cold-crosses, assessed by the number of flies in feeding bottles
787 after the cold coils (*COLDCROSS*). B) Successful hot-crosses, assessed by the number
788 of flies in feeding bottles after the warm coils (*WARMCROSS*). Figure details as in Fig.
789 2.

790

791 **Figure 5.** Count of flies performing a second cross through thermal barriers as a
792 function of time, in a test for behavioral consistency (see main text for details). Bars
793 show the minimum and maximum values, and the median out of four tests. A) shows

the number of second cold-crosses performed by first cold-crossers (blue) or first hot-crossers (red), while B) displays comparable data for second hot-crosses. In both cases, second crosses are compared with a control group of non-previously tested flies (white), which by definition were tested only once for the purpose of these comparisons.

798

Figure 6. Lineage-related differences in crosses through extreme temperatures over time in *D. melanogaster*. The outbred Odd2010 population (black), core of most experiment, was compared with isofemale lines 14 (green), 13 (blue) and 40 (red) of the DGRP (see main text). A) Total number of extreme temperature crosses (i.e., *COLD*CROSS + *WARM*CROSS). B) fraction of total crosses in relation to the number of flies counted in the thermal gradient of the T-System. C) Total number of cold-crosses. D) Total number of hot-crosses. Circles and bars are the mean value $\pm$ 1 SD.

806

Figure 7. Hypothetic populational patterns of risk-taking behaviors across thermal landscapes in small motile ectothermic animals. Individuals exploring thermal landscapes may either retreat from extreme temperatures (shyness) or engage in their exploration (boldness). Polybehavioral populations could differ in the fraction of shy or bold individuals according to ecological and microevolutionary contexts. For instance, population dynamics at a given ancestral thermal niche (e.g., in range-core populations) may reflect directional selection favoring shy individuals (black solid line). Thermally bold individuals may be favored in novel environments with potential for colonization (e.g., edge populations, dotted red line). Transitional states would be possible in microevolutionary time.

817

Figure 8. Hypothetical Thermal Performance Curve (TPC) highlighting cold-to-hot asymmetry (Martin and Huey, 2008). In this figure hatched areas represent thermal barriers based on present data (4-6°C above mean CT_{min} , 5-6°C above mean CT_{max}). Physiological risk would be asymmetrical (dark blue vs. black arrows), from temporarily impaired activity at the cold end (blue fly) to dying individuals at the hot end (black fly). This asymmetry may explain higher exploration of extreme cold by *D. melanogaster* (light blue vs. red arrows), as well as the contrast between voluntarily walking into inhibiting cold (dashed white line within cold barrier) and erratic flying at the hot side (dashed black line within heat barrier). However, CT_{min} and CT_{max} , as

827 typically assessed, do not necessarily reflect limits for voluntary exploration and
828 thermal boldness.

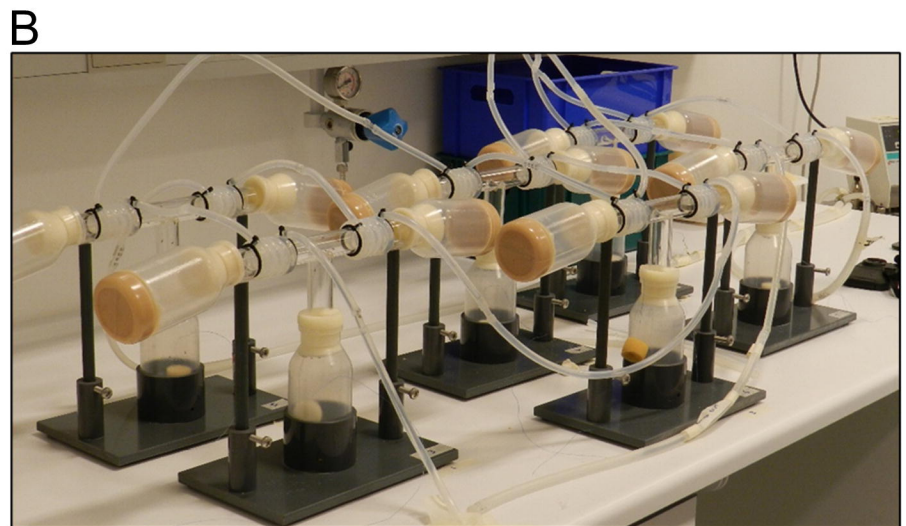
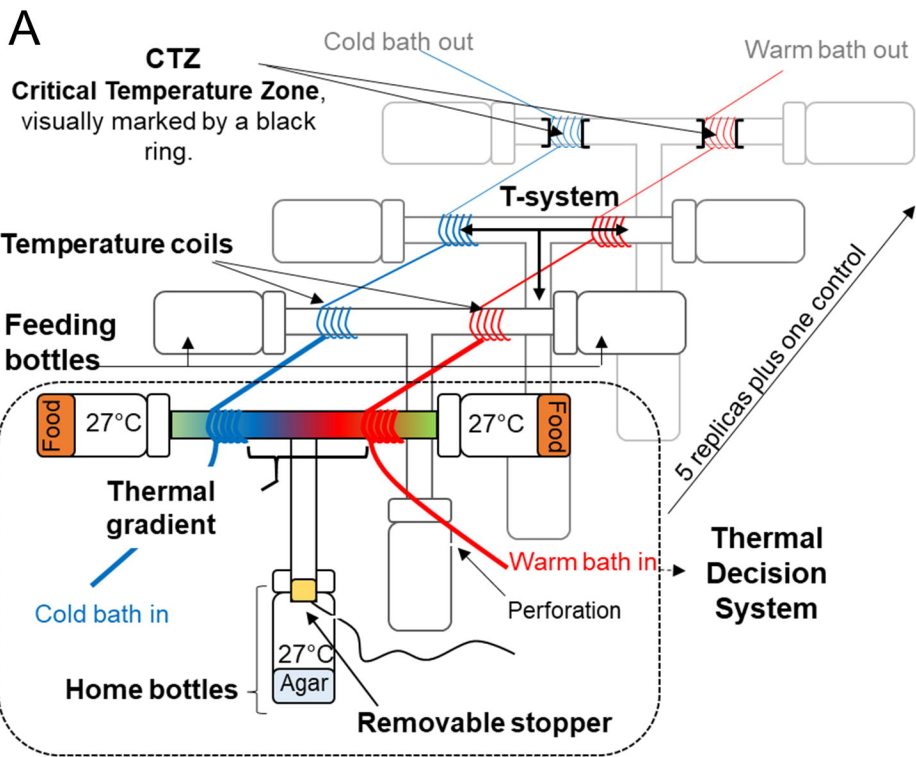


Figure 1

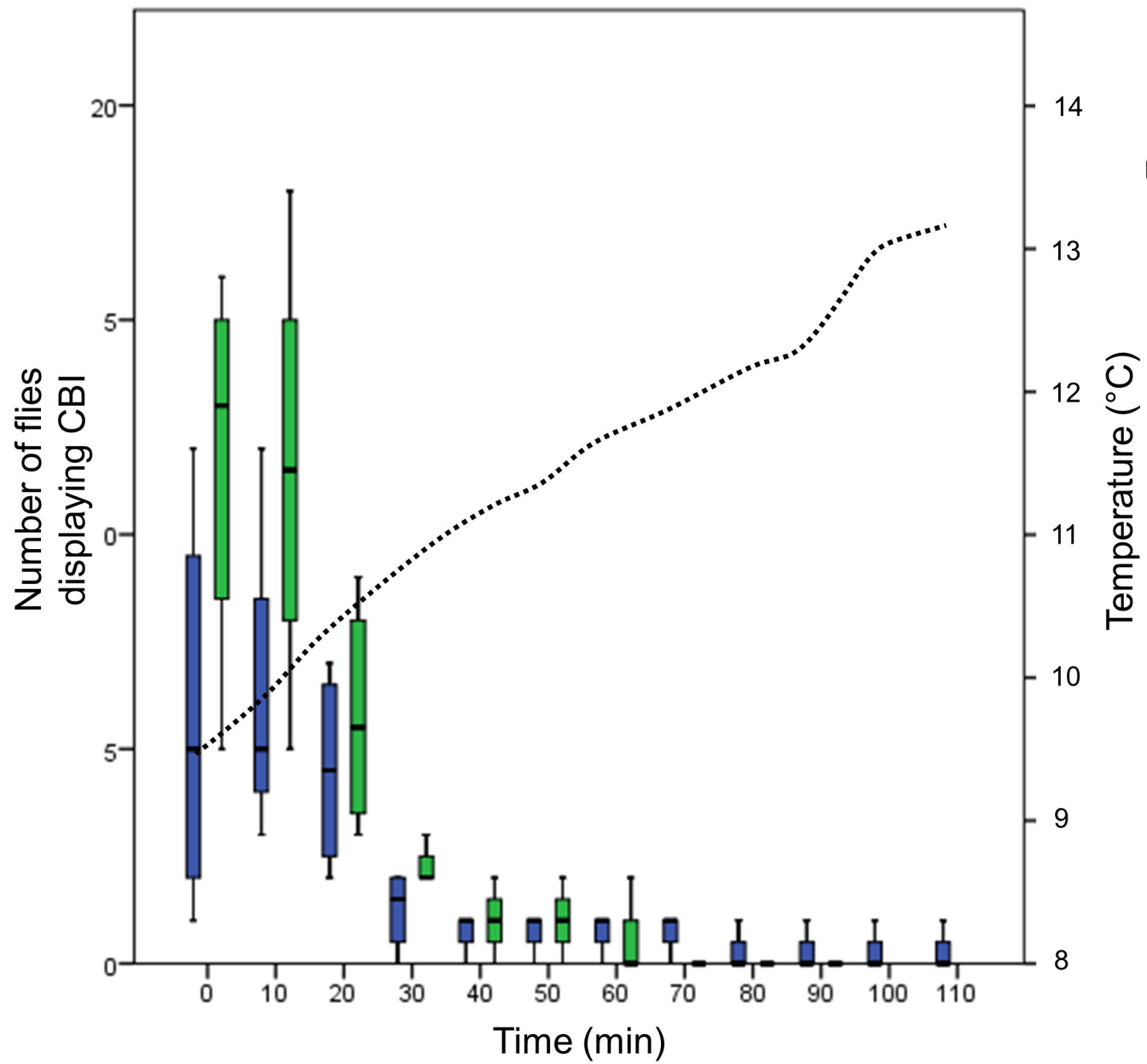


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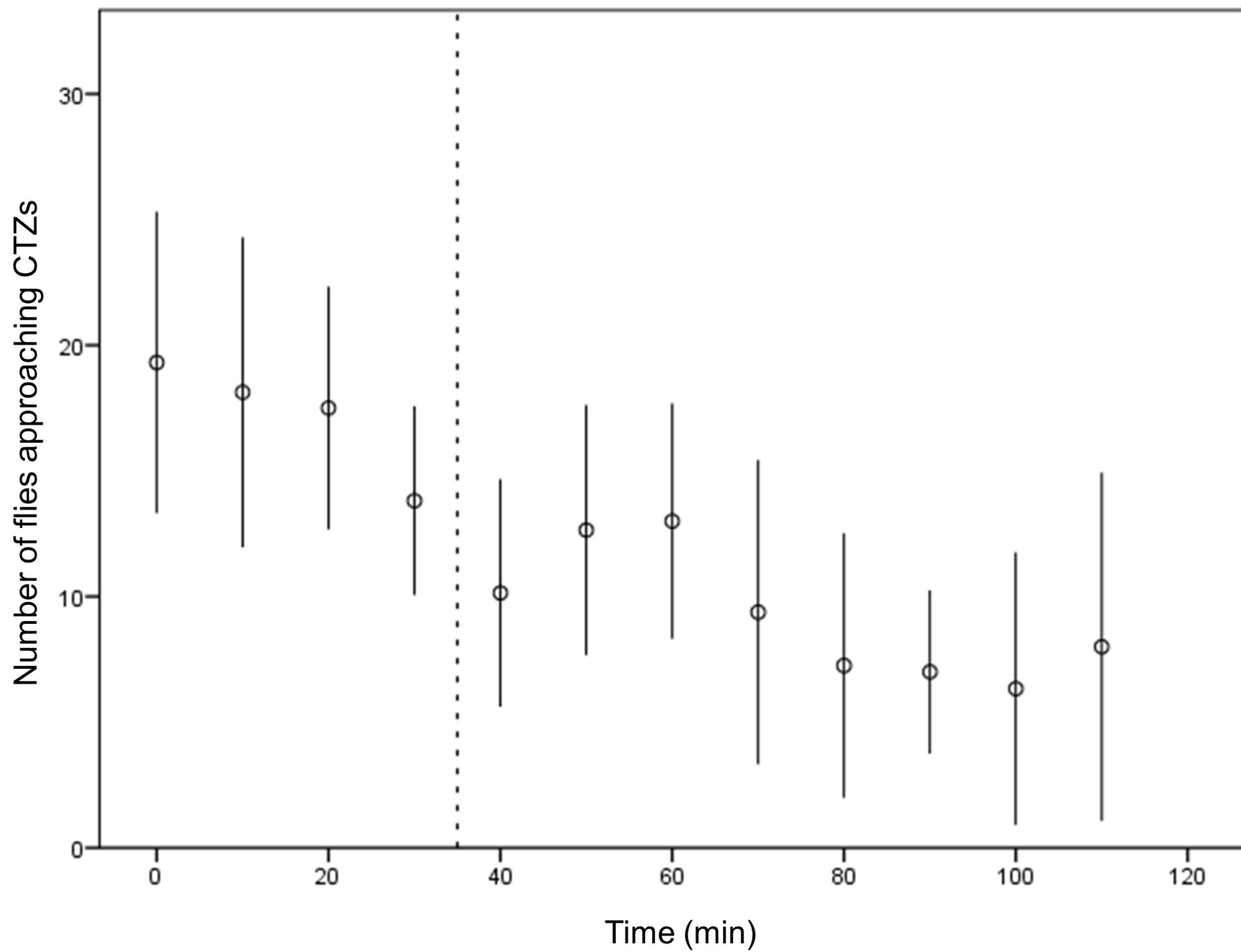


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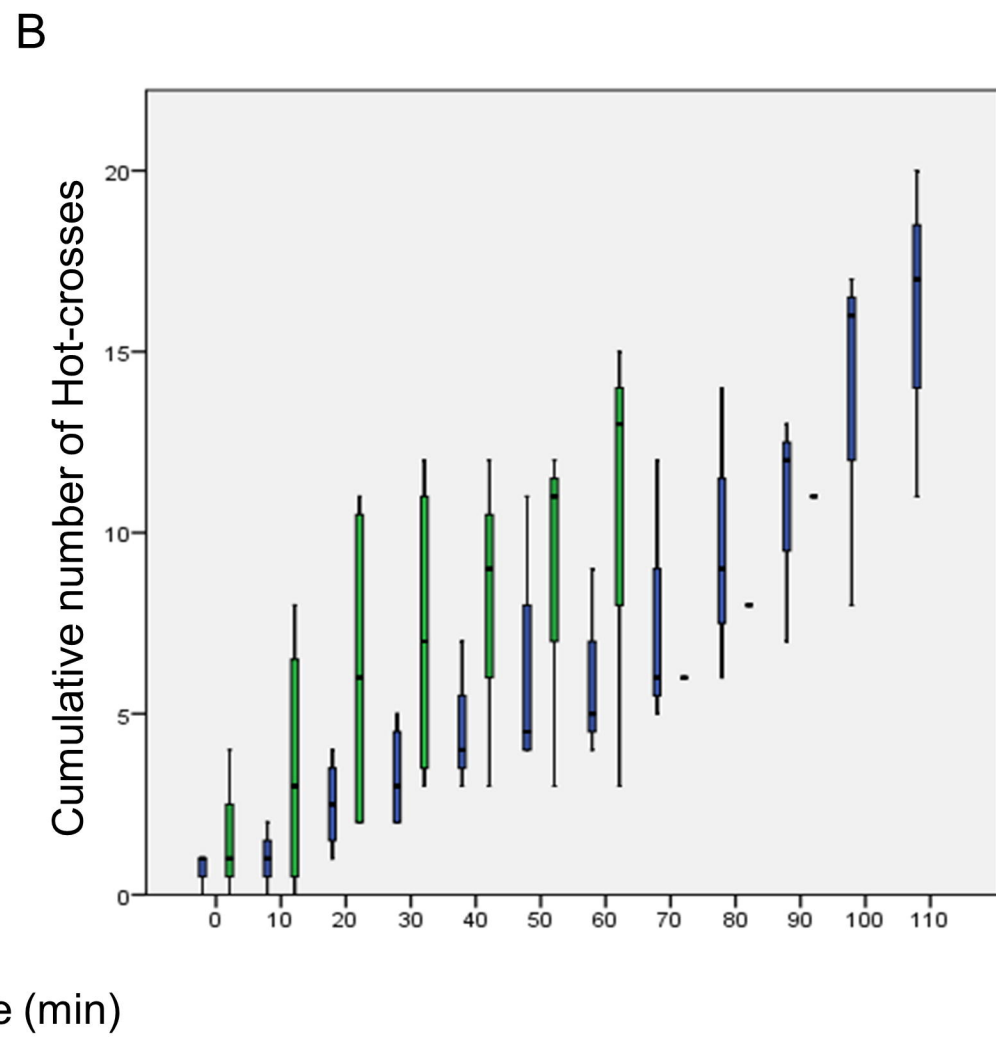
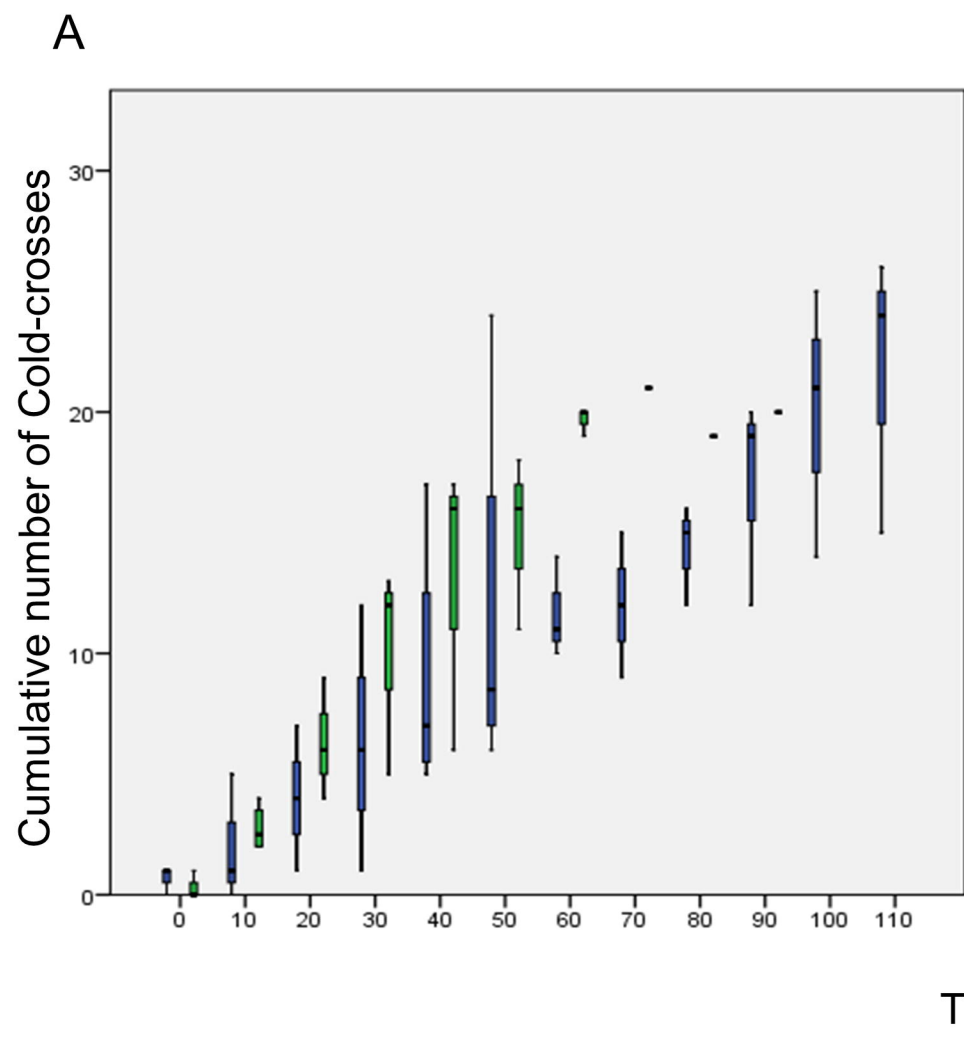


Figure 4

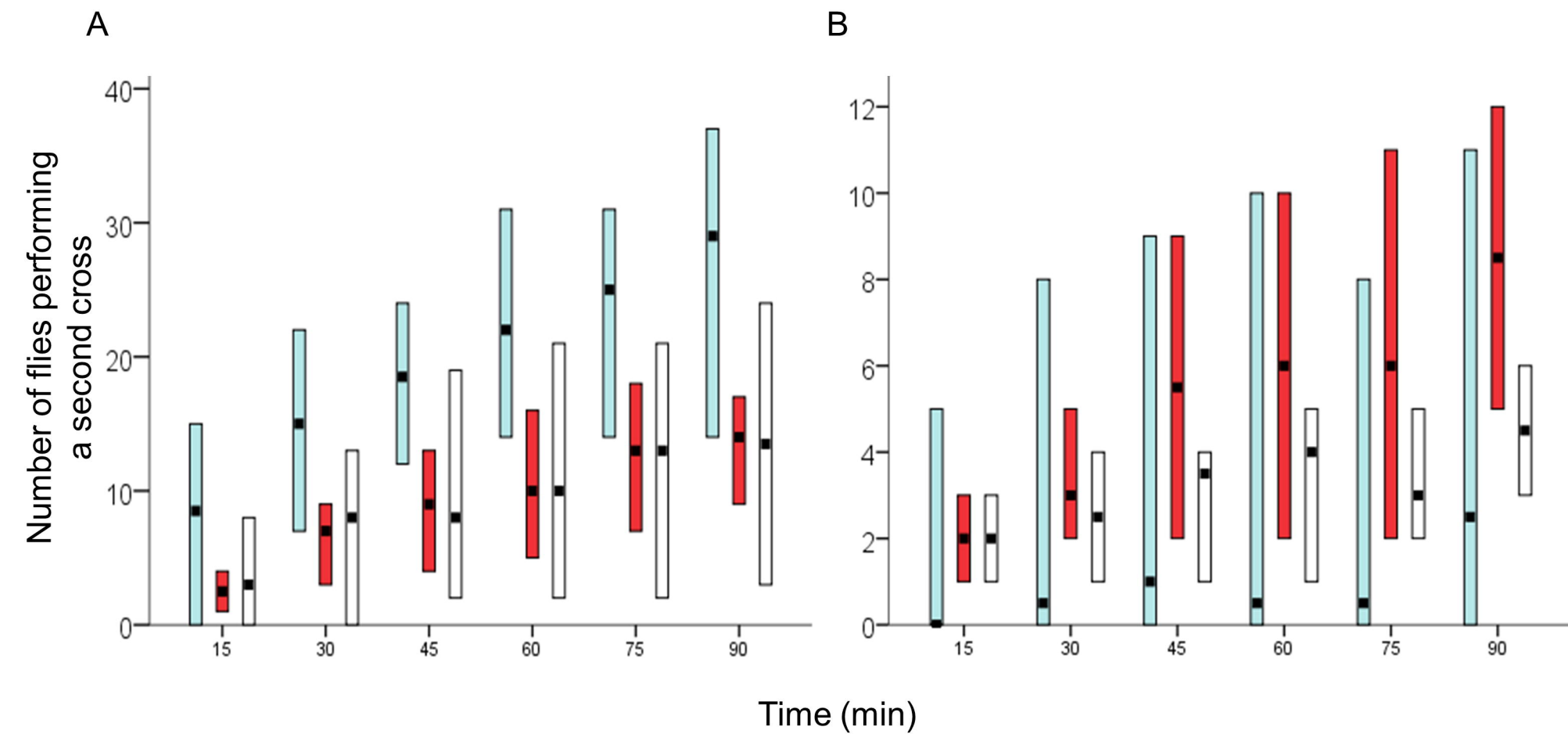


Figure 5

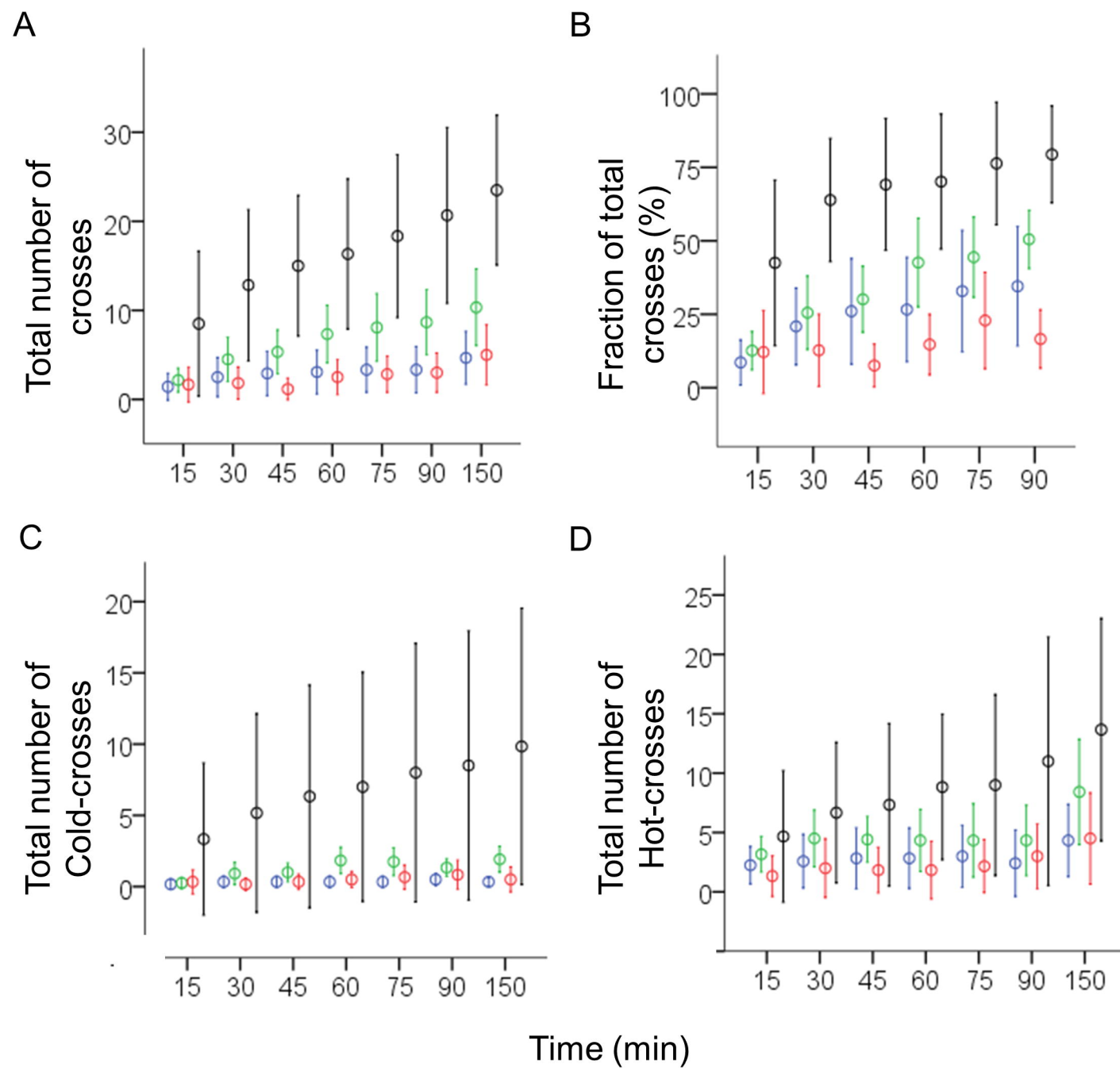


Figure 6

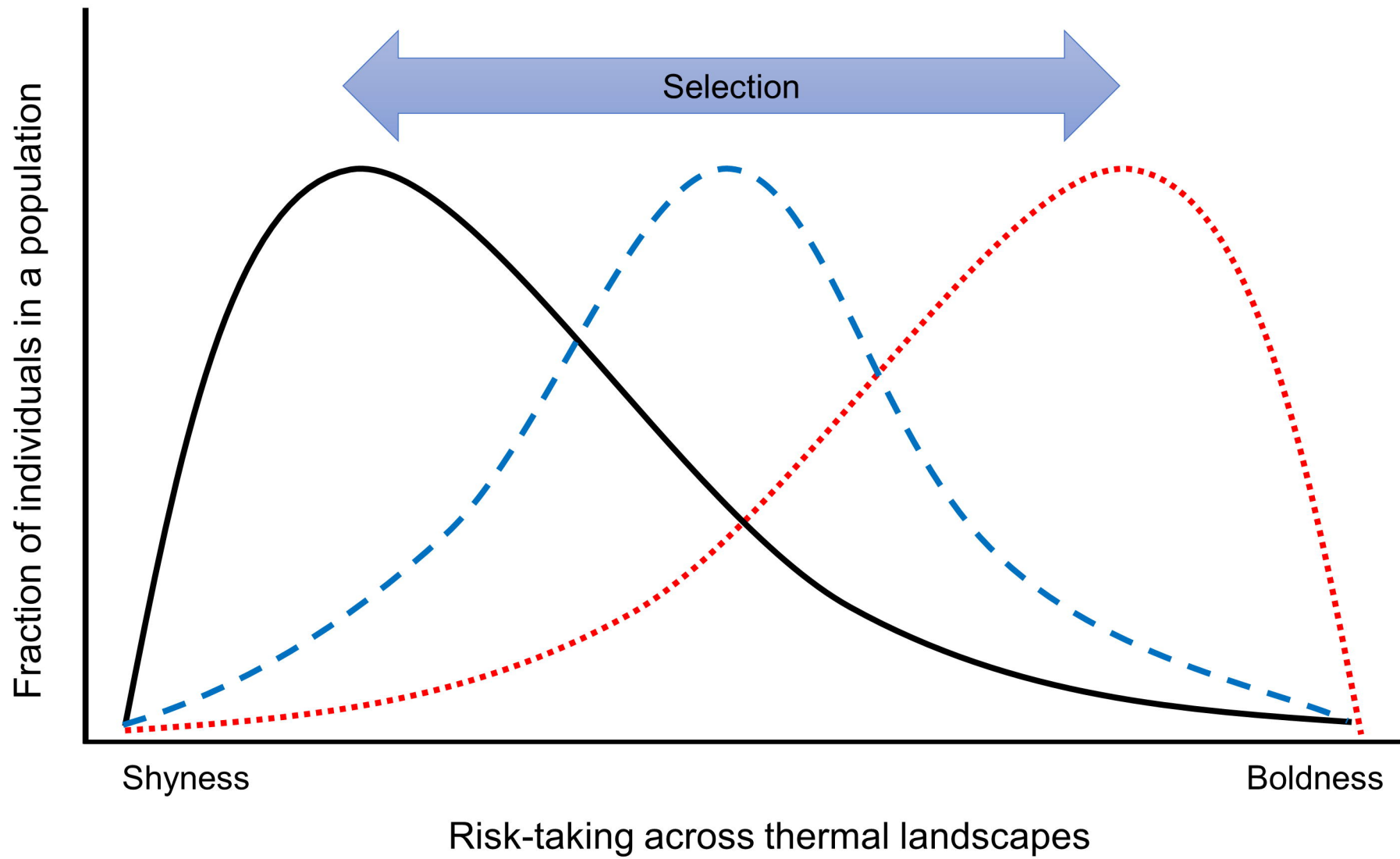


Figure 7

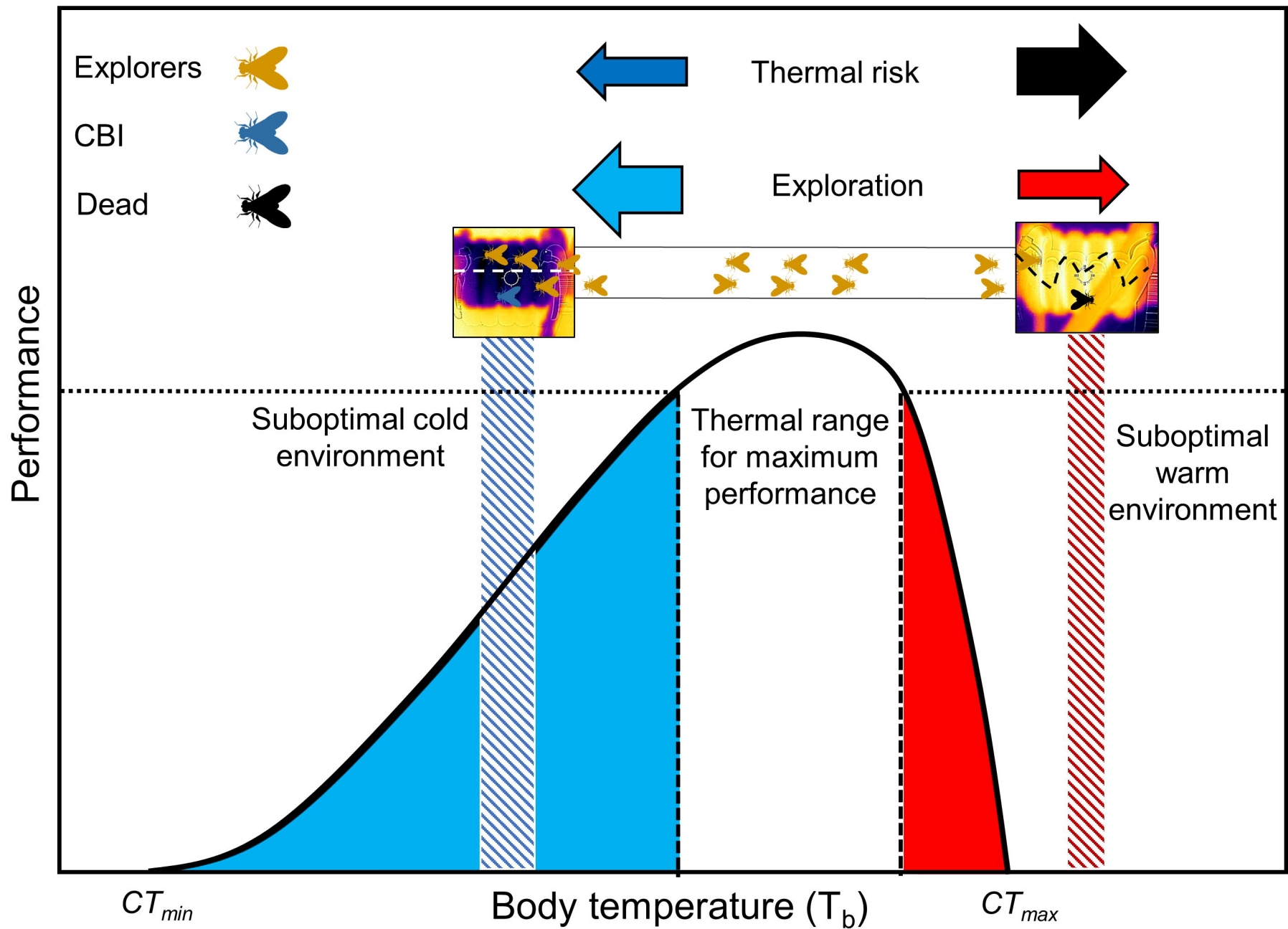


Figure 8