1   **Title: Temperatures that sterilise males better match global species distributions than**

2   **lethal temperatures**

3

4   **Authors:** Steven R Parratt[1*], Benjamin S Walsh[1], Soeren Metelmann[2], Nicola White[1], Andri

5   Manser[1†], Amanda J Bretman[3], Ary A Hoffmann[4], Rhonda R Snook[5], Tom AR Price[1*]

6   **Affiliations:**

7   [1] Department of Evolution, Ecology and Behavior, University of Liverpool, Liverpool, UK.

8   [2] Health Protection Research Unit in Emerging and Zoonotic Infections, University of

9   Liverpool, Liverpool, UK.

10   [3] School of Biology, Faculty of Biological Sciences, University of Leeds, Leeds, UK.

11   [4] School of BioSciences, Bio21 Institute, University of Melbourne, Australia.

12   [5] Department of Zoology, Stockholm University, Stockholm, Sweden

13   *Correspondence to: s.parratt@liverpool.ac.uk/t.price@liverpool.ac.uk

14   †Current Address: Department of Evolutionary Biology and Environmental Studies,

15   University of Zürich, Zürich, Switzerland

16

Abstract

Attempts to link physiological thermal tolerance to global species distributions have relied on lethal temperature limits, yet many organisms lose fertility at sublethal temperatures. Here we show that, across 43 Drosophila species, global distributions better match male-sterilising temperatures than lethal temperatures. This suggests that species distributions may be determined by thermal limits to reproduction, not survival, meaning we may be underestimating the impacts of climate change for many organisms.

24

Main Text:

To preserve biodiversity, we urgently need to understand the physiological, behavioral and evolutionary factors that underpin species' thermal distributions[1]. Laboratory-derived estimates of the highest temperatures at which an organism can survive (critical thermal limits/CTL) provide measures of species' thermal tolerances. Linking CTLs to current distributions has enabled better modelling of future species distributions under climate change scenarios[2], likely to be vital for prioritizing conservation efforts[3] and effectively managing invasive species[4].

Despite CTLs being measured in artificial laboratory conditions, they correlate reasonably well with species' macroecological distributions[5,6] and have been used to estimate species' capacity to tolerate temperature increases across their current range; their 'thermal safety margins'[5,7]. However, CTLs can be higher than the temperatures that cause seasonal population declines in nature[8]. Some of this discrepancy has been attributed to methodological shortcomings[9-11], but could also be due to organisms becoming infertile at sub-lethal temperatures[12]. Sub-lethal temperatures cause losses in fertility in plants[13], insects[14-16], fish[17], corals[18], birds[19] and mammals, including humans[20]. If the temperatures that cause infertility (thermal fertility limits/TFLs) are often lower than CTLs, we may both be generally underestimating organisms' vulnerability to climate change, and misidentifying which organisms are most at risk. If TFLs correlate with natural distributions better than CTLs, incorporating TFLs into models of climate change impacts may improve accuracy.

47 We recorded three measures of upper thermal limits in adult males from 43 species of

48 *Drosophila*. To compare fertility and survival limits under identical heat-stress conditions, we

49 exposed flies to a 4-hour static heat stress at a range of temperatures from benign through

50 to lethal (Supplementary Table 1). From these data we estimated both the temperature that

51 is lethal to 80% of individuals (LT80), and the temperature at which 80% of surviving males

52 are sterilized (TFL80). Measuring thermal traits under static temperature stress rather than

53 slowly increasing temperatures (i.e. ramping) has received criticism[21]. However, ramping

54 assays require an immediate observable response, such as flies losing coordinated motor

55 function. Unfortunately, sterilization is not immediately observable, so we use static

56 temperatures and assay fertility through subsequent matings. We score fertility at two time

57 points: (i) cumulatively over 1-6 days post-heat, to capture any immediate sterilizing effect of

58 heat, and (ii) 7-days after heat-stress to capture any recovery of fertility or delayed sterility.

59 To compare our estimates of TFL80 and LT80 with a measure of lethal temperature under

60 ramping thermal stress, we also assayed the CT_{MAX} of each species. This is the temperature at

61 which males lose coordinated motor function under gradually increasing temperatures.

62 CT_{MAX} is commonly used to predict species' sensitivity to thermal stress associated with

63 climate change[3,5,7].

64

65 We found that 11 of 43 species experience an 80% loss in fertility at cooler-than-lethal

66 temperatures immediately following heat-stress (Supplementary Figure 1). Interestingly,

67 rather than seeing a recovery of fertility over time, the impact of high temperatures on

68 fertility was more pronounced 7-days post heat stress (Figure 2A). Using this delayed

69 measure of fertility, 44% of species (19/43) showed fertility loss at cooler-than-lethal

70   temperatures. The difference between lethal and fertility limits ranged from 0°C to 4.3°C

71   (mean of all species = 1.15 ± 0.22°C), and LT80 and TFL80 predict dramatically different

72   rankings of species' robustness to high temperature (Figure 2B). All three thermal limits

73   significantly, positively correlate with each other (Supplementary Table 2). Despite deriving

74   from different types of heat-stress, the correlation coefficient between CT_{MAX} and LT80 is

75   larger than that between TFL80 and either measure of lethal temperature. Relatively low

76   correlations between survival (measured as CT_{MAX} under dynamic conditions or LT80 under

77   static stress) and fertility (measured under static heat stress) suggests they are distinct

78   phenomena, and measuring both may be important for understanding species responses to

79   thermal stress.

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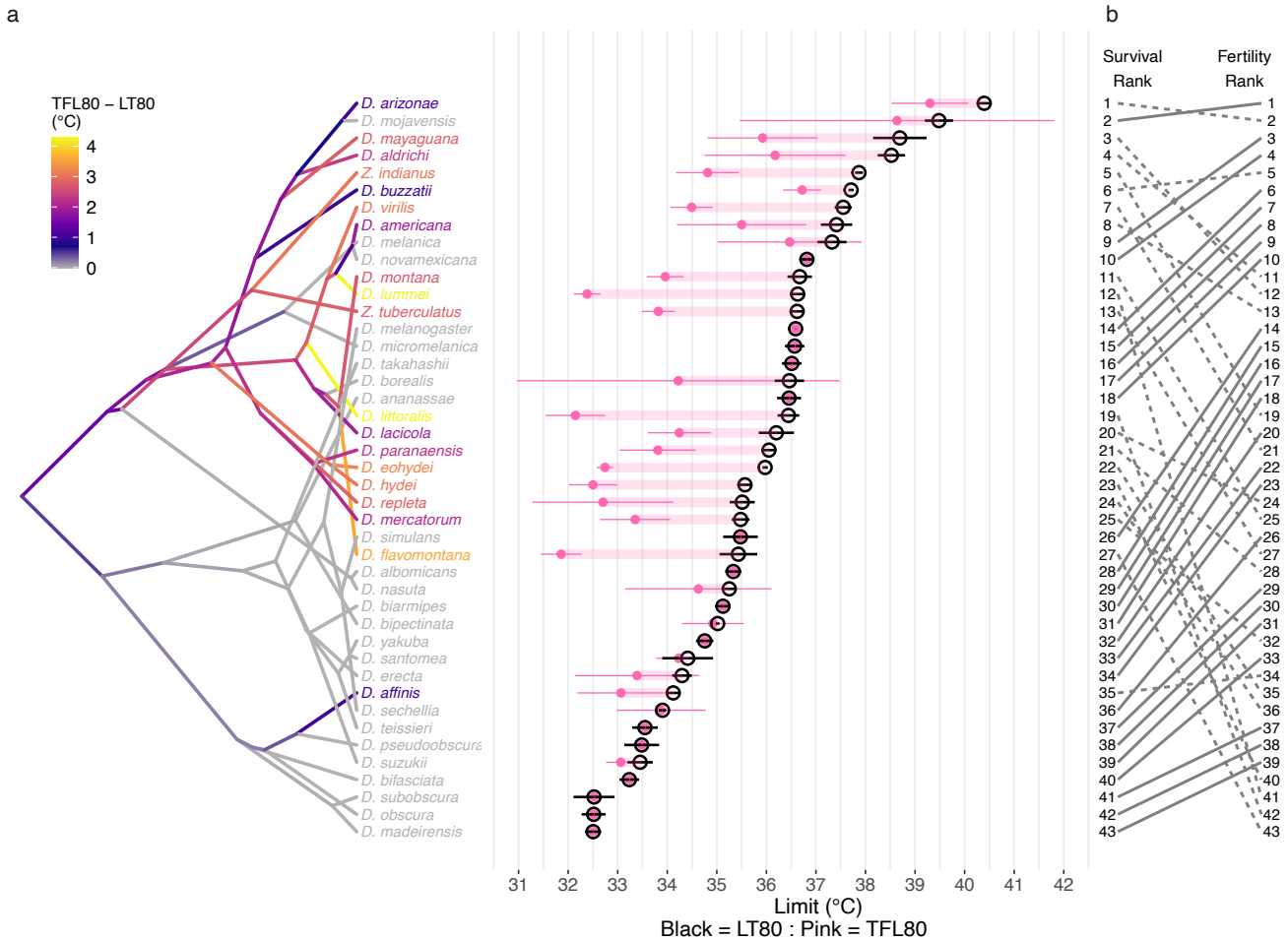
Figure 1: 80% lethal temperatures (LT80) and 80% sterilising temperatures (TFL80) for 43

species of *Drosophila*. Species ranked by LT80 from highest tolerance (top) to lowest

(bottom). a) Upper lethal temperature (LT80, black circles) and upper thermal fertility limits

(TFL80 measured 7-days after heat stress, pink points) of all 43 species. Pale pink bar links

estimates form the same species. 19 of 43 species show significantly lower thermal fertility

limits than lethal limits. 95% CI are shown as error bars for both measures, differences

between a species' TFL80 and LT80 considered to be significant if these bars do not overlap.

Axis phylogeny branches coloured by the difference between species' LT80 and TFL80

measured 7-days post heat stress. Yellower colours indicate larger differences, species with

91   no significant difference indicated in grey. b) Relative ranking of species by each thermal

92   tolerance measure. Dashed lines indicate species with significantly lower TFL80 than LT80.

93   For fertility measured immediately following heat-stress see Supplementary Figure 1.

94   Our data confirm that fertility loss at sub-lethal temperatures is common in *Drosophila*,

95   suggesting that lethal limits alone may overestimate the thermal tolerance of many species.

96   However, the key question is whether TFLs are linked to organisms' distributions in nature. To

97   test this, we integrated existing distribution data of each sampled *Drosophila* species with

98   global climate data. From this we estimated the mean maximum air temperatures species are

99   likely to encounter in natural populations. Our measurement of CT_{MAX} significantly predicted

100   mean maximum environmental air temperature (PGLS: $t_{40} = 2.647$, $P = 0.012$), albeit this

101   relationship negatively interacts with annual rainfall (PGLS: $t_{40} = -2.077$, $P = 0.044$, $_{adj}R^2 =$

102   0.186, $_{partial}R^2 = 0.336$). LT80 also significantly predicted mean maximum environmental

103   temperature (PGLS: $t_{40} = 3.360$, $P = 0.002$) to a similar extent ($_{adj}R^2 = 0.197$, $_{partial}R^2 = 0.337$).

104   However, the relationship between TFL80 and mean environmental maximum temperature

105   was stronger, both when TFL80 was measured immediately following heat-stress (PGLS: $t_{40} =$

106   4.225, $P < 0.001$, $_{adj}R^2 = 0.286$, $_{partial}R^2 = 0.401$) and 7 days later (PGLS: $t_{40} = 5.014$, $P < 0.001$,

107    $_{adj}R^2 = 0.365$, $_{partial}R^2 = 0.455$). Comparing all best–fit models, TFL80 measured 7-days after heat

108   shock most strongly predicted mean maximum air temperatures in species' environments,

109   explaining 36.5% to 45.5% of the variation (Supplementary Table 3). Based on $_{adj}R^2$, TFL

110   improves accuracy by 85.3% and 95.8% compared to CT_{MAX} and LT80 respectively. Based on

111   partial R^2, which account for non-independence in residuals from phylogenetic models[22], TFL80

112   provides a 35.1% and 35.5% improvement over LT80 and CT_{MAX} (Supplementary Table 3). TFLs

113   also outperformed lethal measures when we used a more conservative 50% threshold for LT

114   and TFL estimates (Supplementary Table 4). These analyses suggest that TFLs and species

115   distributions are strongly linked in nature, and that fertility losses due to high temperature

116   may be an important determinant of where species occur.

117

118   Thermal safety margins (the difference between an organism's thermal limit and the

119   maximum temperature it faces in nature) can be used to predict vulnerability to climate

120   change[7]. TFLs produce significantly smaller safety margins than CTLs (Supplementary Figure 2

121   & Table 5). We illustrate the potential implications of TFL-based safety margins with

122   distribution models of *Drosophila flavomontana,* which has one of the largest differences

123   between LT and TFL estimates, a well-documented distribution not associated with urban

124   areas or farms, and a well understood habitat ecology. Safety margins based on TFL80

125   predict a 17.9% reduction in habitable landscape compared to an identical LT80-based model

126   under current climate conditions (Figure 2A). The disparity between predictions based on

127   sterility and lethality grew to 48.0% by the year 2080 under moderately optimistic future

128   climate forecasts (ICCP-AR5 RCP 4.5, Figure 2B), and to 58.9% under pessimistic climate

129   change scenarios (ICCP-AR5 RCP 8.5, Figure 2C). TFL-based models also predict that by 2080

130   the available habitat for *D. flavomontana* will have reduced by 42.3% and 62.9% under
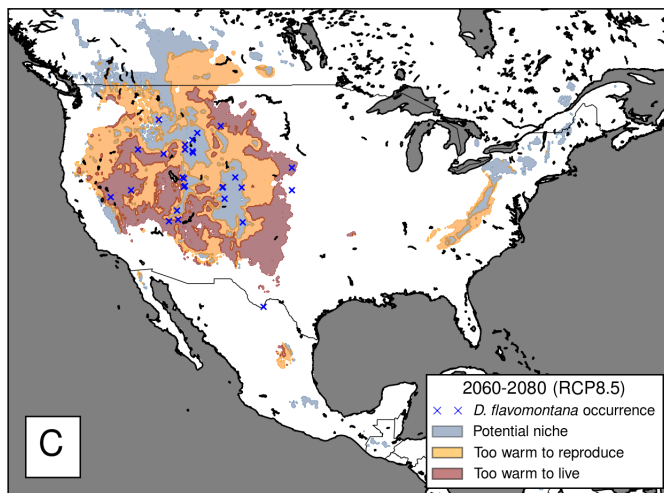
131   RCP4.5 and RCP8.5 respectively.

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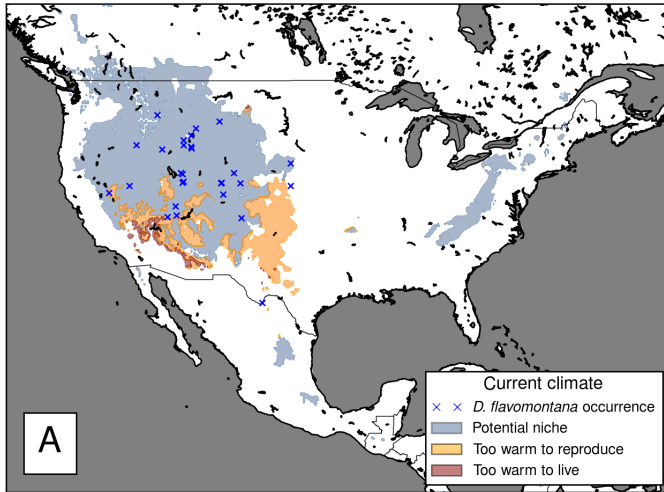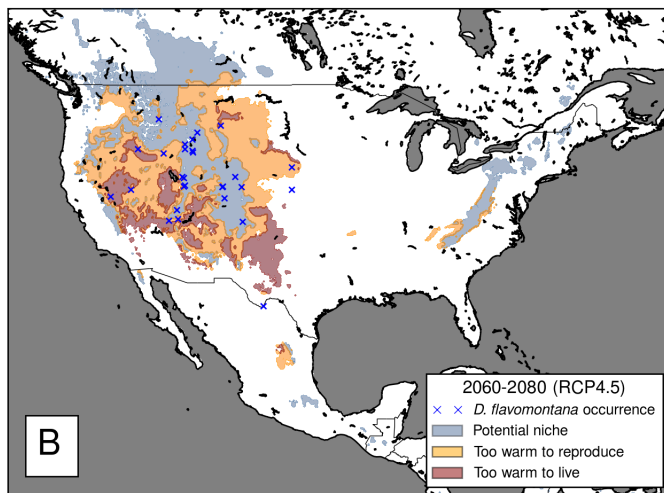
Figure 2: Potential current and future habitat range of *Drosophila flavomontana* (LT80 = 35.4ºC, TFL80 = 31.9ºC). A) current and B & C) possible future climate scenarios (B = RCP4.5

137   'moderately optimistic', C = RCP8.5 'pessimistic', predicted for 2060 - 2080). Colored areas in

138   each panel represent suitable habitat range predicted by a model that excludes maximum

139   temperature. Red areas show regions where maximum summer temperatures exceed LT80.

140   Orange areas show regions where maximum summertime temperatures exceed TFL80. Blue

141   regions are areas where limits for *D. flavomontana* are not exceeded all year.

142

143   How to most accurately measure thermal limits to predict how species will respond to

144   climate change is currently being debated[8,9,11,21]. Tolerance landscape measures of lethal

145   temperatures, which integrate the intensity and duration of heat stress, have been proposed

146   as superior alternatives to point-estimate methods such as CT_{MAX}[8,10]. Here, we step back

147   from this methodological debate and show the importance of identifying and measuring the

148   correct thermally sensitive traits in the first instance. High throughput point-estimates such

149   as we use here for TFL allow cross-species comparison of thermal sensitivity. Importantly,

150   this reveals contrasting patterns of inter-specific variation in survival and fertility, of which

151   fertility loss better matches variation in species natural thermal habitat. Exploration of the

152   physiological, genetic, behavioral and ecological mechanisms that underly thermal fertility

153   limits will now be an important step towards linking temperature-driven sterility with

154   species' responses to climate change.

155

156   If our data for *Drosophila* can be extrapolated to other organisms, then male fertility losses

157   at high temperatures may be common, occurring at substantially lower temperatures than

158   lethality. The limited data on fertility at extreme temperatures supports this, with high

159   temperature losses in male fertility observed in diverse organisms[12], including some high

160   temperature adapted species. For instance, the zebra finch, a desert-dwelling organism with

161   naturally high body temperature and good thermoregulation, shows substantial damage to

162   sperm at temperatures it regularly experiences in nature[19]. Behavioral thermoregulation

163   could potentially reduce the impact of high temperatures on fertility in nature. However,

164   while studies have found that *Drosophila* are able to behaviorally thermoregulate in the

165   lab[23], some evidence suggests that behavioral preferences for cooler microclimates such as

166   leaf litter, shade, or higher altitudes do not necessarily translate into natural settings[24].

167   Further, many species are able to survive high temperature periods by aestivating as adults,

168   eggs or pupae. This may explain why our data predict negative thermal safety margins for

169   some species. Despite these potential mechanisms, we still find that species' distributions

170   are predicted by thermal fertility limits.

171

172   Our work emphasizes that temperature-driven fertility losses may be a major threat to

173   biodiversity during climate change. We urgently need to understand the range of organisms

174   likely to suffer thermal fertility losses in nature, and the traits that predict vulnerability.

175   However, we currently do not understand the physiology underlying variation in TFLs

176   between species, nor the selective forces that created this variation. Ultimately, we need to

177   know whether evolution for higher TFLs will allow species to adapt to a warming

178   environment.

179

180   **Materials and Methods:**

181   We assayed three metrics of upper thermal limits in sexually mature males from 43 species

182   of *Drosophila*: Lethal Temperature (LT), Thermal Fertility Limit (TFL) and Maximum Critical

183   Temperature (CT_{MAX}). We measured LT and TFL under static temperature conditions by

184   exposing flies to four-hour temperature pulses and recording survival and fertility. Using

185   static temperatures to measure thermal tolerances has received criticism[21]. However, in

186   *Drosophila* fertility is internal and has no directly observable marker indicating a male has

187   become sterile, rendering ramping methods impossible. Measuring LT under static

188   temperatures allows us to directly compare measures of fertility loss and lethality under

189   identical conditions. Following heat treatment, males were transferred to fresh vials and

190   allocated to floating racks in pre-heated waterbaths set to a range of temperatures

191   (Supplementary Table 1). Males were heated for 4 hours between ~10am - ~2pm and then

192   returned to temperature-controlled rooms at the species' benign temperature. We scored

193   survival of males the next morning to account for immediate recovery or delayed death.

194   Surviving males were aspirated into separate vials containing 3-4 sexually mature virgin

195   females. Males were kept in these vials at their benign temperature to mate freely for 6 days,

196   then transferred to a second vial with 1-2 more virgin females and allowed to mate for 24

197   hours. This allowed us to score fertility at two time points to capture any recovery or delayed

198   sterilization. Vials were scored as 'fertile' by the presence of larvae or larval tracks. We used

199   dose-response models to estimate the temperatures that kill and sterilize 80% of males; LT80

200   and TFL80 respectively. We only allow TFL80 to be lower than or equal to the species' LT80

201   and we only consider a species' TFL to be statistically lower than its LT if the 95% confidence

202   intervals of these two point-estimates do not overlap. We also measured upper critical limits

203   of our 43 *Drosophila* species under ramping heat conditions (CT_{MAX}). Individual sexually

204   mature males were exposed to temperature increments of 0.1°C/min and the temperature at

205   which flies collapsed for 30 seconds and did not right themselves after tapping the vial was

206   recorded. We explored the correlations between LT80, TFL80 and CT_{MAX} using multiple

207   phylogenetically controlled approaches (supplementary methods).

208

209   We tested how well LT80, TFL80 and CT_{MAX} explained interspecific variation in the mean

210   maximum air temperature species experience in nature. We obtained species distributions

211   from Taxodros.ch and integrated these coordinates with the mean maximum air

212   temperature between the years 1970-2000 form the WorldClim V2 database (Tmax

213   hereafter). We used phylogenetically controlled models to fit each physiological limit as a

214   predictor of Tmax. We compare the adjusted and partial likelihood-based R^2 of each model.

215

216   We predicted future range contraction using TFL and LT for *Drosophila flavomontana*. We

217   used MaxEnt modelling to predict *D. flavomontana's* putative current range based on

218   ecological parameters at its known occurrence in Taxodros. We then constrained this area by

219   matching both LT80 and TFL80 to the maximum annual temperature experienced across this

220   range. We then forecast this to future moderately optimistic (RCP4.5) and pessimistic

221   (RCP8.5) climate change scenarios.

222

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291   **Author Contributions:** Conception: TP, AB, RS, AH, SP. Methodology & data collection: SP,

292   BW, NW. Data curation: SP & SM. Analysis: SP, AM & SM. Original Draft: SP, TP, AB, RS & AH.

293   Review & Editing: All authors. **Data and materials availability:** All data and analysis R code

294   will be deposited on Dryad upon acceptance of this manuscript.

295

296   **Competing Interest:**

297   The authors have no competing interest to declare.

298   Correspondence and requests for materials should be addressed to Steven Parratt or Tom

299   Price (S.parratt@liverpool.ac.uk/T.Price@liverpool.ac.uk).

323   Latitude shown here is the mean absolute latitude i.e. the sign of negative latitudes has

324   been removed to give relative distance from the equator. Fitted lines are predictions

325   from models in which the limit type (TFL, LT or CT_{MAX}) and latitude^2 are fixed effects

326   and species identity is a random effect to account for repeated measures across

327   species (Supplementary Table 4).

328   4. **Supplementary Figure 3: Repeatability of LT80**. Repeatability was high across the 22

329   *Drosophila* species tested. Left panel: There was no significant difference in the estimate

330   of LT80 between full runs and control repeats in any of these species (significance

331   measured as non-overlapping CIs of point estimates of LT80). Pink points = estimate

332   from single species assay, gold point = estimate from simultaneous multispecies control

333   assay. Right panel: The correlation between the two independent measurements of

334   LT80 across these species was strongly positive and explained a high degree of variation

335   in the data (coefficient = 1.06, R^2 = 0.96).

336   5. **Supplementary Figure 4: Repeatability of TFL80 for Drosophila virilis.** We

337   independently repeated the TFL80 assay for one species in which we found a significant

338   difference between LT80 and TFL80. Two independent runs of sexually mature males

339   were conducted by two researchers 6 months apart. Red = original data used to

340   calculate TFL80 for *D. virilis* in this manuscript, blue = repeated assay. Dashed lines

341   intersect at 80% threshold. Line fits predicted by 3-parameter dose-response model. The

342   fly stock and equipment were identical.

343

344   6. **Supplementary Figure 5: Repeatability of knockdown CT_{MAX}.** Left panel; mean CT_{MAX}

345   temperature recorded from species' independent assays (gold points), and in mixed

346   species control blocks (pink points). There was no global significant difference between

347   CT$_{MAX}$ estimates across all species (Block: F$_{1,831}$ = 3.246, P = 0.072). However a significant

348   experimental block*species interaction was found for 4 of the 41 species tested

349   (interaction term: F$_{40,831}$ = 3.7589, P < 0,001); *Drosophila sechelia, D. nasuta, D. yakuba*

350   & *D. tiesseri* all scored significantly higher CT$_{MAX}$ in our mixed species control block than

351   in their own individual CT$_{MAX}$ assays. Right panel: The correlation between estimated

352   CT$_{MAX}$ values from both blocks was strongly positive (coefficient = 1.07, F$_{1,37}$ = 309.84, P

353   < 0.001), and explained 89% of the variation.

354   7. **Supplementary Table 1: Details for species used in this study.** All species either D =

355   Drosophila or Z = Zaprionus.

356   **8. Supplementary Table 2:** Correlations between TFL80, LT80, and CT$_{MAX}$. Multiple

357   methods for phylogenetically controlled correlations were used to test the extent to

358   which these three physiological limits are proxies for one another. In all four methods

359   TFL80 correlates less strongly with CT$_{MAX}$ and LT80 than the two measures of critical

360   limits correlate with each other. Phylogenetic least squares (pgls) allows for estimation

361   of phylogenetic signal (Pagel's λ) in the residuals of the y-variable, thus correlation

362   coefficients from this method are sensitive to which variable is assigned as the predictor

363   and which as the response. To account for this, we present two model outputs for each

364   pair of traits. Phylogenetic independent contrasts (PIC) essentially assume a

365   phylogenetic signal of λ = 1 (complete Brownian motion evolution of the trait). Linear

366   models ('lm') make no adjustment for non-independence in trait values between closely

367   related species. `corphylo` from the `ape` R package allows for the three-way correlation

368   matrix to be estimated simultaneously and produces estimates of phylogenetic signal (d)

369   of each trait under an Ornstein-Uhlenbeck process. TFL80 here measured 7-days post

370   heat-stress. "-" denotes model estimates not shown because they are identical to those

371   in the line above that show the reciprocal x~y configuration. "**" and "***" indicate

372   significance at the *P* < 0.01 and *P* < 0.001 levels respectively.

373   9. **Supplementary Table 3:** Summaries of phylogenetically controlled models that predict

374   maximum environmental temperature (T_{max}) by either CT_{MAX}, LT80 or TFL80

375   independently of each other. Annual precipitation (P_{ANN}) was included as an interaction

376   term with physiological limits. adjR^2 and phylogenetic signal in model residuals (Pagel's

377   λ) given for final best-fit model derived from AICc model selection and inspection of

378   model residuals. Terms retained after model selection shown in italics. All continuous

379   predictors centred and scaled to the mean.

380   10. **Supplementary Table 4: Using 50% thresholds to predict distributions.** Summaries of

381   phylogenetically controlled models that predict maximum environmental temperature

382   (T_{max}) by either CT_{MAX}, LT50 or TFL50 independently of each other. Annual precipitation

383   (P_{ANN}) was included as an interaction term with physiological limits. R^2 and phylogenetic

384   signal in model residuals (Pagel's λ) given for final best-fit model derived from AICc

385   model selection and inspection of model residuals. Terms retained after model selection

386   shown in italics. All continuous predictors centred and scaled to the mean.

387   11. **Supplementary Table 5:** Summary model fits of species' safety margins (SM) to absolute

388   latitude (Lat^2), the physiological limit used in their calculation ('Limit'), and the

389   interaction between 'Lat^2' and 'Limit'. 'Species' identity is included as a random

390   intercept term. We show models of both "Central safety margin" and the "Distribution

391   Safety Margin" *as per* Kellermann[5]. Central safety margins are calculated as the

392   difference between the physiological limit and the mean maximum temperature

393   experienced in every know location in the species' distribution. "Distribution safety

394　　　margins" capture the environmental conditions at the upper thermal edge of specie's

395　　　known distribution by adding 1 standard deviation to the mean maximum temperature.

396　　　R^2 given for final best-fit model derived from AICc model selection and inspection of

397　　　model residuals. Significance of main terms given by Type II sum of squares F-tests with

398　　　Kenward-Rogers degrees of freedom. Terms retained after model selection shown in

399　　　italics. Post-hoc Tukey tests were run with the `emmeans` package to identify significant

400　　　differences between levels of "Limits". TFL80 measured 7-days post heat shock.

401　　　**12. Supplementary References**