

1 **Neural dysfunction correlates with heat coma and CT_{max} in *Drosophila* but does not set the**
2 **boundaries for heat stress survival**

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11 **Keywords:** CNS, heat stress, heat death, spreading depolarisation, heat sensitivity, hyperthermia

12 **Running title:** Neural dysfunction in heat stress

13

14 **Summary statement**

15 Hyperthermic failure of the *Drosophila* central nervous system causes heat coma, a phenotype varying in
16 temperature between drosophilids, but neural failure is likely not the primary cause of heat mortality.

17 **Abstract**

18 When heated, insects lose coordinated movement followed by the onset of heat coma (CT_{max}). These
19 phenotypes are popular measures to quantify inter- and intraspecific differences in insect heat tolerance, and
20 CT_{max} correlate well with current species distributions. Here we examined the function of the central nervous
21 system (CNS) in five species of *Drosophila* with different heat tolerances, while they were exposed to either
22 constant high temperature or a gradual increasing temperature (ramp). Tolerant species were able to preserve
23 CNS function at higher temperatures and for longer durations than sensitive species and similar differences
24 were found for the behavioral indices (loss of coordination and onset of heat coma). Furthermore, the timing
25 and temperature (constant and ramp exposure, respectively) for loss of coordination or complete coma
26 coincided with the occurrence of spreading depolarisation (SD) events in the CNS. These SD events disrupt
27 neurological function and silence the CNS suggesting that CNS failure is the primary cause of impaired
28 coordination and heat coma. Heat mortality occurs soon after heat coma in insects and to examine if CNS
29 failure could also be the proximal cause of heat death, we used selective heating of the head (CNS) and
30 abdomen (visceral tissues). When comparing the temperature causing 50% mortality (LT_{50}) of each body
31 part to that of the whole animal, we found that the head was not particularly heat sensitive compared to the
32 abdomen. Accordingly, it is unlikely that nervous failure is the principal/proximate cause of heat mortality in
33 *Drosophila*.

34 **Introduction**

35 Thermal tolerance is arguably among the most important traits in defining the biogeographical distribution of
36 ectothermic species (Addo-Bediako *et al.*, 2000; Sunday *et al.*, 2014). This is also the case for insects
37 (Gaston & Chown, 1999; Vorhees *et al.*, 2013), including *Drosophila* where tolerance to both low and high
38 temperature shows a high correlation to the current species distributions (Andersen *et al.*, 2015; Jørgensen *et al.*,
39 *et al.*, 2019; Kellermann *et al.*, 2012; Kimura, 2004). In the case of insect cold tolerance there is a general
40 understanding of the processes causing cold coma and cold mortality (Andersen *et al.*, 2018; Bayley *et al.*,
41 2018; Košťál *et al.*, 2004; MacMillan & Sinclair, 2011), and many physiological adaptations that underlie
42 differences in cold tolerance between species and populations have been uncovered (Feder & Hofmann,
43 1999; Overgaard & MacMillan, 2017; Sinclair *et al.*, 2003; Yi & Lee, 2004; Zachariassen, 1985). In contrast,
44 it is generally less clear which physiological perturbations cause heat coma and heat mortality, and
45 accordingly there is a poorer understanding of the adaptations that result in intra- and interspecific variations
46 in insect heat tolerance (but see Bowler (2018) and Neven (2000)).

47 Heat tolerance of insects and other ectotherms is typically measured by recording the onset of characteristic
48 behaviours (or endpoints) during heat exposure. These measures include the loss of equilibrium or righting
49 response, onset of spasms, entry into a comatose state or heat mortality (Cowles & Bogert, 1944;
50 Lutterschmidt & Hutchison, 1997a; Lutterschmidt & Hutchison, 1997b; Terblanche *et al.*, 2011). The term
51 ‘CT_{max}’ (critical thermal maximum) is frequently and indiscriminately used for all of these endpoints
52 although the different behavioural phenotypes represent the responses to different intensities or durations of
53 heat stress. Thus, mortality is most often preceded by a progressive loss of motor-control (Friedlander *et al.*,
54 1976; Gladwell *et al.*, 1975; Lutterschmidt & Hutchison, 1997a) and some of the endpoints, such as heat
55 coma, can be reversed if the animal is removed from the heat stress immediately after the endpoint is
56 observed (Fraenkel, 1960; Hamby, 1975; Heath *et al.*, 1971; Martinet *et al.*, 2015; Rodgers *et al.*, 2010, but
57 see O’Sullivan *et al.*, (2017)). It can be difficult to discriminate the heat coma and heat death (Larsen, 1943;
58 Mellanby, 1954), as the rate of heat injury accumulation responds strongly to small changes in temperature.
59 Accordingly, slightly longer exposures to high temperatures than those causing coma can result in the
60 accumulation of lethal amounts of heat injury (Bigelow, 1921; Jørgensen *et al.*, 2019; Kingsolver &
61 Umbanhowar, 2018).

62 There are a number of physiological dysfunctions that have been suggested to cause heat coma and heat
63 mortality in insects. These include a mismatch between demand and supply of oxygen to active tissues
64 (described in the hypothesis of oxygen and capacity limited thermal tolerance – OCLTT) (Pörtner, 2001),
65 hemolymph hyperkalaemia which would impair muscle function (Gladwell, 1975; Gladwell *et al.*, 1975;
66 O’Sullivan *et al.*, 2017), cellular heat injury to the membranes (Bowler, 1981; Bowler, 2018; Bowler *et al.*,
67 1973; Hazel, 1995) and breakdown of central nervous function (Hamby, 1975; Larsen, 1943; Prosser &

68 Nelson, 1981; Robertson, 2004). The evidence to support acute heat failure or mortality due to oxygen
69 limitations is not strong for terrestrial insects (Klok, 2004; Mölich *et al.*, 2013; Verberk *et al.*, 2015) and
70 there is also limited support for hemolymph hyperkalaemia as the proximal cause of heat coma/mortality
71 (O'Sullivan *et al.*, 2017). Accordingly, the strongest candidate mechanisms underlying heat coma are tied to
72 breakdown of nervous function. Silencing of nervous function has been observed in heat exposed fruit flies
73 and locusts where heat stress causes a spreading depolarisation (SD) in the central nervous system (CNS)
74 (Money *et al.*, 2009; Robertson, 2004; Rodgers *et al.*, 2007). Spreading depolarisation is triggered by failure
75 to maintain ion gradients between the intra- and extracellular compartments within the CNS, which results in
76 depolarization of neurons and glial cells and a surge of potassium ions in the extracellular space of the brain,
77 preventing neural activity (Robertson, 2004; Robertson *et al.* (submitted); Spong *et al.*, 2016). Furthermore,
78 studies have shown that inter- and intraspecific differences in cold coma are highly correlated with the loss
79 of CNS function in insects (Andersen *et al.*, 2018; Robertson *et al.*, 2017). Given the similarity in the
80 behavioural phenotypes of heat and cold coma there is an obvious possibility that the onset of heat coma is
81 also caused by CNS failure in insects.

82 In most insects, heat mortality follows closely after the onset of heat coma (Mellanby, 1954) and the
83 hypothesis about hyperthermic loss of CNS function could therefore also be extended to be the proximal
84 cause of heat mortality. In goldfish, heating either the cerebellum or the water caused similar behavioural
85 responses, that progressed from hyperactivity to coma (Friedlander *et al.*, 1976). A recent study revisited the
86 work of Friedlander *et al.*, and here the authors selectively cooled the brain of Atlantic cod while the fish
87 were subjected to heat stress, and found that this resulted in increased heat tolerance (measured as loss of
88 equilibrium), compared to controls and instrumented controls (Jutfelt *et al.*, 2019). Accordingly, it appears
89 that controlling the temperature of the CNS can mimic whole-animal exposure to a specific temperature.

90 In the present study we used a comparative study system of five *Drosophila* species with pronounced
91 interspecific differences in heat tolerance. The most heat sensitive species goes into coma at a temperature
92 6°C lower than the most tolerant species in a ramping assay, and similarly the constant temperature estimated
93 to cause onset of coma after a 1-hour exposure is almost 6°C lower in the sensitive species compared to the
94 most heat tolerant species used here (Jørgensen *et al.*, 2019). To investigate the relation between neural
95 dysfunction and the two behavioural heat stress phenotypes, loss of coordinated movement (T/t_{back}) and onset
96 of heat coma (T/t_{coma}), we measured DC potentials in the central nervous system of the five species during
97 heat exposure to record spreading depolarisation as an indication of neuronal failure. These experiments
98 were performed with both gradual heating (a dynamic ramping assay) and constant (static) heat exposure to
99 constant temperature. The loss of coordinated movement, the onset of heat coma and heat mortality occur in
100 rapid succession in many insects. To examine if the onset of heat mortality is caused proximately by failure
101 in the CNS, we designed a simple experiment in which we compare the heat sensitivity of flies that are

102 heated over their entire body with specimens heated specifically in the head (CNS) or abdomen (visceral
103 tissues). This experiment was performed in three of the *Drosophila* species and was designed to evaluate if
104 some body sections (head with primarily neuronal tissue vs abdomen with primarily visceral tissue) were
105 more sensitive to heat stress than others.

106 **Materials and methods**

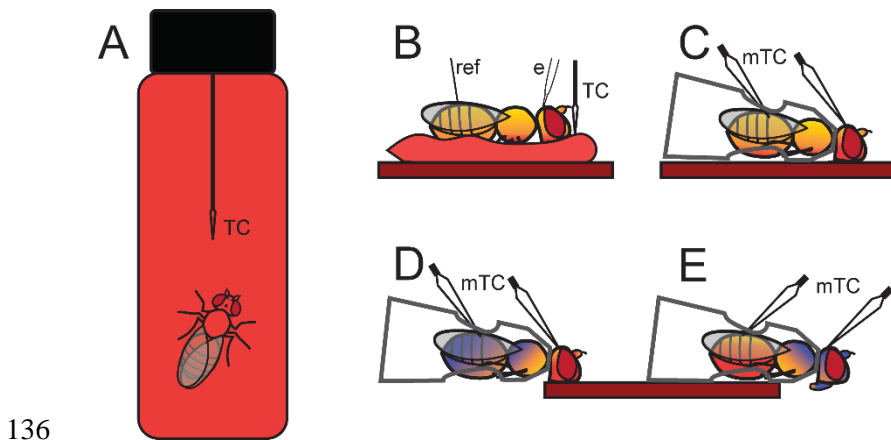
107 ***Experimental animals***

108 Five species of *Drosophila* (*D. immigrans*, Sturtevant 1921; *D. subobscura*, Collin 1936; *D. mercatorum*,
109 Patterson and Wheeler 1942; *D. melanogaster*, Meigen 1830 and *D. mojavensis*, Patterson 1940) were used
110 in this study. The least heat tolerant species *D. immigrans* can survive 35.4°C for 1 hour while the most
111 tolerant species *D. mojavensis* can survive 41.2°C for 1 hour (Jørgensen *et al.*, 2019) and collectively these
112 five species represent a broad range of heat tolerances within *Drosophila*. Flies were reared and maintained
113 under common garden conditions in 250-mL bottles containing 70 mL of oat-based Leeds medium (see
114 Andersen *et al.* (2015)) in a 19°C room with constant light. Maintenance bottles with adults that parented the
115 experimental flies were changed twice a week, and newly eclosed adults from rearing bottles were collected
116 and transferred to fresh vials with fly medium every 1-3 days. Experimental flies were produced by
117 transferring a tablespoon of used medium (including eggs) to another 250-mL bottle with 70 mL new
118 medium. 2-4 days post-eclosion flies were anaesthetised with CO₂, sexed and female flies were moved to
119 new medium vials, and allowed to recover from the CO₂ anaesthesia for at least two days before
120 measurements (MacMillan *et al.*, 2017). All experiments were performed on 4-9 days-old non-virgin female
121 flies, because of their larger size.

122 ***Heat tolerance assays***

123 Behavioural heat tolerance phenotypes were characterised with a ramping and a static assay using the same
124 setup as previously described in Jørgensen *et al.* (2019). In this setup the fly was exposed to homogenous
125 heat exposure within a glass vial that was submerged in a water tank with a controlled temperature (Fig. 1A).
126 In the ramping assay, temperature was increased by 0.25 °C min⁻¹ from 19 °C. Two behavioural phenotypes
127 were recorded during this experiment: 1) the temperature at which the fly would lose coordination and fall on
128 its back (T_{back}) and 2) the temperature at which the fly was completely still (T_{coma}). T_{coma} was verified by
129 poking the vial lids with a stick to agitate the flies and check for reflexes. The static assay used a similar
130 setup and method to record knockdown, but instead of increasing the temperature gradually, the flies were
131 placed in the bath pre-set to 38 °C, after which the exposure durations causing loss of coordinated movement
132 (t_{back}) and heat coma (t_{coma}) were noted (here the lowercase “t” represents time). The “static” assay was only
133 static for 1 hour at 38 °C after which the temperature was increased by 0.25 °C min⁻¹ to ensure that more heat

134 tolerant flies would also succumb to heat stress. 7 flies were measured for each species in each assay, except
135 *D. subobscura* in the ramping assay (n=6).



137 Fig. 1 Overview of heating methods used for the experiments. Colour of the fly body indicates the assumed
138 heat distribution, with red indicating warmer over yellow to blue for colder (eyes are red to characterise
139 *Drosophila*). Spear-shaped arrows show the placement of thermocouples for each method, normal size (1.5
140 mm tip) thermocouples are marked with TC, micro thermocouples (25 μ m tip) with mTC. (A) For
141 behavioural phenotype assessment the fly was placed in a glass vial which was submerged in a temperature-
142 controlled water bath. A uniform heat distribution around the fly was expected. (B) To measure spreading
143 depolarisation the fly was fastened in a bed of wax (lighter red) on top of a Peltier element heating stage
144 (darker red). The wax bed is assumed to give a relatively uniform heat distribution across the ventral body
145 surface, but the dorsal side is possibly cooled slightly by the surrounding air. For these experiments,
146 temperature was measured on top of the wax, adjacent to the head. The placement of the reference (ref) and
147 measuring electrode (e) is also shown. (C) To assess heat sensitivity following whole-body heat exposure the
148 fly was tethered inside a pipette tip, which was placed on the heating stage (dark red). The ventral side was
149 warmer than the dorsal side, and the head tended to be slightly warmer than the abdomen. For these
150 experiments we measured temperature on the dorsal side of the head and abdomen using micro-
151 thermocouples. (D) In selective heating of the head, the fly was tethered but here only the head was in
152 contact with the heating stage. Consequently, the abdomen and thorax were maintained at a lower
153 temperature. (E) Selective heating of the abdomen resulted in a lower temperature of the thorax and head,
154 notice that the non-measuring parts of thermocouples are oriented away from the heating plate.

155 *Measuring spreading depolarisation*

156 Electrophysiological measurements of DC potentials in the CNS (a proxy for nervous function) were carried
157 out as described by Andersen *et al.* (2018). Filamented borosilicate glass capillaries (1 mm diameter; 1B100-
158 F-4, World Precision Instruments, Sarasota, Florida, USA) were pulled to low tip resistance (5-7 M Ω) using
159 a Flaming-Brown PC-84 micro-pipette puller (Sutter Instruments, Novato, CA, USA) and back-filled with
160 500 mM KCl solution. The glass electrodes were connected to a Duo 773 intracellular differential amplifier
161 (World Precision Instruments, Sarasota, Florida, USA) using the low impedance channel and probe, and a
162 chlorinated Ag/AgCl wire was used as reference electrode to ground the preparation. An MP100 data-
163 acquisition system was used to digitalize the voltage output which was recorded using AcqKnowledge
164 software (Biopac Systems, Inc., CA, USA).

165 A fly was prepared for measurement by gently fastening its ventral side to a bed of wax on a glass cover
166 slide. Using a small pair of scissors, a small hole was cut in the abdomen between the second and third-to-
167 last tergites for placement of the ground electrode. Another cut was made along the head midline just
168 posterior to the ocelli to insert the glass recording electrode. The cover slide with the fly was placed onto a
169 Peltier plate pre-set to 30 °C which could be thermoelectrically heated (PE120, Linkam Scientific
170 Instruments, Tadworth, United Kingdom), and temperature was monitored continuously using a type K
171 thermocouple (integrated with the MP100 data-acquisition system) placed on top of the wax, adjacent to the
172 head of the fly (Fig. 1B). This heating method was expected to heat the ventral side of the fly
173 homogeneously, but also result in a small temperature gradient from the ventral to the dorsal side. The glass
174 electrode and the reference (Ag/AgCl) electrode were placed in their designated holes using
175 micromanipulators, and the voltage was zeroed. To test the quality of the preparation, a flow of humidified
176 N₂ was passed over the fly to elicit an anoxic spreading depolarisation (SD). The single depolarisation
177 triggered by anoxia, persists throughout the exposure to N₂, but has been found to be completely reversible in
178 *Drosophila* (Armstrong et al., 2011; Rodríguez & Robertson, 2012) and locusts (Rodgers *et al.*, 2007), and
179 additionally we did not find any difference in timing of SD in heating experiments with and without prior
180 anoxia treatment. We therefore used this anoxia test to discard preparations that failed to depolarise
181 (suggesting that there was a problem with the electrode placement). This test also gave an indication of the
182 size of depolarisation that could be expected from that particular preparation as this is also dependent on the
183 quality of impalement and location of the recording electrode. If the preparation had depolarised ≥ 20 mV in
184 response to anoxia, the voltage was zeroed again, and the preparation was either used for ramping, static or
185 control experiments.

186 In ramping experiments, the temperature of the thermal stage was increased from 30 °C by 0.25 °C min⁻¹ and
187 the temperature (at the half-amplitude of the negative DC shift associated with SD) of the first and last SD
188 event (SD_{first} and SD_{last}, respectively) along with the number of SD events was recorded. The ramping
189 continued until it was clear that no more depolarisations would occur, which was concluded when the
190 preparation could no longer maintain a stable base line DC potential (see example traces in Fig. 2). In static
191 heat exposure experiments, temperature was rapidly increased from 30 °C to 38 °C (mean heating time: 73 s,
192 approx. 6.6 °C min⁻¹), and the timing of SD_{first} and SD_{last} and the number of depolarisation events were noted
193 as above. The stage was kept at 38 °C until no more depolarisations were anticipated (same criterion as in
194 ramping experiments). In preparations for which no depolarisations had occurred during the 1-hour exposure
195 (only in *D. melanogaster* and *D. mojavensis*), the stage temperature was increased by 0.25 °C min⁻¹ after the
196 first hour at 38 °C and this heating was continued until depolarisations were measured. Some of the
197 preparations elicited only a single SD event, and accordingly the temperature/time reported was the same for
198 SD_{last} as SD_{first} (see Fig. 2C).

199 A number of pilot studies were conducted to test if the starting condition at 30 °C or the handling of the fly
200 was stressful enough to elicit SDs by keeping a few *D. immigrans* (the least heat tolerant species) and *D.*
201 *mojavensis* (the most heat tolerant species) at 30 °C for 1 hour, but these conditions failed to elicit SDs in
202 either species. These experiments were concluded by increasing temperature by 1 °C min⁻¹ until SD events
203 were observed, leading us to conclude that the preparations were responsive but that the handling and
204 starting conditions (30 °C) alone were unable to evoke this response.

205 ***Selective heating of head and abdomen***

206 To further examine the role of nervous function in heat tolerance, we performed a series of experiments in
207 which we selectively heated the head or the abdomen of flies and compared their survival after 24 hours to
208 that of flies that had been heated more uniformly (See Fig. 1C-E). The motivation for this study was to
209 examine if the head (dominated by nervous tissue) was more heat sensitive than the abdomen (dominated by
210 fat-body and intestinal tissue). Only three species (*D. subobscura*, *D. melanogaster* and *D. mojavensis*) were
211 used for these experiments as they represent low, medium and high heat tolerance, respectively. *D.*
212 *subobscura* was chosen to represent low heat tolerance rather than *D. immigrans* due to its smaller size,
213 which made it more appropriate for the method.

214 For these experiments the flies needed to be restrained in a way that allowed one end of the fly to be
215 held closer to the heating stage, and as survival was used as the measure of sensitivity, the restraining
216 method fixation should also allow for the flies to be moved from the heating stage without inflicting injury to
217 the animals. Accordingly, flies were fastened in 200 µL pipette tips, using a device originally designed for
218 hemolymph extraction (MacMillan & Hughson, 2014). With a stream of air, the fly was manipulated
219 headfirst into the pipette tip, and the airflow was blocked once the fly was stuck in the tip (taking care not to
220 injure it). The pipette tip was removed from the device and the tip was cut off just anterior to the head
221 followed by two cuts (one from the dorsal and one from the ventral view of the fly) that were made in
222 roughly a 45° angle towards the anterior part of tip (Fig. 1C-E). These angled cuts allowed better
223 contact between the head and the heating stage on the ventral side and room for the thermocouple to measure
224 head temperature on the dorsal side. Using a scalpel, some of the plastic covering the abdomen was gently
225 “shaved” off, while making sure that no holes were made. The tip was then reattached to the air pressure
226 device and the fly was “pushed” until the head protruded from the tip. The area that had been thinned before
227 was now cut away, leaving the abdomen exposed, thereby decreasing the distance to the heating stage on the
228 ventral side (Fig. 1C-E). Another cut was made in the dorsal side of the tip allowing placement of a micro
229 thermocouple directly on the dorsal side of the abdomen (here it was often necessary to move the wings to
230 the side) (Fig. 1C-E). Flies that were injured (other than severed wings) were discarded. The preparations
231 were used for either whole-body heating, selective heating of the head, selective heating of the abdomen or

232 as un-heated controls. Flies were generally heated on the ventral side, but we also tested some flies exposed
233 to whole body heating from the dorsal side (see Supplements Fig. S1).

234 For ventral whole-body heating, the pipette tip was placed on the Peltier plate (PE120, Linkam
235 Scientific Instruments, Tadworth, United Kingdom) with the wide end of the tip at a slightly positive angle,
236 to facilitate closer contact between the heating stage and the ventral side of the head and abdomen (Fig. 1C).
237 When the tip was staged, two micro K type Fine thermocouples (tip diameter 25 μ m, KFG-25-100-100,
238 ANBE, Genk, Belgium) were placed on the surface of the head and the abdomen, respectively (Fig. 1C).
239 This method gave a relatively homogenous heating of the fly when measured on the dorsal side, with a
240 tendency for slightly higher temperatures measured on the head (possibly due to closer contact with Peltier
241 plate). For every sample, the tip was turned 180° horizontally, such that the head and abdomen switched
242 location on the heating stage, to minimise any differences in heating across the stage. The transversal
243 temperature gradient that arose from ventral heating was measured in *D. mojavensis* by gradually moving
244 thermocouples through head and abdomen from the dorsal towards the ventral side, in flies that had been
245 killed before the experiment. This transverse difference was recorded at 2.51 ± 0.22 °C and did not differ
246 between head and abdomen (one sample t-test, $t=11.05$, $df=11$, $p<0.001$). Similar measurements were made
247 for a few *D. melanogaster* and *D. subobscura*, with comparable results.

248 To test heat tolerance, the temperature of the heating stage was quickly increased to the desired test
249 temperature (~1.5 min), and once the temperature was stable the fly was left at this condition for 15 minutes.
250 After heating, temperature would rapidly drop to room temperature (~1 min) when the thermal stage was
251 turned off. The tip was then removed from the Peltier plate, and the fly was immediately checked for
252 movement. After 15 minutes, the fly was again checked for movement, released by cutting the tip and then
253 transferred to a 2-mL Eppendorf tube with fly medium in the bottom and air holes in the lid. Flies were
254 checked for movement after one day of recovery following the heat exposure (recovery at 19 °C), and their
255 status (live/dead) here was used for further analysis. Flies were regarded as “dead” if they were unable to
256 move after the 24-hour recovery period.

257 Selective heating of either head (Fig. 1D) or abdomen (Fig. 1E) was performed using the same
258 preparation as above, but with the body part to be heated placed on the heating stage while the rest of the
259 body was placed away from the stage. This heating method resulted in large temperature differences between
260 body parts, with heating of the head giving a larger difference than heating of the abdomen (Table 1).

261 Table 1. Temperature difference between abdomen and head measured topically on the dorsal side with
262 ventral heating. Values reported as mean \pm s.e.m.

	$T_{\text{abdomen}} - T_{\text{head}} (\text{°C})$		
	<i>D. subobscura</i>	<i>D. melanogaster</i>	<i>D. mojavensis</i>
Heating whole-fly	-0.92 ± 0.15	-2.06 ± 0.19	-1.63 ± 0.17
Heating abdomen	3.35 ± 0.28	4.6 ± 0.22	4.79 ± 0.29
Heating head	-6.44 ± 0.28	-9.16 ± 0.41	-10.19 ± 0.36

263

264 Control experiments were performed to test if the manipulation of the flies resulted in any mortality. In these
265 experiments, the flies were prepared similarly to flies used for heating, but instead of heat exposure they
266 were kept at room temperature and assessed for survival following the same protocol.

267 **Data analysis**

268 All data analyses were performed in R version 3.5.2 (R Core Team, 2018). Unless otherwise stated all results
269 are reported as mean \pm s.e.m., and the critical value for statistical significance was 0.05. Onset of the
270 phenotypes (T_{back} and T_{coma}) and SD events (SD_{first} and SD_{last}) were tested for co-occurrence using two-way
271 ANOVAs for each assay type (ramp and static) with species and measured variable (T_{back} , T_{coma} , SD_{first} ,
272 SD_{last}) in ramp and (t_{back} , t_{coma} , SD_{first} , SD_{last}) in static assays as factor variables. Tukey's HSD *post hoc* test
273 was used to examine differences in onset of phenotypes and SD events within species. The correlation
274 between heat stress phenotypes and onset of SD events was examined *between* species *within* assay type
275 using linear regressions (lm()-function in R). The regression lines were compared to the line of unity
276 (intercept = 0, slope =1) with the function linearHypothesis in the Car-package (Fox & Weisberg, 2011).

277 The survival assessments from the selective and whole-body heating experiments were paired with the
278 temperatures measured from the thermocouples placed on head and abdomen. The temperature causing 50%
279 mortality (LT_{50}) after 24 hours was estimated through a non-linear least square-model using the nls()-
280 function in R. The nls()-function was given the following equation of a sigmoidal curve:

$$281 \quad \text{Survival}(T) = \frac{1}{1 + \exp(-a*(T-b))} \quad \text{Eqn 1}$$

282 Where $\text{Survival}(T)$ is survival at the temperature T , a is the slope of the descending part of the sigmoidal
283 curve and b is the estimate of LT_{50} . 95% level confidence intervals were calculated for each survival curve
284 around the estimated LT_{50} using confint2() from the nlstools-package (Baty *et al.*, 2015). Curves with non-
285 overlapping confidence intervals were regarded significantly different.

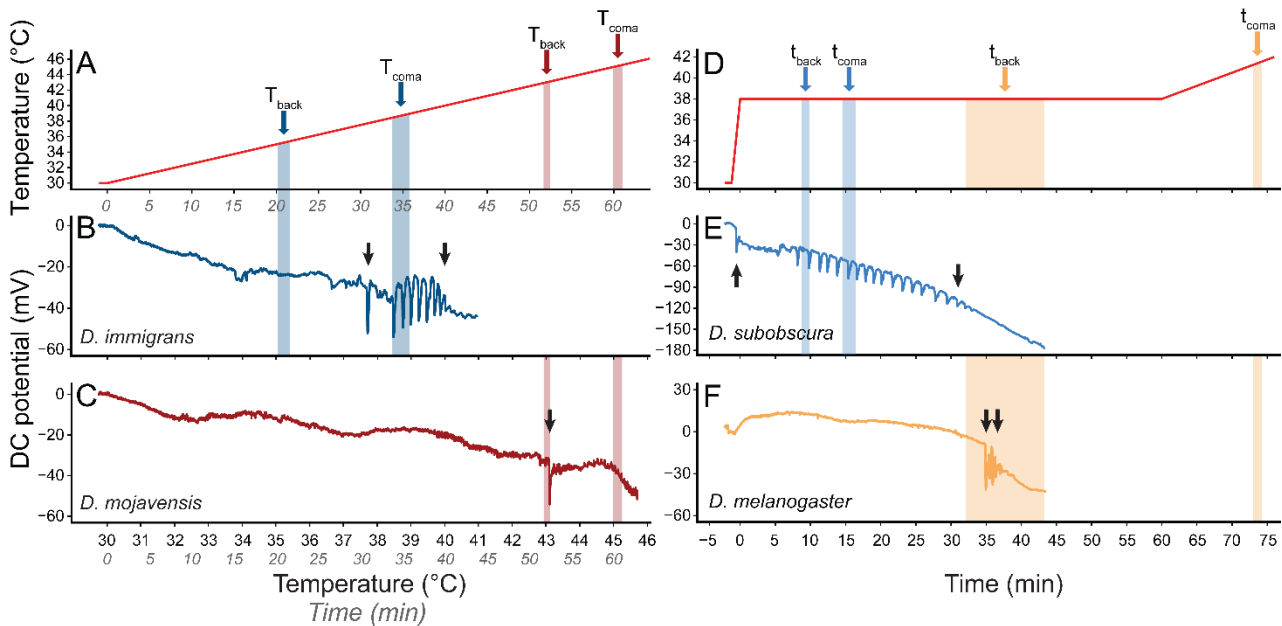
286 **Results**

287 ***Loss of CNS function and onset of heat stress phenotypes***

288 Neural function during heat exposure was examined by measuring negative DC shifts associated with
289 spreading depolarisation (SD) in the central nervous system (CNS) in the head of five *Drosophila* species
290 representing a range of heat tolerances. Flies were heated using either a ramping assay during which
291 temperature (i.e. stress intensity) was gradually increased, or a static assay during which temperature was
292 kept constant at 38 °C. The temperature (ramp) or time (static) of the first or last SD (SD_{first} and SD_{last} ,
293 respectively) were then compared to the timing or temperature of two behavioural heat stress phenotypes
294 measured using similar heating protocols (the phenotypes measured were the loss of coordinated movement
295 (T/t_{back}) and onset of heat coma (T/t_{coma}), Fig. 2). These experiments were used to examine 1) if heat stress
296 phenotypes correlate with signs of neural dysfunction, and 2) if this putative correlation is affected by the
297 way heat stress is inflicted.

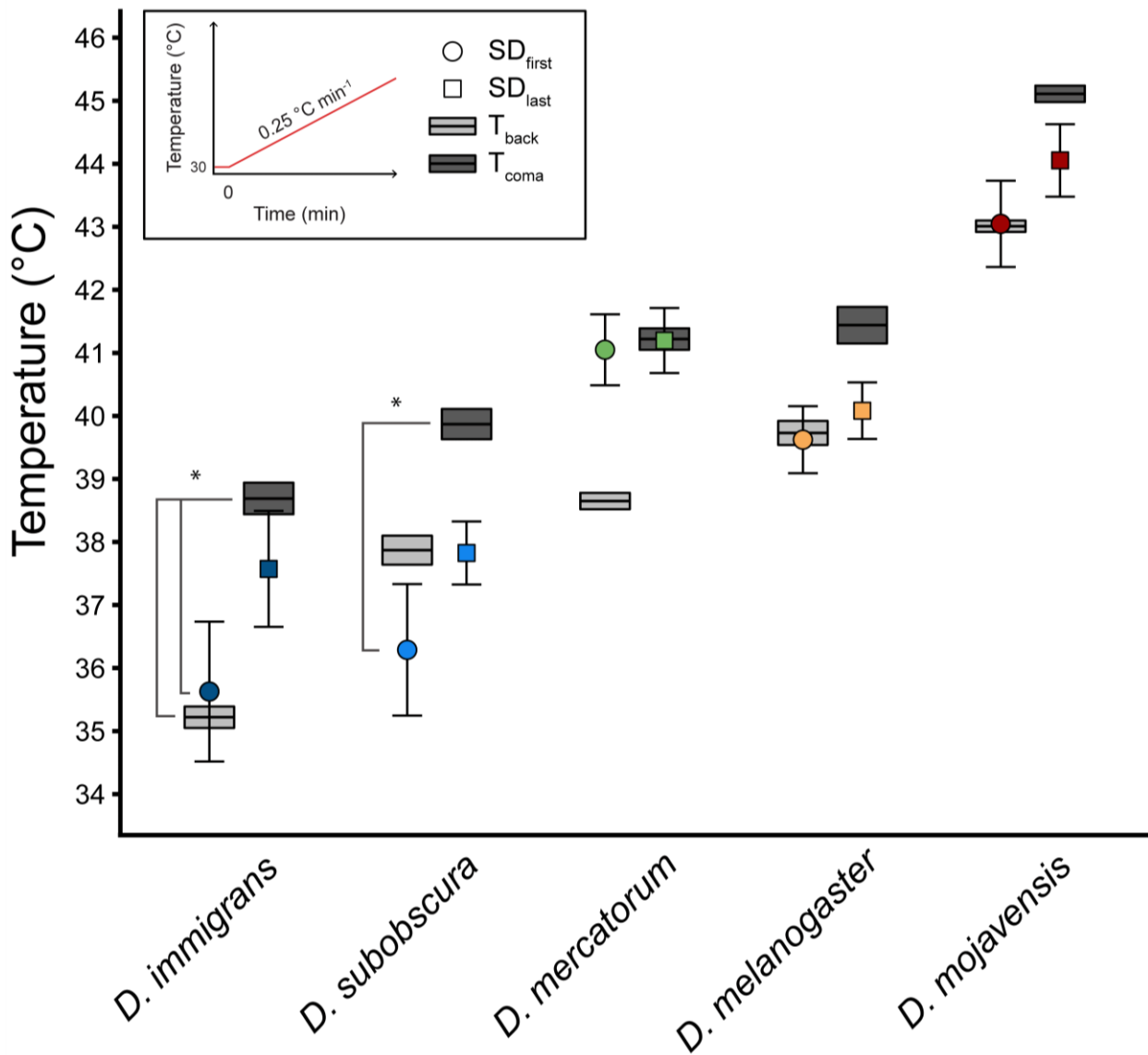
298 When flies were exposed to gradually increasing temperatures in a ramp, there were clear interspecific
299 differences in the temperatures where the behavioural heat stress phenotypes were observed. For example,
300 the least heat tolerant species (*D. immigrans*) showed loss of coordination (T_{back}) at 35.22 ± 0.45 °C and went
301 into heat coma (T_{coma}) at 38.69 ± 0.25 °C, while the most heat tolerant species (*D. mojavensis*) reached T_{back}
302 at 43.01 ± 0.24 °C and T_{coma} at 45.11 ± 0.34 °C, giving the species system a range of T_{back} of 7.8 °C and T_{coma}
303 of 6.4 °C. Similarly, the temperatures at which SD events were observed gave interspecific differences of
304 7.4 °C for SD_{first} and 6.5 °C for SD_{last} between the least and most tolerant species (again *D. immigrans* and *D.*
305 *mojavensis*). Generally, we found that the temperature of T_{back} and T_{coma} coincided with perturbation of
306 nervous function as indicated by SD_{first} and SD_{last} (Fig. 3). For three of the species (*D. mercatorum*, *D.*
307 *melanogaster* and *D. mojavensis*) the two-way ANOVA followed by a Tukey HSD *post hoc* test did not
308 reveal any significant differences in temperature between either of the behavioural phenotypes and the SD
309 events. For the remaining two species (also the two least tolerant), T_{coma} was observed at a significantly
310 higher temperature than the first SD event (Fig. 3). In *D. immigrans* it was also possible to separate the two
311 heat stress phenotypes from each other, as T_{back} was observed at a significantly lower temperature than T_{coma} .
312 However, we caution that the means of heating differed between the phenotype experiments and the
313 neurological experiments, and that this could be a source of experimental noise (see Methods and Discussion
314 for further arguments). To test if there was a general co-occurrence of phenotypic and neurological events,
315 we performed linear regressions of the mean temperatures of either of the two behavioural phenotypes and
316 the two neuronal phenotypes (Table 2). All regression combinations yielded high coefficients of
317 determination (R^2 : 0.73-0.9), and only one of the four regressions (SD_{first} against T_{coma}) was significantly
318 different from the line of unity (Table 2, see Supplements Fig. S2). The regression analysis indicated that

319 across species there were generally only small differences between the temperature where behavioural and
 320 neurological collapse was observed.



321

322 Fig. 2 Representative temperature and DC potential traces from ramping (A-C) and static (D-F) heat
 323 exposures. The temperature profiles during (A) ramping and (D) static assays are marked with species-
 324 coloured arrows and transparent boxes for the two phenotypes, T/t_{back} and T/t_{coma} (mean \pm s.e.m.), for two
 325 species from each assay (The phenotypes and DC potential traces were not recorded from the same
 326 individuals). (B) The heat sensitive *D. immigrans* experienced spreading depolarisation at a lower
 327 temperature than the (C) heat tolerant *D. mojavensis* during a ramping assay. (E) Similarly, the heat sensitive
 328 *D. subobscura* experienced spreading depolarisation sooner than the (F) more heat tolerant *D. melanogaster*
 329 in the static assays. In (A-C), the x-axis show both measured temperature and the corresponding time
 330 (italicised) according to the ramping rate of $0.25\text{ }^{\circ}\text{C min}^{-1}$, and in (D-F) the time scale is adjusted such that
 331 time = 0 when the temperature reached $38\text{ }^{\circ}\text{C}$. Black arrows in (B-C, E-F) mark the SD_{first} (left) and SD_{last}
 332 (right) SD event, notice the example of a single SD event in *D. mojavensis* (C).



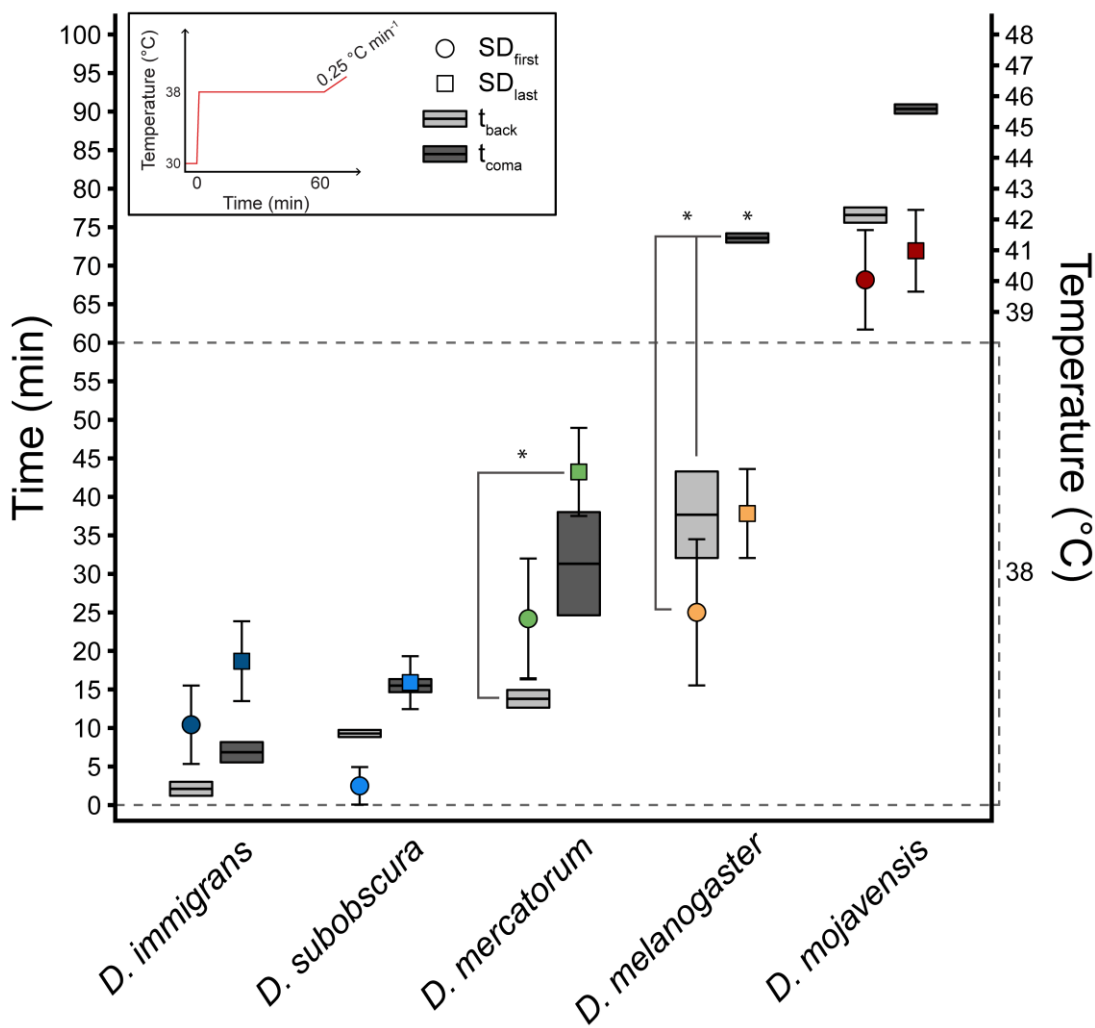
333

334 Fig. 3 Temperature of SD_{first} (circle) and SD_{last} (square) and the temperature of the two behavioural heat
335 stress phenotypes T_{back} (light grey bars) and T_{coma} (dark grey bars) in a ramping assay. SD_{last} coincide with
336 SD_{first} in cases where only a single SD event was observed. SD measurements were performed on a Peltier
337 element while the whole animal knockdown phenotype were observed from flies in glass vials submerged in
338 a temperature-controlled water bath. Asterisks mark significant differences between either of the four
339 phenotypes ($p < 0.05$), $n=7$ for each species and data are reported as mean \pm s.e.m.

340 Table 2 Coefficients of determination (R^2) from linear regressions between behavioural phenotypes and SD
 341 measurements, p -values are from the test comparing the linear regressions to the line of unity (i.e. p -values
 342 above 0.05 indicate that the compared phenotypes occur at the same temperature/time). The highest R^2 in
 343 each assay type is marked in bold italics, and linear regressions which were different from the line of unity (p
 344 < 0.05) are underlined. See Supplements Fig. S2-S3 for a graphical representation of the linear regressions.

	Dynamic		Static	
	T_{back} ($^{\circ}\text{C}$)	T_{coma} ($^{\circ}\text{C}$)	t_{back} (min)	t_{coma} (min)
SD_{first} ($^{\circ}\text{C}$ or min)	$R^2 = 0.73, p = 0.699$	$R^2 = 0.8, p = \underline{0.041}$	$R^2 = \mathbf{0.86}, p = 0.812$	$R^2 = 0.65, p = 0.309$
SD_{last} ($^{\circ}\text{C}$ or min)	$R^2 = 0.78, p = 0.260$	$R^2 = \mathbf{0.9}, p = 0.089$	$R^2 = 0.77, p = 0.392$	$R^2 = 0.65, p = 0.632$

345
 346 During constant heat exposure (38 $^{\circ}\text{C}$, Fig. 4), we recorded the timing of SD events and behavioural heat
 347 stress phenotypes and again we found these behavioural and neurological measures to coincide. Note that for
 348 some species we started to increase the temperature by 0.25 $^{\circ}\text{C min}^{-1}$ after 1 hour of exposure, but that all
 349 measures are reported in minutes of exposure. Between species there was a clear increase in the heat
 350 exposure duration that the nervous system could uphold function with increasing heat tolerance of the
 351 species (according to the timing of behavioural heat stress phenotype onset), although the least tolerant
 352 species in terms of neuronal failure (*D. subobscura*) was the second least tolerant when assessed for
 353 behavioural phenotype (*D. immigrans* was the least tolerant on this term, as in the ramping assay) (Fig. 4). A
 354 two-way ANOVA followed by a Tukey HSD *post hoc* test revealed that it was not possible to separate the
 355 timing of behavioural heat stress phenotypes and the neurological perturbations in *D. immigrans*, *D.*
 356 *subobscura* and *D. mojavensis*. In *D. mercatorum* and *D. melanogaster* significant differences between the
 357 timing of behavioural and neurological phenotypes were found, with a delayed coma onset for *D.*
 358 *melanogaster* relative to both t_{back} and the SD events, and a relatively long time span between the loss of
 359 coordinated movement and the last SD event in *D. mercatorum* (Fig. 4). However, linear regressions on the
 360 mean time of the four possible combinations of SD events and behavioural phenotypes showed a high
 361 correlation between both SD_{first} and SD_{last} with t_{back} (R^2 : 0.77-0.86), while the correlations between SD types
 362 and t_{coma} were slightly weaker (R^2 : 0.65) (Table 2, see Supplements Fig. S3). When the four regression lines
 363 were compared to the line of unity, none of them were significantly different, again suggesting that across
 364 the species system there were generally an overlap between the exposure durations that resulted in
 365 behavioural and neurological phenotypes.



366

367 Fig. 4 Exposure time in a static assay until SD_{first} (circle) and SD_{last} (square) and loss of coordinated
 368 movement (t_{back}, light grey bars) and onset of coma (t_{coma}, dark grey bars). The time scale is adjusted such
 369 that time = 0 when the temperature reached 38 °C (average time to heat from room temperature to 38 °C was
 370 73 s for SD measurements). After 1 hour at 38°C the temperature was increased by 0.25 °C min⁻¹, and SDs
 371 and phenotypes that occurred during the ramp is here presented on the time scale (with the corresponding
 372 temperature on the secondary y-axis). SD measurements were performed on a Peltier plate while behavioural
 373 phenotypes were assessed from flies in glass vials submerged in a temperature-controlled water bath.
 374 Asterisks mark significant differences between either of the four phenotypes ($p < 0.05$), $n=7$ for each species
 375 and data are presented as mean \pm s.e.m.

376 Examination of the DC potential measurements showed considerable variance between preparations. Some
 377 preparations were characterised by only eliciting a single SD event (meaning that SD_{first} and SD_{last} occurred
 378 at the same time/temperature, Fig. 2C) while other specimens showed multiple (2-30) SD events (see
 379 examples in Fig. 2). Comparison between the ramping and constant heat exposures showed that single SD
 380 events were much more prevalent during the ramping heat exposure (40% of individuals showed single SD,
 381 $n=35$) than in the constant heat exposure (9% showed single SD, $n=29$) (see Supplements Fig. S4).
 382 Furthermore, when the constant heat exposure for 1 hour was followed by a ramping increase in temperature,
 383 flies would mostly elicit just a single SD (66%, $n=6$). All five species were able to display both single and

384 repeated SD events and in roughly the same proportion (2-4 preparations of each species (out of 7) showed a
385 single SD during ramping). The number of SD events observed in “multiple” SD events also differed with
386 heat exposure assay. In static assays, preparations with multiple SDs elicited 11.38 ± 1.56 SD events while
387 preparations with multiple SDs during ramping assays only had 5.95 ± 1.12 SD events (two sample t-test,
388 $t=2.83$, $df=43.15$, $p=0.007$).

389 ***Selective heating of the head and abdomen***

390 As heat coma and heat death often occur in close succession, we performed an experiment designed to
391 investigate and compare the heat sensitivity of the head (site of nervous function measurements from the first
392 experiment) and the abdomen (consisting more of visceral tissues) (see Fig. 1C-E). This test involved
393 restraining flies in pipette tips and non-heated controls for handling showed 0% mortality for *D. subobscura*
394 and *D. melanogaster*, and 13% mortality for *D. mojavensis* after 24 hours ($n=14/16/39$, respectively). For
395 these experiments the temperature estimated to cause 50% mortality in the flies 24 hours after heat exposure
396 (LT_{50}) was used to compare heat sensitivity between body parts.

397 Both whole-fly and selective heating showed that the heat tolerant *D. mojavensis* had higher values of
398 LT_{50} than the moderate heat tolerant *D. melanogaster*, which in turn also had higher values of LT_{50} than the
399 heat sensitive *D. subobscura* (Fig. 5). When the whole fly was heated simultaneously, we did record
400 differences between head and abdominal temperature (measured topically on the dorsal side), but these
401 differences were generally less than 2 °C (see Table 1 and Supplements Fig. S1). In experiments using
402 selective heating of either the head or abdomen the flies were characterised by much larger regional
403 differences in temperature (ΔT ranging 3.35-10.19 °C depending on species and body part heated, see Table
404 1).

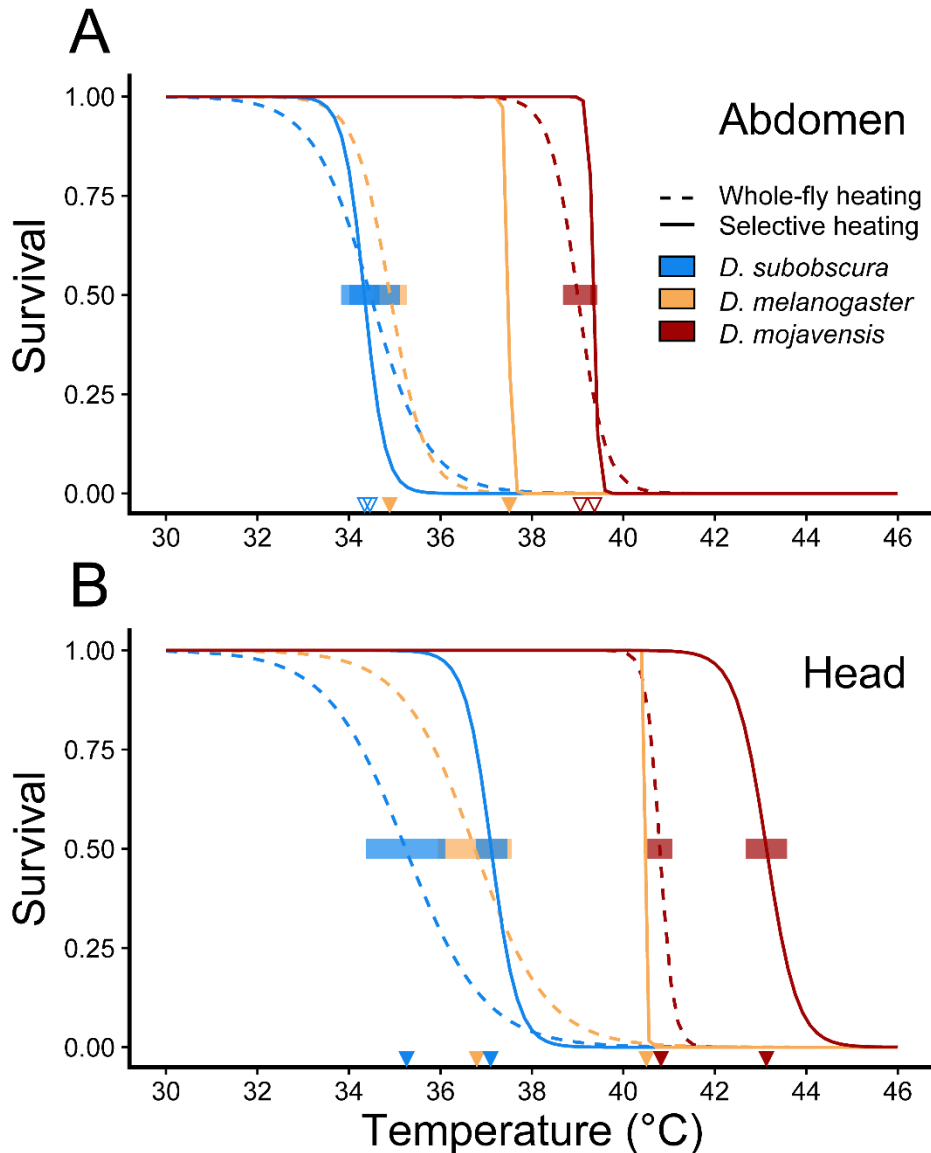
405 The experiments revealed species specific differences in the relation between LT_{50} estimates during
406 whole animal heating and selective heating. For *D. mojavensis*, heating the abdomen (and maintaining the
407 head at a lower temperature, $\Delta T=4.79 \pm 0.29$ °C) did not change the LT_{50} compared to abdominal
408 temperature when the whole fly was heated (LT_{50} was 0.35 °C higher but the estimates have overlapping
409 95% confidence intervals, Fig. 5A). Thus for *D. mojavensis*, LT_{50} was the same irrespective if the head was
410 kept cool or warm during heating of the abdomen. When the head of *D. mojavensis* was heated selectively
411 (with the abdomen considerably cooler: $\Delta T=10.19 \pm 0.36$ °C), LT_{50} increased by 2.33 °C compared to flies
412 experiencing whole animal heating (non-overlapping 95% confidence interval, Fig. 5B). Thus, a higher head
413 temperature was needed to evoke mortality in *D. mojavensis* when the abdomen was relieved from heat
414 stress.

415 Performing the experiments on *D. melanogaster* we observed slightly smaller differences between
416 body parts than in *D. mojavensis*, both when the head was selectively heated (abdomen maintained at a lower

417 temperature, $\Delta T=9.16 \pm 0.41$ °C) and when the abdomen was heated (head kept cooler, $\Delta T=4.6 \pm 0.22$ °C).
418 For *D. melanogaster* we found LT_{50} to increase when applying selective heating on the abdomen (LT_{50} was
419 2.59 °C higher, Fig. 5A) and the head (LT_{50} was 3.77 °C higher, Fig. 5B), compared to LT_{50} resulting from
420 whole-fly heating. Accordingly, maintaining one end of a *D. melanogaster* at a lower temperature than the
421 other, increases heat tolerance of the fly.

422 In experiments with *D. subobscura*, the temperature differences between body parts were smaller than
423 for the other two species. Selectively heating the abdomen made the abdomen 3.35 ± 0.28 °C warmer than
424 the head but did not change the LT_{50} of the abdomen when compared to that of whole-fly heating (LT_{50} was
425 0.13 °C lower for the selective heating, likely attributed to the shape of the survival curve, but with
426 overlapping 95% confidence intervals). When selectively heating the head, resulting in a 6.44 ± 0.28 °C
427 colder abdomen, head LT_{50} increased by 1.87 °C compared to head LT_{50} of whole-animal heated flies.

428



429

430 Fig. 5 Survival curves and LT_{50} estimates for whole-fly and selective heating of *D. subobscura* (blue), *D.*
431 *melanogaster* (yellow) and *D. mojavensis* (red). (A) Survival curves are related to the temperature measured
432 topically on the abdomen during selective heating of the abdomen (full lines) and whole-fly heating (dashed
433 lines). (B) Survival curves are related to the temperature measured topically on the head during selective
434 heating of the head (full lines) and whole-fly heating (dashed lines). Note that whole-fly heating curves are
435 slightly different in A and B because they are based on the temperature measurements from the abdomen and
436 head, respectively. LT_{50} , the temperature that resulted in 50% mortality, was estimated for all survival
437 curves, and is marked on the temperature axis by a species coloured triangle. If the 95% confidence intervals
438 of selective heating and whole-fly heating LT_{50} (shaded, species coloured areas) within a species did not
439 overlap, a closed triangle was used, and conversely, if confidence intervals overlapped, open triangles were
440 used. Whole-fly heating and selective heating of abdomen and head were performed on $n=24/15/18$ for *D.*
441 *subobscura*, $n=24/17/16$ for *D. melanogaster* and $n=35/17/17$ for *D. mojavensis*, respectively. Selective
442 heating of *D. melanogaster* yielded very steep survival curves where the confidence intervals could not be
443 determined.

444 **Discussion**

445 Inter- and intraspecific differences in heat tolerance have been demonstrated for *Drosophila* in multiple
446 studies (Castañeda et al., 2015; Jørgensen et al., 2019; Kellermann et al., 2012; Kimura, 2004; Overgaard et
447 al., 2014; Stratman & Markow, 1998). These differences have often been measured using the onset of
448 reversible behavioural phenotypes such as loss of coordinated movement and entry into heat coma, or by
449 measuring heat induced mortality in animals exposed to high temperatures (Lutterschmidt & Hutchison,
450 1997a). However, it is still unclear which physiological perturbations are the proximate cause of the different
451 heat tolerance endpoints (but see Robertson (2004) and Rodgers *et al.* (2010)), and this has been particularly
452 difficult to discern because of the close proximity of the endpoints at high temperatures. Multiple
453 physiological mechanisms have been suggested as the proximate cause of heat mortality, including oxygen
454 transport limitations, protein denaturation, loss of membrane integrity or ion homeostasis, and mitochondrial
455 dysfunction (Bowler, 2018; Davison & Bowler, 1971; Gladwell, 1975; Pörtner, 2001; Somero, 1995). The
456 endpoint prior to mortality, the onset of heat coma, has instead been suggested to be caused by either
457 muscular or nervous failure (Bowler, 1963; Gladwell *et al.*, 1975; Robertson, 2004). In locusts exposed to
458 increasing temperature, ventilation failed concurrently with an abrupt surge in extracellular $[K^+]$, which has
459 been related to a drop in DC potential that is a reliable marker of spreading depolarisation in the CNS (SD)
460 (Robertson, 2004; Rodgers *et al.*, 2007). Once the locust was returned to benign temperatures, extracellular
461 $[K^+]$ surrounding the neurons returned to baseline levels, and the motor pattern ventilation resumed (Rodgers
462 *et al.*, 2007; Rodgers *et al.*, 2010).

463 To our knowledge there has been no comprehensive comparative studies investigating species differences in
464 CNS function at high temperature and the aim of this study was to examine the role of the nervous system in
465 relation to heat tolerance in five *Drosophila* species. The temperatures at which two behavioural phenotypes
466 (loss of motor control (T_{back}) and loss of motor function (T_{coma})) were observed were compared to the
467 temperature of neuronal failure (SD) as assessed by electrophysiological measurements of DC potentials in
468 the fly brain during ramping heat exposure, and likewise the timing of SD and behavioural phenotypes
469 during constant heat exposure. These experiments revealed a good correlation between the failure of motor
470 control/function and neuronal failure, however it is unclear if failure of the CNS is also causing heat
471 mortality. Thus, we designed an experiment to test the sensitivity to heat exposure on different parts of the
472 fly body to further examine if the nervous system could be limiting heat stress survival.

473 ***Heat stress phenotypes correlate with onset of nervous failure***

474 Measurements of spreading depolarisation (i.e. large negative shifts in DC potential) during both ramping
475 and static assays, showed that, overall, perturbation of nervous function correlated well with the two
476 behavioural heat stress phenotypes (t/T_{back} and t/T_{coma}) (Fig. 3-4). Onset times and temperatures of the
477 behavioural coma phenotype were similar to the values previously reported in the five species measured in

478 similar heat tolerance assays (Jørgensen *et al.*, 2019). The loss of motor function was assessed on untethered
479 flies in glass vials with a homogeneous temperature, whereas SD measurements required the flies to be
480 fastened and furthermore a hole was cut in the head and abdomen to insert measurement electrodes (Fig. 1).
481 The invasive preparation required for SD measurements could potentially alter heat tolerance, and we also
482 observed a surprisingly large internal thermal gradient in the fly (sometimes more than 2 °C) when using the
483 Peltier plate for heating. The differences in experimental protocols between behavioral and neurological
484 experiments are likely to introduce some noise in the comparison between these experiments, particularly
485 because we know already that the rate of heat injury accelerates extremely quickly at high temperature (Q_{10}
486 of heat injury accumulation rate is often >10.000). Thus, very small differences in exposure temperature (or
487 time) can separate tolerance and death during heat exposure (Jørgensen *et al.*, 2019). Considering these
488 sources of variation, it would be unexpected to find a perfect correlation between the two experiment types.
489 Despite these “experimental challenges” we found clear patterns of association between loss of motor control
490 and the occurrence of SD events in the CNS (Figs. 3 and 4).

491 Generally, the characteristics of heat stress phenotypes follow a progressive loss of motor control,
492 from first hyperactivity, through loss of coordinated movement and spasms to the onset of heat coma or heat
493 stupor where the animal is unresponsive (Cossins & Bowler, 1987; Heath & Wilkin, 1970; Lutterschmidt &
494 Hutchison, 1997a). Accordingly, for these experiments it follows that the two behavioral phenotypes t/T_{back}
495 and t/T_{coma} are bound in a way such that t/T_{back} will occur prior to (or at a lower temperature) compared to
496 t/T_{coma} . Similarly, the first SD must precede the last SD, unless only a single SD event is observed (in which
497 case the first and last SD are the same). It is therefore tempting to conclude that SD_{first} is linked to t/T_{back} and
498 likewise SD_{last} to t/T_{coma} but with the lack of clear statistical support for this, we will only conclude that it is
499 likely that the two closely occurring behavioural phenotypes (t/T_{back} and t/T_{coma}) are linked to the
500 simultaneously occurring SD events (SD_{first} and SD_{last} , respectively). The relation between behavioural
501 phenotypes and nervous dysfunction has also been examined at low temperatures in different species of
502 *Drosophila*, where temperature of cold coma onset is also highly correlated with the temperature of SD in
503 the CNS of *Drosophila* (Andersen & Overgaard, 2019; Andersen *et al.*, 2018). However, similar to our heat
504 experiments it is difficult to determine specifically how first and last SD events are linked to loss of motor
505 control (T_{back}) or loss of movement (T_{coma}). Importantly, there is no association between cold-induced SD
506 events and cold mortality as insects can survive cold in a “comatose” state for long periods of time
507 (MacMillan & Sinclair, 2011; Overgaard & MacMillan, 2017).

508 The present study found that single SD events (instead of multiple events) were more prevalent in
509 ramping experiments than during static heat exposure (Supplements Fig. S4). Additionally, the number of
510 SD events that occurred in preparations with more than one SD, was significantly higher during ramping heat
511 exposure compared to static. In hyperthermic locusts single continuous SD events that persist until the heat

512 exposure is removed are the most prevalent, but repetitive SD events have been observed in locusts treated
513 with ouabain (Rodgers *et al.*, 2009; Spong *et al.*, 2014) and in hyperthermic brain slices from immature rats
514 (Wu & Fisher, 2000). Contrary to hyperthermia, which is thought to lead to accumulation of $[K^+]$, ouabain is
515 limiting K^+ clearance through its inhibition of the Na^+/K^+ -ATPase (Rodgers *et al.*, 2009). According to
516 Rodgers *et al.* (2009) the repetitive SD events are caused by transient surges in extracellular $[K^+]$ that are
517 resulting from imbalances between accumulation and clearance of K^+ . A speculative explanation for the
518 increased prevalence of single SD events in ramps could be that when temperature is gradually increased, the
519 mitigation of the physiological conditions resulting in SDs (high extracellular $[K^+]$ in the space surrounding
520 the CNS) cannot keep up as heat stress increases exponentially (Jørgensen *et al.*, 2019), resulting in a total
521 silencing of the CNS. Conversely, the static exposure may allow the fly to remove some of the $[K^+]$ that has
522 accumulated in the extracellular space. This could relieve the condition causing the SD event and
523 temporarily restore some nervous function until a new SD events occurs when K^+ clearance is surpassed by
524 the accumulation (Rodgers *et al.*, 2010). Despite differences in experimental protocols we here clearly
525 demonstrate that SD events in the CNS and the loss of motor function or entry into coma coincide in
526 *Drosophila* species with different levels of heat tolerance. This indicates that loss of CNS function is the
527 proximal cause to the onset of heat coma (CT_{max}), a behavioural phenotype that is commonly used to
528 describe animal heat tolerance. However, as found in cold *Drosophila*, it is also important to emphasise that
529 the significance of nervous dysfunction in the onset of coma does not necessarily mean that the loss of
530 nervous function directly results in heat death.

531

532 ***Selective heating of the head and abdomen suggests interspecific differences in body part heat sensitivity***

533 To investigate the role of the CNS failure for heat mortality, we designed an experiment to estimate heat
534 sensitivity of the head and the abdomen when either the whole fly was heated, or when one body part was
535 selectively exposed to a higher temperature than the rest of the fly. If CNS failure at high temperatures is the
536 main cause of heat mortality, then we would expect that maintaining the head at a lower temperature than the
537 abdomen should also lower mortality. Conversely, if the head was heated selectively, we would expect
538 mortality to occur at the same temperature as when the whole fly was heated. Manipulations of body
539 compartment temperatures have previously been used successfully in crayfish (Bowler, 1963), goldfish
540 (Friedlander *et al.*, 1976) and Atlantic cod (Jutfelt *et al.*, 2019) to investigate the heat sensitivity of either
541 heat coma or heat mortality. To our knowledge this is the first study to attempt such a study in small insects
542 such as *Drosophila*.

543 Using the experimental setup with a fly tethered in a pipette tip, we found clear differences in heat
544 tolerance (measured as LT_{50}) between species, such that the desert species *D. mojavensis* was more heat
545 tolerant than the cosmopolitan *D. melanogaster*, which in turn was more heat tolerant than the temperate *D.*

546 *subobscura*. This finding is entirely consistent with the other heat stress phenotypes measured in the present
547 study and with findings from previous studies (Jørgensen *et al.*, 2019; Kellermann *et al.*, 2012). The
548 tethering of the flies was not in itself invasive as attested by no mortality of controls in *D. subobscura* and *D.*
549 *melanogaster*, and low mortality in *D. mojavensis* controls. Selective heating of abdomen and head suggests
550 interspecific differences in body part sensitivity (Fig. 5). All three species showed increased heat tolerance of
551 the head when the abdomen was simultaneously kept at a lower temperature (i.e. heating only the head, Fig.
552 1D). This suggest that the head may not be the most heat sensitive body part (Fig. 5B). When the head was
553 maintained at a lower temperature (abdomen was heated, Fig. 1E), the species differed in response (Fig. 5A).
554 *D. subobscura* and *D. mojavensis* maintained a similar LT_{50} for the abdomen when only the abdomen was
555 heated compared to heating of the whole animal, suggesting that the abdomen is a heat sensitive body part in
556 these two species since selective heating of abdomen gives the same heat tolerance as heating the whole fly.
557 *D. melanogaster* showed a different response as LT_{50} increased in flies when only the abdomen was heated
558 (i.e. a similar response as when the head was selectively heated). This suggest that for *D. melanogaster* both
559 body parts are injured through heat exposure and that the damage may be additive such that it is the total
560 amount of accumulated injury that determines heat tolerance. Overall these experiments showed that the
561 head was not a particular heat sensitive region and the higher LT_{50} values in flies with selective heating of
562 the head suggest that neuronal tissue can survive some degrees beyond the temperature causing SD events.

563 The increase in LT_{50} for flies with selective heating of the head support the notion that spreading
564 depolarisation is an adaptive mechanism to protect the organism during stress (Robertson, 2004; Rodgers *et*
565 *al.*, 2010). We observed in multiple cases where flies used for the LT_{50} experiments would enter a heat coma
566 (they were completely unresponsive immediately following heat exposure), but they would later resume
567 movement and often recover normal behaviour. Likewise, we observed in the initial behavioural phenotype
568 assays that flies removed from the heat immediately after t/T_{coma} had been observed would recover
569 subsequently. Together these data indicate that SD events are not directly associated with mortality and that
570 nervous failure is not a proximal cause of heat death. Nevertheless, thermal sensitivity of the nervous system
571 could impose a critical challenge to fitness if critical behaviours, such as escape responses, are impaired at
572 stressful temperatures (Montgomery & Macdonald, 1990).

573 In conclusion, experiments performed for this study show clear interspecific differences in the extent
574 (time/temperature) that the flies can tolerate heat stress, which is related to the overall heat tolerance of the
575 species. Based on the first experiments we find that loss of nervous function is likely to be the cause of the
576 characteristic loss of coordinated movement and coma that is classically used to assess heat tolerance in
577 insects (CT_{max}). Our experimental conditions did not allow us to conclude specifically if it is the first or last
578 SD event that is the cause of these phenotypes, and it is also possible that related neuronal failure in other
579 ganglia could play a role. Our second set of experiments with selective heating showed that the head (mainly

580 neuronal tissue) is not particularly heat sensitive compared to other parts of the body. Thus, entry into
581 (reversible) coma and heat mortality are likely different physiological processes and loss of brain function is
582 not the proximal cause of heat death.

583 The temperature and time span from when the most heat-sensitive species suffered from neural failure
584 to when the CNS of the most heat tolerant species succumbed was large, inviting further studies to
585 investigate adaptations in the CNS to alter heat sensitivity. Our results strongly suggest that hyperthermic
586 loss of CNS function and loss of motor coordination and function (coma) are correlated, which is of clear
587 interest to uncover the physiological perturbations limiting heat tolerance. The role of muscle and
588 neuromuscular synapses in loss of function was not examined in the present study, and although they may
589 also coincide with loss of coordinated movement and heat coma, the correlation between the upstream CNS
590 silencing and loss of function is striking. However, it is also important to appreciate that even small
591 disturbances in nervous function at less stressful temperatures could mean the difference between life and
592 death to an unrestrained animal in nature if its escape response is retarded by nervous dysfunction.

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597 **Competing interests**

598 No competing interests declared

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