A Unifying Model to Estimate Thermal Tolerance Limits in Ectotherms Across Static, Dynamic and Fluctuating Exposures to Thermal Stress

Lisa Bjerregaard Jørgensen (lbj@bio.au.dk)
Zoophysiology, Department of Biology, Aarhus University, 8000 Aarhus C
Hans Malte
Zoophysiology, Department of Biology, Aarhus University, 8000 Aarhus C
Michael Ørsted
Zoophysiology, Department of Biology, Aarhus University, 8000 Aarhus C
Nikolaj Andreasen Klahn
Zoophysiology, Department of Biology, Aarhus University, 8000 Aarhus C
Johannes Overgaard
Zoophysiology, Department of Biology, Aarhus University, 8000 Aarhus C

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A unifying model to estimate thermal tolerance limits in ectotherms across static, dynamic and fluctuating exposures to thermal stress

Lisa Bjerregaard Jørgensen*, Hans Malte, Michael Ørsted, Nikolaj Andreasen Klahn & Johannes Overgaard

Zoophysiology, Department of Biology, Aarhus University, 8000 Aarhus C, Denmark

**Author for correspondence (lbj@bio.au.dk)*

Abstract

Temperature tolerance is critical for defining the fundamental niche of ectotherms and researchers classically use either static (exposure to a constant temperature) or dynamic (ramping temperature) assays to assess tolerance. The use of different methods complicates comparison between studies and here we present mathematical model (and *R*-scripts) to reconcile thermal tolerance measures obtained from static and dynamic assays. Our model uses input data from several static or dynamic experiments and is based on the well-supported assumption that thermal injury accumulation rate increases exponentially with temperature (recently re-introduced as Thermal Tolerance Landscapes). The model also assumes thermal stress at different temperatures to be additive and using experiments with *Drosophila melanogaster*, we validate these central assumptions by demonstrating that heat injury attained at different heat stress intensities and durations is additive. In a separate experiment we demonstrate that our model can accurately describe injury accumulation during fluctuating temperature stress and further we validate the model by successfully converting literature data of ectotherm heat tolerance (both static and dynamic assays) to a single, comparable metric (the temperature tolerated for 1 hour). The model presented here has many promising applications for the analysis of ectotherm thermal tolerance and we also discuss potential pitfalls that should be considered and avoided using this model.

Introduction

Tolerance to temperature extremes is arguably among the most important traits defining the fundamental niche of ectotherms [1–5]. However, studies of thermal tolerance are difficult to compare due to the plethora of static (constant temperature) and dynamic (ramping temperature) assays used to assess tolerance limits [3,6,15–19,7–14]. Furthermore, it is difficult to relate laboratory measures of thermal tolerance to the temperature stress experienced under natural conditions where the duration and intensity of temperature stress fluctuates unpredictably.

Static and dynamic methods differ in their temperature protocol (constant and changing temperature, respectively) but also in duration of the stress during testing (see discussions in Jørgensen et al. [11] and Terblanche et al. [19]). A solution to unify many of these different tests can be found in the exponential relation between tolerance time and temperature (see Fry et al. [9], Jørgensen et al. [11], Kilgour & McCauley [12] and discussion below). Thus, a linear relation between $\log_{10}$(tolerance time) and temperature in ectothermic organisms has been known for more than a century [7,9,10,12,20–25]. This analysis of thermal tolerance has also recently been revisited as a "thermal tolerance landscape" (TTL) [11,26]. In this study, we expand this analysis further and present a theoretical and mathematical framework that allows researchers to directly compare thermal tolerance measurements obtained during constant and dynamic experiments. Specifically, this model (and associated *R*-scripts) allows researchers to convert assessments of tolerance from static to dynamic assays (and *vice versa*) and to use lab measurements of tolerance to assess the severity of thermal stress experienced under temperature fluctuations.

Theoretical foundation

Assessment of thermal tolerance uses various endpoints (loss of righting response, onset of spasms, coma, death [27,28]), but irrespective of the endpoint used, the estimate of tolerance will depend critically on the duration and intensity of stress exposure. From here and onwards we discuss this as coma during heat stress, but the model is likely also applicable to other endpoints and to cold stress phenotypes. In static measurements, thermal tolerance is recorded as the duration until onset of coma (t_{coma}) (Fig. 1A). The TTL describes thermal tolerance of a species/population using the slope of the relation between assay temperature and $\log_{10}(t_{coma})$ and a point on the line (here we use $sCT_{max\ (1h)}$ which is the temperature causing heat coma after a 1-h exposure) (Fig. 1B). The slope represents a thermal sensitivity factor that describes the temperature change resulting in a one order of magnitude change in t_{coma}. To be consistent with TTL terminology the slope is described by the parameter *z* where *z*=-1/slope (cf. Rezende et al. [26]), which is analogous to Q_{10} where $Q_{10}=10^{10/z}$. The TTL parameters from the linear regression can be used to

estimate the exposure duration (t_{coma}) tolerated at a specific temperature or to calculate the maximal static temperature (sCT_{max}) that can be tolerated for a specific duration (but see below and associated *R*-script for details).

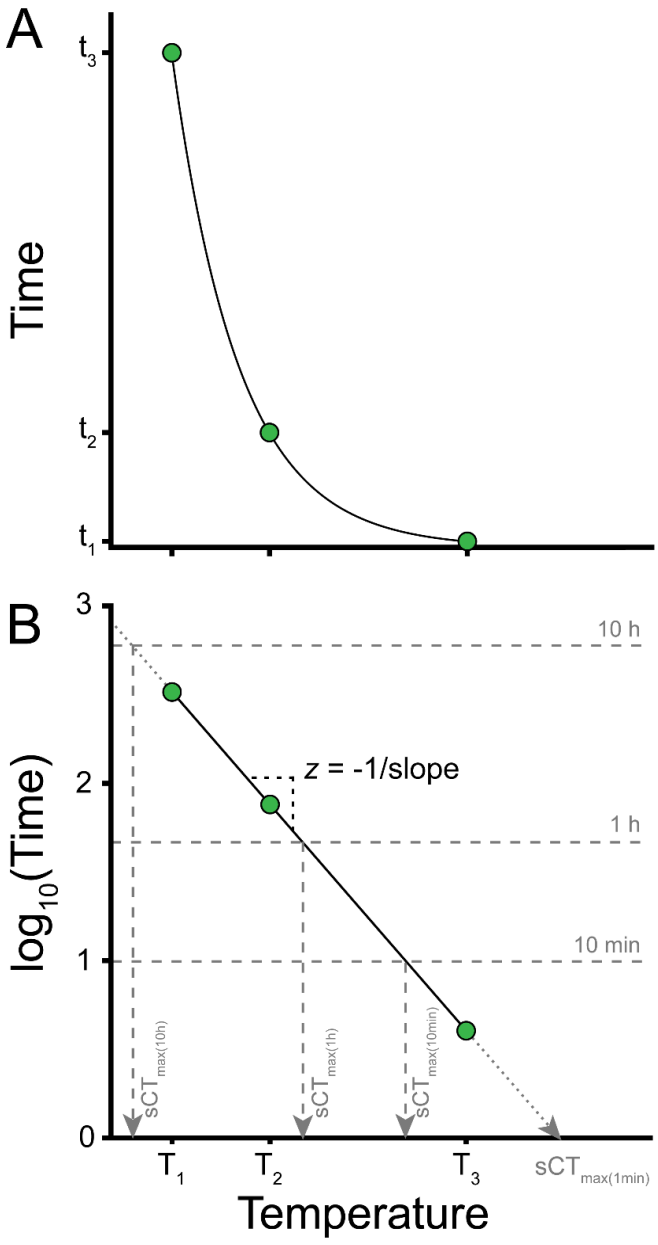


Fig. 1 Exponential relation between temperature and t_{coma} presented as a thermal tolerance landscape (TTL). A) Time to coma (t_{coma}) decreases exponentially with temperature. **B)** Observations from (A) presented as a TTL where the linearity of log_{10}-transformed t_{coma} vs. temperature indicates the exponential relation (full line). The negative inverse slope of the TTL is used as the thermal sensitivity parameter *z* (*z*= the change in temperature required to change t_{coma} by an order of magnitude). Intersections between the TTL and horizontal lines show the temperature (sCT_{max}) at which coma is expected to occur after a fixed time (e.g. 1 h, $sCT_{max\ (1h)}$). Extrapolations in either end of the TTL (dotted lines) are tempting but should be met with caution as the relationship is only linear in a certain time-temperature interval (see discussion).

Another perspective of TTL analysis is to consider temperature as the determinant of an injury accumulation rate (Fig. 2Aii) [9,11,12]. From this perspective injury accumulation rate increases exponentially with temperature and t_{coma} is then reached when the animal has accumulated the critical amount of injury, illustrated graphically as the area under the curve in Fig. 2. Thus, applying the concept of injury accumulation rates, it is possible to use TTL parameters to find the tolerance limits using other combinations of static, dynamic or fluctuating stress exposure. To validate this model empirically, we use *Drosophila melanogaster* to parameterize a TTL with static experiments and then use the TTL parameters to predict (and test) the accumulated injury from two sequential stress exposures at two different temperatures (as in Fig. 2C) and to predict (and test) heat failure in flies exposed to randomly fluctuating temperatures (as in Fig. 2D).

To make this method accessible to researchers we describe the mathematical foundation and provide *R*-scripts to directly derive TTL parameters and use them to assess tolerance limits. The scripts can derive TTL parameters from static data sets (where time to failure, t_{coma}, is measured at different constant temperatures), or from dynamic data sets (where the maximal temperature tolerated, CT_{max}, is measured using different ramping rates). The scripts also allow for construction of a TTL using only a single static or dynamic estimate of heat tolerance, but we caution that the model will be extremely sensitive to the (untested) assumed value of *z* that must be provided. Irrespective of the type of input data, the scripts can predict heat failure at various static and dynamic measurements from TTL parameters or integrate TTL parameters with data of temperature fluctuations to predict heat stress under fluctuating conditions. To test the applicability of the model we compare estimates of heat tolerance in nine ectotherm species from many different literature sources with the assumption that each of these species are characterised by a reasonably constant species-specific heat tolerance. Specifically, we use the original experimental data to generate TTL parameters and then estimate the temperature required to cause heat failure in 1 hour.

As it applies to nearly all models, the quality of the input determines the quality of the model output and more importantly all models have boundaries and limitations for their applications. This is also true for this model and we therefore discuss important considerations including: *i)* The importance of using experimental data for model parameterization within the time and temperature domain of interest, *ii*) The risks of model extrapolation, and *iii*) The need to consider model boundaries in time and temperature and to consider interactions with repair and hardening.

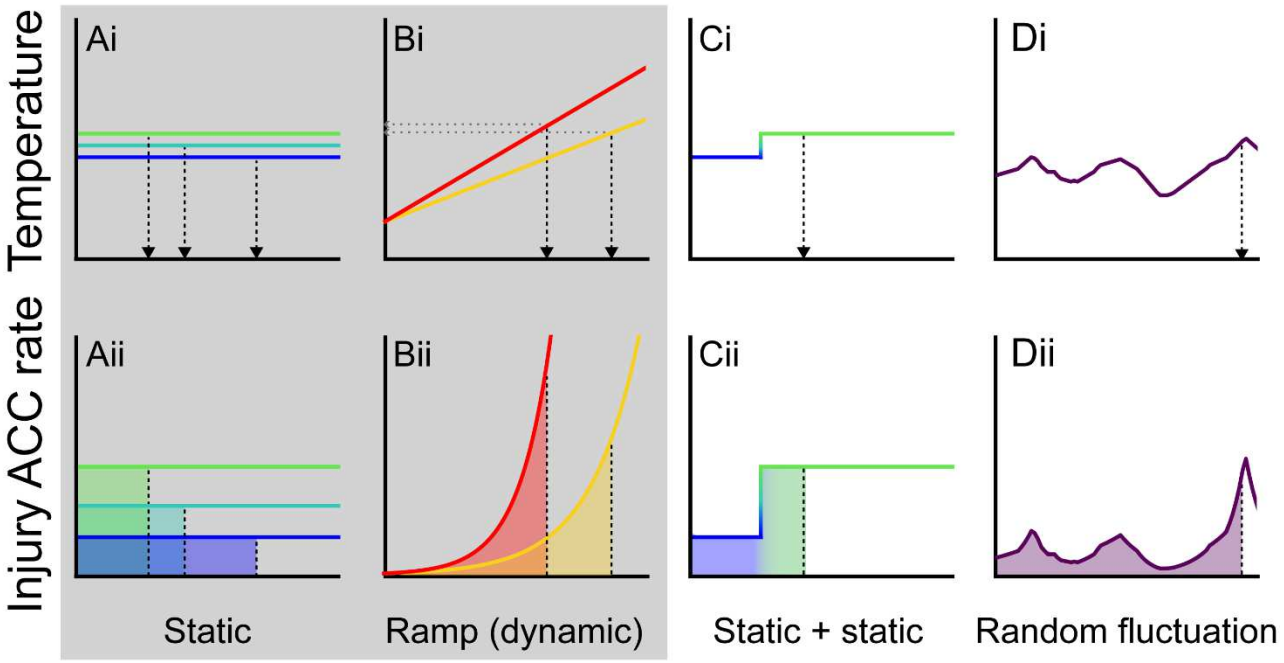


Fig. 2 Temperature profiles of heat tolerance experiments and their corresponding temperature-dependent injury accumulation (ACC) rates based on parameters from the thermal tolerance landscape. x-axes represent a time scale. Shaded areas under the curves represent identical amounts of accumulated injury that result in heat failure (lethal dose, Aii-Dii). The exposure duration resulting in accumulation of the lethal dose is reported as t_{coma} (static) or CT_{max} (dynamic), marked by vertical and horizontal arrows, respectively, in Ai-Di. The grey box highlights the two classical assay types (static and dynamic). **Ai-Aii)** Exposure to a static temperature yields a constant temperature-dependent injury accumulation rate. **Bi-Bii)** Exposure to a dynamic ramp where temperature changes by a fixed rate resulting in an exponentially increasing injury accumulation rate with time. The fast ramping rate (red) results in a shorter exposure before CT_{max} is observed than the slow rate (yellow), but as the injury accumulation rate increases more slowly for the low ramping rate, the final CT_{max} is lower. **Ci-Cii)** During initial exposure to a static temperature (blue), some injury is accumulated, but not sufficient to trigger coma onset. Successive exposure to another static temperature (green) yields the critical amount of injury, but as some injury had already accumulated, the exposure to the second temperature is shorter than if only this temperature had been used (compare with Aii). The prediction of the model will only hold true if injury is additive at the two static temperatures. **Di-Dii)** Varying (natural or laboratory generated) temperature changes that cannot be described by a simple fixed ramping rate. However, knowing the temperature-time profile permits calculations of the injury accumulation at any time and thus estimation of t_{coma}.

Materials and methods

1) Mathematical foundation

If we assume that the acute, temperature-related injury accumulation rate of an animal depends on temperature by some function *R(T)* then the amount of accumulated injury/damage (*d*) at some time *t* is given by:

$$d(t) = \int_{t_c}^{t} R[T(\tau)]\, d\tau \qquad \text{Eqn. 1}$$

Here $T(\tau)$ is the time-varying temperature regime and t_c is the time where injury accumulation starts. The temperature at t_c is T_c, and up to this temperature the repair of injury can match the generation of injury. For equation 1 to hold true, however, we must require that once temperature surpasses T_c it will stay above T_c. At some time t_{Ld} (the dynamic knockdown time, vertical arrow in Fig. 2Bi), a lethal dose d_L (area under the curve in Fig. 2Bii) has been attained and the animal is knocked down. If injury generation rate depends exponentially on temperature, and if injury repair rate is maximized at T_c (reached at t_c), then the accumulated lethal dose is related to the knockdown time t_{Ld} by:

$$d_L = R_0 \int_{t_c}^{t_{Ld}} (e^{k(T(t)-T_c)}-1)\, dt \qquad \text{Eqn. 2}$$

Due to the potent (high *k*) relation between injury accumulation rate and temperature, and because the true T_c for any species is rarely known, we simplified this to (see Fig S1 for further justification):

$$d_L \approx R_0 \int_{t_{c^*}}^{t_{Ld}} e^{k(T(t)-T_{c^*})}\, dt \qquad \text{Eqn. 3}$$

Where t_{c^*} is some start time for integration where the temperature *T* attains some convenient value T_{c^*} (e.g. rearing temperature) below the true T_c. In the event that exposure temperature is constant (and above T_{c^*}) we get:

$$d_L = t_{Ls}\, R_0 e^{k(T-T_{c^*})} \qquad \text{Eqn. 4}$$

where t_{Ls} is the static knockdown time. Rearranging this leads to an exponential relation between the static knockdown time and temperature, which is here called sCT_{max} (static CT_{max}):

$$t_{Ls} = \alpha\, e^{-k(sCT_{max}-T_{c^*})} \qquad \text{Eqn. 5}$$

where $\alpha = d_L/R_0$. This is identical to the usual form of the TTL if $log(\alpha) = sCT_{max}/z$ and $k = ln(10)/z$.

Assuming that the accumulated injury is always the same at knockdown then, once knowing knockdown time and temperature in one experimental setting, will allow calculation of expected knockdown temperatures and/or times in other experimental settings. Thus, In the case of a linearly increasing temperature ramp of the form $T(t)=T_0+b\cdot t$, where T_0 is the ramp start temperature and b is the ramping rate, the dynamic knockdown time and temperatures can be calculated by: (Jørgensen et al. [11] – note that in the original paper the "*ln*" and the *k* in front of $(T_{c*}-T_0)$ was lost in typesetting):

$$t_{Ld}=\frac{1}{kb}\ln\left[kbt_{Ls}e^{k(sCT_{max}-T_0)}+e^{k(T_{c*}-T_0)}\right] \qquad \text{Eqn. 6}$$

$$dCT_{max}=T_0+\frac{1}{k}\ln\left[kbt_{Ls}e^{k(sCT_{max}-T_0)}+e^{k(T_{c*}-T_0)}\right] \qquad \text{Eqn. 7a}$$

One can also express sCT_{max} as a function of dCT_{max}:

$$sCT_{max}=T_0+\frac{1}{k}ln\left[\frac{1}{kbt_{Ls}}\left(e^{k(dCT_{max}-T_0)}-e^{k(T_c-T_0)}\right)\right] \qquad \text{Eqn. 7b}$$

If the temperature varies randomly (and therefore is not monotonically increasing) it is no longer possible to calculate an expected knockdown temperature from knockdown temperatures obtained in static or dynamic ramp experiments. However, the expected knockdown time can still be found by evaluating (usually numerically) the integral of Eq. 3 as a function of time.

Using the equations

We provide two *R*-scripts (https://github.com/MOersted/Thermal-tolerances, see guide in Supplementary Information) to aid TTL parameterization from static and dynamic assays. One script establishes the TTL parameters from the knockdown time at two or more static temperatures (Fig. 1), while the other script derives TTL parameters from two or more values of dCT_{max} obtained by different ramping rates and/or start temperatures (Fig. 2B). Using dynamic input data, sCT_{max} and *z* is estimated either through nonlinear curve fitting in case dCT_{max} is available for three or more ramping rates, or by solving two equations (either 7a or 7b) with two unknowns when dCT_{max} is known for two ramping rates (see Supplementary Information for details).

Knowing only one dCT_{max} or sCT_{max} will not suffice to establish a TTL since *k* (and thus *z*) in equations 7a and 7b will be left unknown. In such cases, an estimated value of *z* must be supplied (some guidance is provided in Supplementary Information, Table S1), to allow the establishment of a crude TTL, which will be subject to uncertainty and sensitive to extrapolation (see discussion).

Once the TTL is parameterized, both scripts have four available outputs; *i*) estimated exposure duration (t_{coma}) tolerated at specific temperatures, *ii*) estimated static temperature (sCT_{max}) that can be tolerated for specific durations, *iii*) estimated maximal dynamic temperature dCT_{max} that can be tolerated in experiments with specific ramp rates, and *iv*) estimated injury accumulation under fluctuating temperatures (Fig. 2D), and thus the exposure duration tolerated before a given percent of lethal injury has accumulated.

2) Empirical experiments for model validation

Animal husbandry

Drosophila melanogaster (collected in Denmark, 2011) were kept in bottles with oat-based Leeds medium [6] (19°C, constant light). Experimental flies were produced by transferring a spoonful media containing egg/larvae to fresh bottles (23°C, 14:10 L:D cycle). Emerging flies were collected every two days and after 2-5 days flies were briefly anaesthetised with CO_2 (<5 min), then separated by sex and returned to fresh vials. Flies were allowed >2 days to recover from anaesthesia before experiments [29], resulting in 4-8 days old experimental flies.

Thermal Tolerance Landscape (TTL)

TTLs were generated by exposing flies to different stressful static temperatures and measuring the time to coma (t_{coma}, like Fig. 1). Experiments were conducted at temperatures resulting in heat coma onset ranging from ~ 10 min to 5 h using the experimental setup described in Jørgensen et al. [11]. Briefly, flies were placed individually in 5-mL glass vials with a droplet of Leeds medium in the cap (food and water source), then mounted to a rack and submerged in a preheated temperature-controlled water bath. The median value of t_{coma} was reported for each temperature allowing for long experiments (> 30 min) to be terminated once more than half of the flies had entered heat coma (See Jørgensen et al. [11] for use of median values). A TTL was parameterized for each sex by linear regression of the $\log_{10}$-transformed median t_{coma} against the assay temperature as described above (Fig. 1).

Test of injury additivity between two static temperatures

To investigate whether heat injury acquired at different temperatures is additive, flies were exposed to one stressful temperature (T_1) and successively transferred to another stressful temperature (T_2) (Fig. 2C). The two temperatures were selected from the TTL: a 'low' temperature (~36.5°C) with expected t_{coma} >4 h and a 'high' temperature (~39.5°C) with expected t_{coma}<1 h. Experiments were performed with both the 'high' and 'low' temperature as T_1 to examine whether additivity was independent of the heat stress intensity during the first exposure. In each experimental run, six groups (n=10) of both sexes were placed at T_1 and

199 then transferred to T_2 at regular intervals for recording of t_{coma}. For each combination of sex and treatment
200 order ('high' or 'low' temperature as T_1) the median exposure time at T_1 and T_2 was recalculated as
201 fractions of the t_{coma} predicted by the TTL. As an example, an initial exposure of 15 min to the higher test
202 temperature (39.5°C, TTL-predicted t_{coma} = 50 min) corresponds to a fraction of 15 min/50 min = 0.3 of
203 injury resulting in coma. The hypothesis of additivity predicts that the fractions of accumulated injury
204 resulting in coma should sum to 1, and accordingly the predicted fraction of time to coma at T_2 should be
205 0.7. Thus, if the lower test temperature (36.5°C) has a TTL-predicted median t_{coma} of 300 min, a fraction of
206 0.7 corresponds to 300 min·0.7=210 min.

207 *Test of injury additivity during temperature fluctuations*

208 Animals in the field experience temperature fluctuations. If injury is additive, it should be possible to
209 predict t_{coma} under such conditions by considering fluctuations as many additive exposures to different
210 temperatures each characterised by an injury accumulation rate (calculated from TTL, Fig. 2D). To
211 investigate whether heat injury during fluctuating temperatures can be modelled from TTL parameters,
212 t_{coma} was observed in flies exposed to randomly fluctuating temperatures and compared to a TTL-predicted
213 t_{coma}. Flies were divided into 13 experimental groups (n=16-18) for each sex. Groups were introduced to the
214 water bath at different times, which was randomly and sequentially programmed to heat or cool.
215 Consequently, each group experienced a unique temperature profile (range: 34.5-42.5°C). The median t_{coma}
216 for each sex and experimental group was compared to the t_{coma} predicted by the TTL. Specifically, to predict
217 t_{coma} under these conditions the associated *R*-script was given the average temperature for each 10-second
218 period and injury accumulation for each period was then calculated from the TTL. Injury accumulated
219 during each 10-second interval was summed until the critical amount of injury had accumulated resulting in
220 predicted t_{coma}.

221 ***3) Model validation using literature data***

222 Measures of heat tolerance from different publications are difficult to compare as they are based on
223 different experimental procedures or conditions. To examine whether our model can improve comparison
224 of published tolerance estimates, we collected heat tolerance measures from both static and dynamic
225 assays for nine ectothermic species including a crustacean (*Daphnia magna* Straus 1820), insects (*D.*
226 *melanogaster* Meigen 1830, *Drosophila subobscura* Collin 1936, *Glossina pallidipes* Austen 1903, *Tenebrio*
227 *molitor* L. 1758), collembolans (*Folsomia candida* Willem 1902, *Orchesella cincta* L. 1758) and fishes
228 (*Gambusia affinis* Baird & Girard 1853, *Salmo salar* L. 1758). Literature search was aided by an overview of
229 dynamic assays (with at least three ramping rates) [30], the GlobTherm database [31] and a review of CT_{max} in
230 aquatic ectotherms [32]. For static assays the time of failure and assay temperature were recorded while the

ramping rate, CT_{max} and the initial temperature (t_0) were noted for dynamic assays. Acclimation temperature was recorded if specified. The aim was to gather multiple heat tolerance measures from the same publication to provide input for TTLs, but publications with fewer assay temperatures or ramping rates were also included to examine how the model operated using assumed values of *z*. The data set included 155 static entries and 227 dynamic entries from 55 publications. For all studies included the associated *R*-scripts were used to derive TTL parameters to estimate the static temperature that causes heat failure after a 1-hour exposure ($sCT_{max(1h)}$). For studies with single tolerance measures we assumed the mean species value of *z* calculated from the other publications in our analysis (Table S1).

Results

Thermal Tolerance Landscape (TTL)

Median time to heat coma (t_{coma}) ranged from 7 to 289 min (~5 h) in flies exposed to constant, stressful temperatures ranging 36.30-42.75°C (Fig. 3A). The resulting TTL was well-fitted (R^2>0.98) for both sexes, showing that the exponential function is appropriate to describe the negative relation between temperature and t_{coma}. These TTL parameters (*z* and $sCT_{max(1h)}$) were used to characterise temperature-specific injury accumulation rates in subsequent experiments.

Test of injury additivity between two static temperatures

To investigate whether heat injury acquired at two stressful temperatures is additive, groups of flies were exposed to a static temperature (T_1) and subsequently exposed to another static temperature (T_2) (Fig 3B-C). Injury accumulated at the two temperatures was scored as the fraction of injury required to enter coma at T_1 and T_2, respectively (calculated from TTL, Fig. 3A), and these fractions summed to ~ 1 (graphically points are scattered around the line of additivity, R^2>0.78, Fig. 3B-C). This indicates that heat injury at two static stressful temperatures is additive, which is supported statistically as the linear regressions of the fractional injury accumulation to t_{coma} at T_1 *vs*. T_2 were not significantly different from the line of additivity for neither sex nor temperature sequence (*P*>0.05, *linearHypothesis(intercept=1, slope=-1)*, Fig. 3B-C).

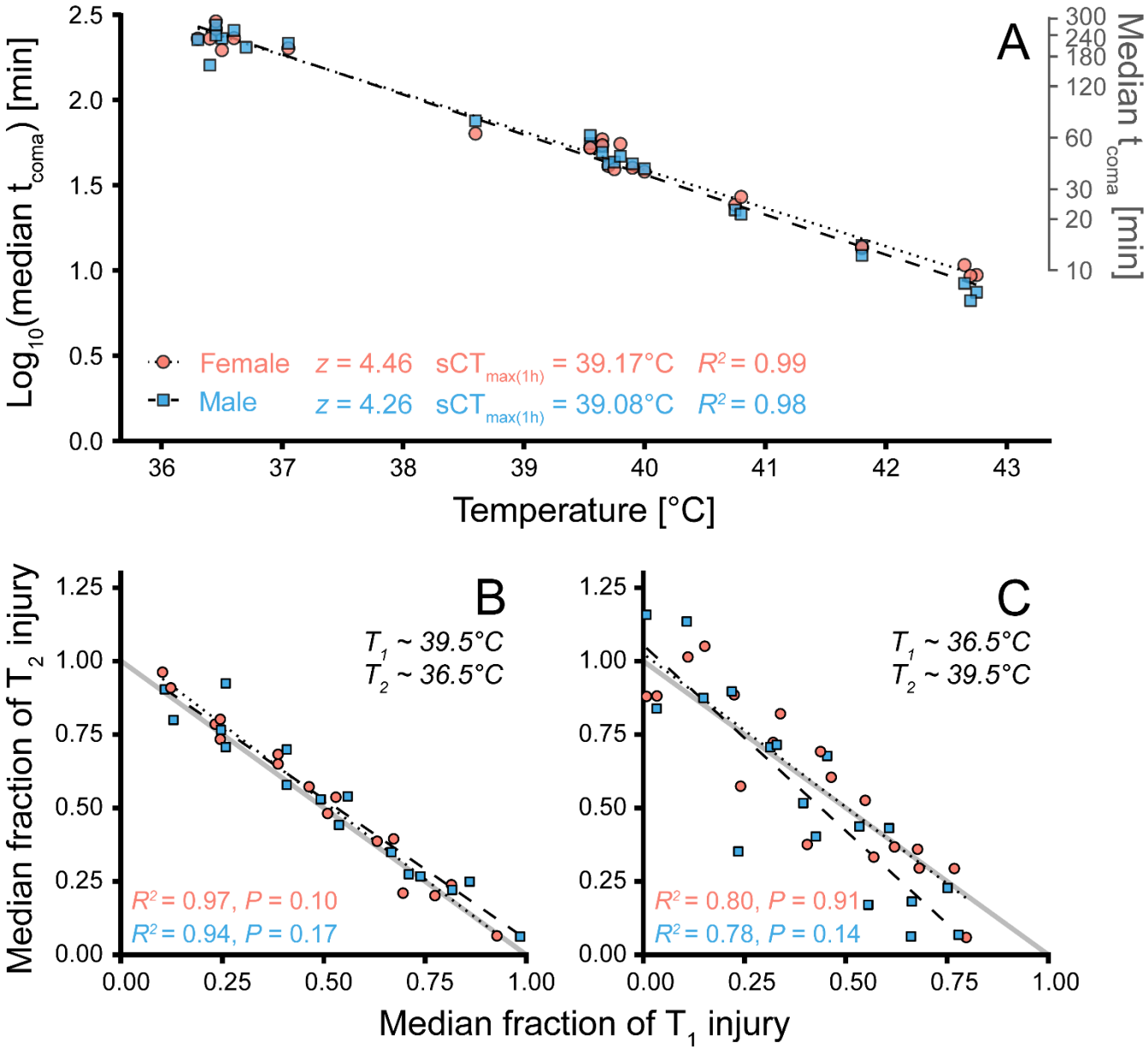


Fig. 3 Thermal tolerance landscapes and test of injury additivity between two temperatures. A) Flies were exposed to a static stressful temperature until coma onset (t_{coma}). Each point is the median t_{coma} of a group (n=2-10) and TTLs are made for each sex by linear regressions of median $\log_{10}(t_{coma})$ vs. assay temperature. **B)** Groups of flies (n=5-6 per sex) were exposed to a high temperature (~39.5°C) and then, after different durations, transferred to a lower temperature (~36.5°C) where median t_{coma} was recorded. The exposures are shown as fractions of injury resulting in coma at T_1 (x-axis) and T_2 (y-axis), where small and large fractions indicate brief and long exposurs, respectively (i.e. brief exposure results in small accumulation of injury relative to the critical amount of injury). If injury acquired at two different temperatures is additive, the two fractions should sum to 1, i.e. follow the (full) line of additivity. **C)** A similar experiment as (B), but with initial exposure to ~36.5°C and the subsequent exposure to ~39.5°C.

Test of injury additivity during random temperature fluctuations

Groups of flies were exposed to randomly fluctuating temperatures to investigate whether injury accumulation was also additive under these conditions (Figs. 2D, 4A). Each group experienced a unique sequence of stressful temperatures resulting in exposures spanning 33-245 min before the last fly entered coma. For each of the random temperature exposures the accumulated amount of injury was predicted using TTL parameters and the accompanying *R*-script (Fig. 4B). Median t_{coma} was recorded for each sex in the 13 test groups and compared to the predicted t_{coma} for each specific fluctuating temperature sequence (Fig. 4C). Predicted and observed t_{coma} correlated strongly in both sexes (R^2>0.94), and though the linear regressions deviated from the line of unity (P<0.02, *linearHypothesis(intercept=1, slope=-1)*), the predicted and observed t_{coma} were not significantly different (male: W=96, P=0.58; female: W=88, P=0.84, *two-sample Mann-Whitney U test*, inset in Fig. 4C). The model calculated the mean amount of injury accumulated at the median observed t_{coma} to be 1.10 (range: 0.75-1.36) and 1.09 (range: 0.86-1.46) for males and females, respectively, where 1.00 is the TTL predicted amount causing coma (Fig. 4C).

Using TTL parameters to compare heat tolerance measures from the literature

Literature values of heat tolerance were obtained for nine ectotherm species and for each publication we used values as model input to calculate a common heat tolerance estimate; the static temperature estimated to result in heat failure after a 1-hour exposure ($sCT_{max\ (1h)}$, Fig. 5). Input data included both dynamic and static experiments and after conversion to a common metric $sCT_{max\ (1h)}$ we generally found overlap within species and it is for example possible to discern large interspecific differences in heat tolerance (compare *D. melanogaster* and *D. subobscura* or *G. affinis* and *S. salar*, Fig. 5). In species with a relatively large intraspecific variation, higher acclimation temperatures tend to increase heat tolerance, although this was not formally tested.

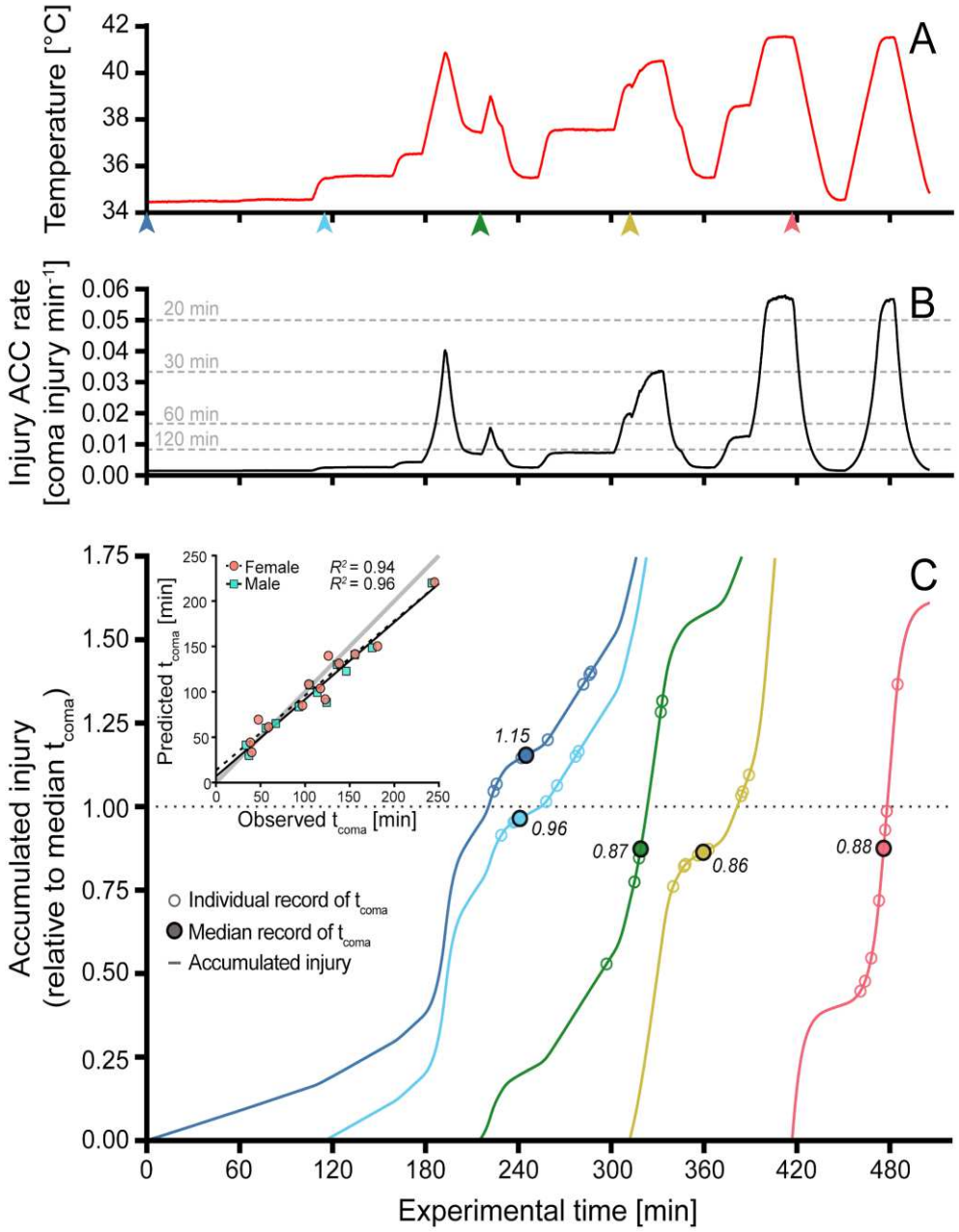


Fig. 4 Test of injury additivity during random temperature fluctuations. A) Fluctuating temperature profile from one of the experiments. **B)** The corresponding temperature specific injury accumulation (ACC) rates for each 10-second interval calculated from the TTL (Fig. 3A). Rates are presented as a fraction of coma injury min^{-1} (i.e. 0.01 corresponds to an injury rate that would cause coma in 100 min), and dashed lines show the injury accumulation rate that, if held constant, would result in coma after 20, 30, 60 and 120 min. **C)** Groups of flies (n=8-9) were introduced at different times during the assay (arrowheads in (A)), and thus each group experienced a unique sequence of fluctuating temperatures. The temperature-specific injury accumulation rates were used to predict the gradual injury accumulation (coloured lines), and accordingly, *when* each experimental group should have accumulated the critical amount of injury according to the TTL (fraction = 1, dotted line). Numbers accompanying median points are the calculated amount of injury accumulated at that specific time for this group. **Inset)** Observed vs predicted t_{coma} from all experiments (grey line of unity).

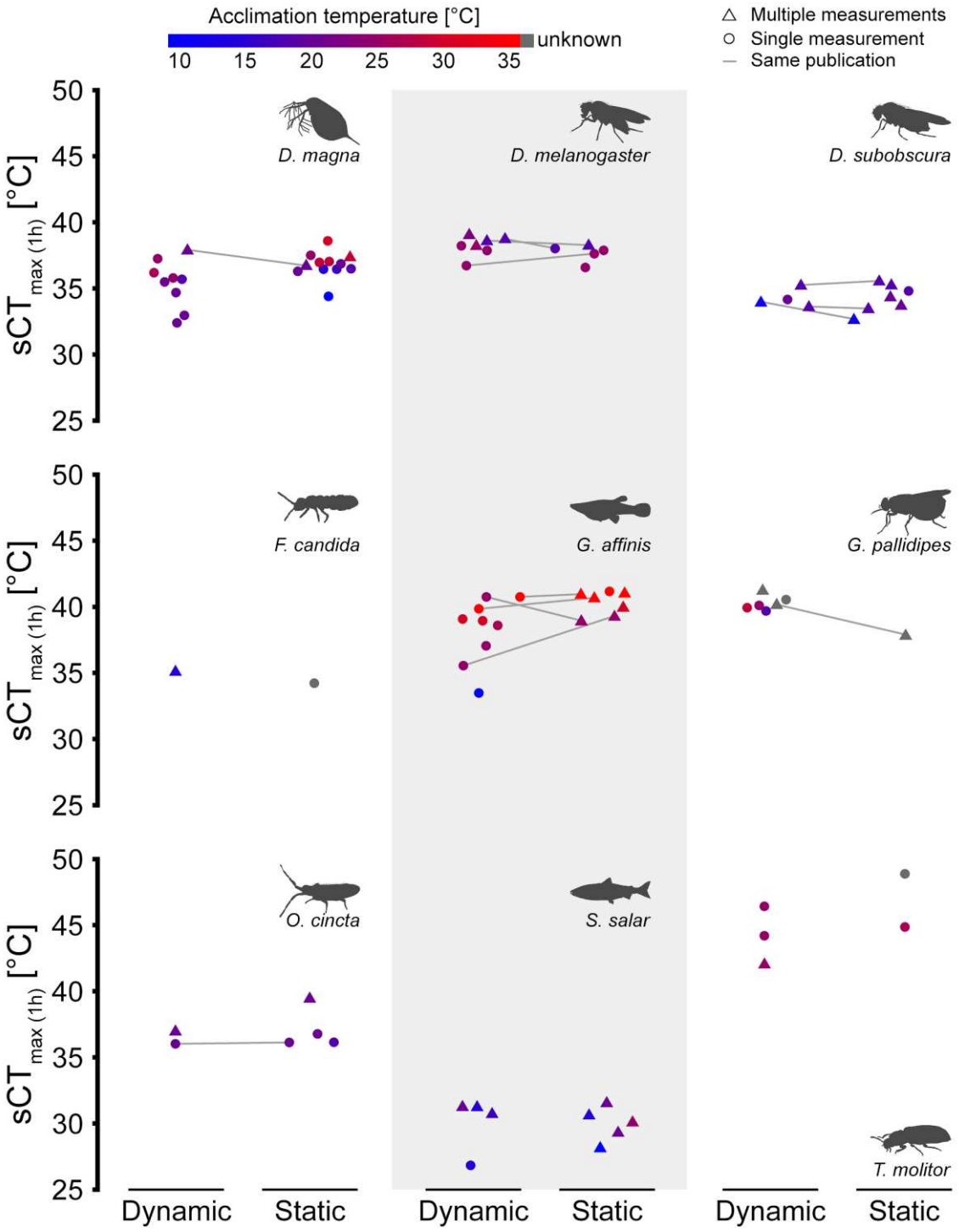


Fig. 5 Heat tolerance measures from the literature re-calculated to the temperature resulting in heat failure after a 1-hour exposure ($sCT_{max\ (1h)}$). Heat tolerance measurements from both dynamic and static assays were obtained from the literature for nine ectothermic species and used as input for the associated *R*-scripts. If multiple measures of the same assay type were available in a publication (triangles), these measurements were used for TTL parameterization and then calculate $sCT_{max\ (1h)}$. If only a single measurement was available (circle) an estimated value of *z* was supplied to create a TTL for calculation of $sCT_{max\ (1h)}$ (see main text). Connected points represent publications that provided both dynamic and static measures and colour indicates acclimation temperature.

Discussion

Fitting tolerance time vs. temperature to build a thermal tolerance landscape

The high coefficients of determination found in the *D. melanogaster* TTLs (Fig. 3A) are not uncommon and the exponential relation has consistently been found to provide a good fit of tolerance time vs. temperature in ectotherms [3,11,21–23,26]. Tolerance time vs. temperature data are also well fitted to Arrhenius plots which are based on thermodynamic principles (e.g. Armstrong et al. [33]) and the absence of breakpoints in such plots provides a strong indication (but not direct proof) that the cause of coma/heat failure under the different intensities of acute heat stress is related to the same physiological process regardless whether failure occurs after 10 min or 10 hours [2,3] (But see discussion below). Despite the superior theoretical basis of Arrhenius analysis, we proceed with simple linear regressions of $\log_{10}$-transformed t_{coma} (TTL) as this analysis likewise provides a high R^2 and is mathematically more straightforward. The physiological cause(s) of ectotherm heat failure are poorly understood [34,35] but we argue that they are founded in a common process where heat injury accumulates at a temperature-dependent rate until a species-specific critical dose is attained (area below the curve in Fig. 2). It is this reasoning that leads to TTLs and explains why heat stress can be additive and thus also determines the boundaries of TTL modelling.

Injury is additive across different stressful assay temperatures

If heat stress acquired at intense and moderate stress within the span of the TTL acts through the same physiological mechanisms or converges to result in the same form of injury, then it is expected that injury is additive at different heat stress intensities. This hypothesis was tested by exposing flies sequentially to two static temperatures (different injury accumulation rates) and observe whether coma occurred as predicted from the summed injury (Fig. 2C). The accumulated heat injury at the two temperatures was found to be additive regardless of the order of temperature exposure (Fig. 3B-C). Accordingly, the mode of heat injury accumulation is not important, provided that the relation between temperature and injury accumulation rate is always the same. This finding is consistent with a conceptually similar study using speckled trout (Fry et al., 1946) which also found strong support for additivity of heat stress at different stressful temperatures.

If injury accumulation is additive irrespective of the order of the heat exposure, we can extend the model to fluctuating temperature conditions. We have previously done this by accurately predicting dynamic CT_{max} from TTL parameters obtained from static assays for 11 *Drosophila* species (Fig. 6A, see discussion below and Jørgensen et al. [11]) but here we extend this to temperature fluctuations that cannot be described by a simple mathematical ramp function. Specifically, groups of flies were subjected to

randomly fluctuating temperatures and the observed t_{coma} was then compared to t_{coma} predicted using integration of heat injury based on TTL parameters (Fig. 4). The injury accumulation (Fig. 4C) was calculated by introducing the fluctuating temperature profiles in the associated *R*-script and the observed and predicted t_{coma} was found to correlate well (R^2>0.94) across the 13 groups tested for each sex. These results further support the idea that injury is additive across a range of fluctuating and stressful temperatures and hence that similar physiological perturbations are in play during moderate and intense heat stress. It is important to note that in these experiments, temperatures fluctuated between 34.5 and 42.5°C and accordingly the flies were never exposed to benign temperatures that could allow repair or hardening (see below).

In conclusion, empirical data (present study; Bigelow [7]; Fry et al. [9]; Hollingsworth [21]; Jacobs [10]) support the application of TTLs to assess heat injury accumulation under fluctuating temperature conditions both in the lab and field for vertebrate and invertebrate ectotherms. Potential applications could be assessment of injury during foraging in extreme and fluctuating environments (e.g. ants in the desert [36] or lizards in exposed habitat [37]) or for animals experiencing extreme conditions [38,39]. The associated *R*-scripts allow assessment of percent lethal damage under such conditions if the model is provided with TTL parameters and information of temperature fluctuations (But see discussion of model limitations below).

Model application for comparison of static vs. dynamic data

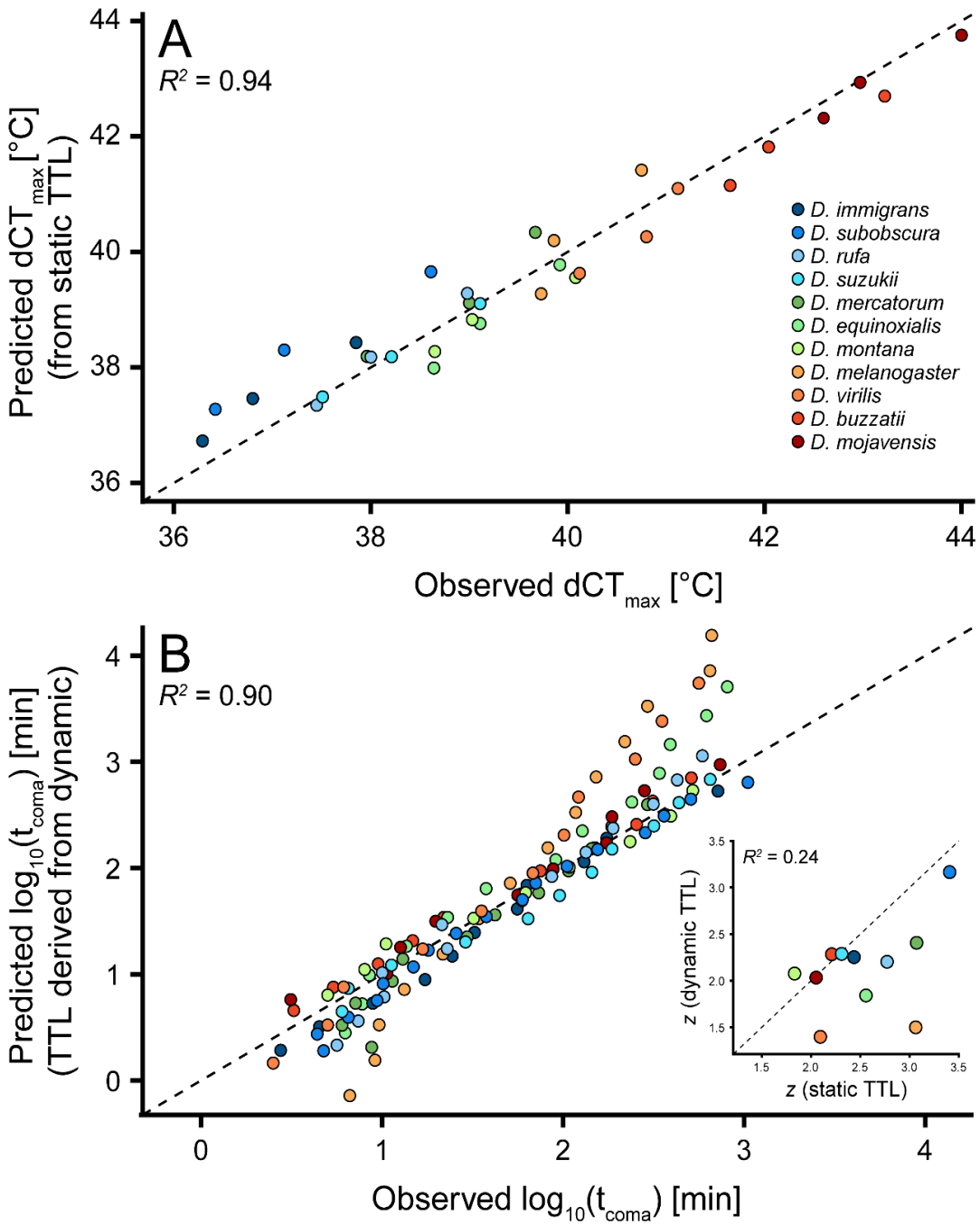
There is little consensus on the optimal protocol to assess ectotherm thermal tolerance and many different types of static or dynamic tests have been used to assess heat tolerance. TTLs represent a mathematical and theoretical approach to reconcile different estimates of tolerance as the derived parameters can subsequently be used to assess heat injury accumulation at different rates (temperatures) and durations [9,11,12]. Here we provide *R*-scripts that enable such reconciliation and to demonstrate the ability of the TTL model to reconcile data from static vs. dynamic assays we used data from Jørgensen et al. [40] where heat tolerance was measured for 11 *Drosophila* species using three dynamic and 9-17 static measurements for each species. Introducing data from only static assays we derived TTL parameters and subsequently used these to predict dynamic CT_{max} that were compared to empirically observed CT_{max} for three ramp rates (Fig. 6A). In a similar analysis, TTL parameters were derived from the three dynamic (ramp) experiments to predict t_{coma} at different static temperatures which were compared to empirical measures from static assays (Fig. 6B). Both analyses found good correlation between the predicted and observed values regardless whether the TTL was parameterized from static or dynamic experiments (Fig. 6). However, predictions from TTLs based on three dynamic assays were characterised by more variation, particularly

when used to assess tolerance time at very short or long durations. Furthermore, *D. melanogaster* and *D. virilis* which had the poorest correlation between predicted and observed t_{coma} in Fig. 6B had values of *z* from the TTLs based on dynamic input data that were considerably different from values of *z* derived from TTLs based on static assays (Fig. 6B inset). In conclusion TTLs (and associated *R*-scripts) are useful for conversion between static and dynamic assessment of tolerance. The quality of model output depends on the quality and quantity of data used as model input, and in this example the poorer model was parameterized from only three dynamic assays while the stronger model was based on 9-17 static assays (see also discussion below).

Model application for comparison of published data

Thermal tolerance is important for defining the fundamental niche of animals [1,2,4] and the current anthropogenic changes in climate has reinvigorated the interest in comparative physiology and ecology of thermal limits in ectotherms. Meta-analyses of ectotherm heat tolerance data have provided important physiological, ecological and evolutionary insights [5,41,42], but such studies are often challenged with comparison of tolerance estimates obtained through very different methodologies.

Species tolerance is likely influenced by acclimation, age, sex, diet, etc. [43] and also by the endpoint used (onset of spasms, coma, death, etc. Lutterschmidt & Hutchison [27]). Nevertheless, we expected heat tolerance of a species to be somewhat constrained [42], so here we tested the model by converting literature data for nine species to a single and species-specific estimate of tolerance, $sCT_{max\ (1h)}$, the temperature that causes heat failure in 1 hour (Fig. 5). The overwhelming result of this analysis is that TTL parameters are useful to convert static and dynamic heat tolerance measures to a single metric, and accordingly, the TTL model and *R*-scripts presented here have promising applications for large-scale comparative meta-analyses of ectotherm heat tolerance where a single metric allows for qualified direct comparison of results from different publications and experimental backgrounds. While this is an intriguing and powerful application, we caution that careful consideration should be put into the limitations of this model (see discussion below).



399

400 **Fig. 6 Conversion of heat tolerance measures between static and dynamic assays in *Drosophila*.** Data from
401 Jørgensen et al. [40]. **A)** Heat tolerance (dCT_{max}, *d* for dynamic assays) plotted against predicted dCT_{max}
402 derived from species-specific TTLs created from multiple static assays. Data are presented for three
403 different ramping rates (0.05, 0.1 and 0.25°C min^{-1}). Note that this graph is adapted from Fig. 4b in
404 Jørgensen et al. [11]. **B)** TTL parameters based on dCT_{max} from three dynamic tests were used to predict t_{coma}
405 in static assays. Each point represents an observed vs. predicted value of species- and temperature-specific
406 $\log_{10}(t_{coma})$. **Inset)** Species values of the thermal sensitivity parameter *z* parameterized from TTLs based on
407 static assays (x-axis) and from TTLs based on dynamic assays (y-axis). The dashed line represents the line of
408 unity in all three panels.

Practical considerations and pitfalls for model interpretation

As shown above it is possible to convert and reconcile different types of heat tolerance measures using TTL parameters and these parameters can also be used to model heat stress under fluctuating field conditions. Modelling and discussion of TTL predictions beyond the boundaries of the input data has recently gained traction (see examples in Rezende et al. [44] and Buckley [45]) but we caution that the potent exponential nature of the TTL requires careful consideration as it is both easy and enticing to misuse this model.

Input data: The quality of the model output is dictated by the input used for parameterization. Accordingly, we recommend TTL parameterization using several (>5) static experiments that should cover the time and temperature interval of interest, e.g. temperatures resulting in t_{coma} spanning 10 min to 10 hours, thus covering both moderate and intense heat exposure. Such an experimental series can verify TTL linearity and allows modelling of temperature impacts across a broad range of temperatures and stress durations [9,11,21]. It is tempting to use brief static experiments (high temperatures) for TTL parameterization, but in such cases, we recommend that the resulting TTL is only used to describe heat injury accumulation under severe heat stress intensities. Thus, the thermal sensitivity factor *z* represents a very powerful exponential factor (equivalent to Q_{10} = 100 to 100,000; Jørgensen et al. [11]) which should ideally be parametrized over a broad temperature range (see below). We also include a script that allows TTL parametrization from multiple ramping experiments and again we recommend a broad span of ramping rates to cover the time/temperature interval of interest. A drawback of ramping experiments is the relatively large proportion of time spent at benign temperatures where there is no appreciable heat injury accumulation. Thus, dynamic experiments can conveniently use starting temperatures that are close to the temperature where injury accumulation rate surpasses injury repair rate (see discussion of "true" T_c below, in Supplemental Information and Overgaard et al [15] for other considerations regarding ramp experiments).

A final methodological consideration relates to body-temperature in brief static experiments where the animal will spend a considerable proportion of the experiment in a state of thermal disequilibrium (i.e. it takes time to heat the animal). To avoid this, we recommend direct measurement of body temperature (large animals) or container temperature (small animals), and advise against excessive reliance on data from test temperatures that results in coma in less than 10 min.

Extrapolation: Most studies of ectotherm heat tolerance include only a single measure of heat tolerance which is inadequate to parameterize a TTL. However, a TTL can still be generated from a single measure of tolerance (static or dynamic) if a value of *z* is assumed (see Supplemental Information). As *z* differs within species and between phylogenetic groups (Table S1, Jørgensen et al. [11], Rezende et al. [26]), choosing the appropriate value may be difficult and discrepancies between the 'true' and assumed *z*

represent a problem that should be approached with care. In Fig. 7A we illustrate this point in a constructed example where a single heat tolerance measurement is sampled from a 'true' TTL (full line; t_{coma} = 40 min at 37°C). Along with this 'true' TTL we depict the consequences for model predictions if the assumed value of *z* is misestimated by ±50 %. Extrapolation from the original data point is necessary if an estimate of the temperature that causes coma after 1 hour is desired, however due to limited extrapolation (from 40 to 60 min), estimation of $sCT_{max\ (1h)}$ values based on the 'true' and *z*±50 % are not very different (<±0.22°C in this example). Accordingly, moderate extrapolations are associated with minor latent errors and such assumptions were the basis for many data points in our comparative analysis (Fig. 5). If extensive extrapolation is used (here forty-fold from 40 min to either 1 or 1600 min, Fig. 7A), the assumed *z* results in sCT_{max} estimates varying ±2°C from the true value and even more dramatic discrepancies are seen if t_{coma} is calculated for the temperatures resulting in the 'true' sCT_{max} for 1 min or 1600 min (41 and 33°C, respectively, table in Fig. 7A). Due to the powerful exponential nature of the TTL, extrapolation to 41 or 33°C with values of *z*±50 % gives predicted t_{coma} of 1.5 s-3.42 min ('true' t_{coma}=1 min) and 8 h-44 days ('true' t_{coma}~1 day). Accordingly, excessive extrapolation of TTLs should be avoided as even moderate errors in the estimate of *z* can result in dramatic output errors if the TTL is extrapolated beyond the domain of the input data.

Breakpoints and incipient lethal temperature: TTLs are established at critically high temperatures and another cause of concern for extrapolation of TTLs relates to the boundaries of the model. Below some temperature (incipient lethal temperature *c.f.* Fry et al. [9], here termed T_c (see *Mathematical foundation*)) the processes related to acute heat injury will no longer determine the duration of survival, and graphically this is represented as a breakpoint in the TTL (analogous to an Arrhenius breakpoint)(Fig. 7B). The premise of the TTL is not valid below T_c and hence other processes will limit survival below this temperature resulting in a breakpoint. For most species T_c is unknown, and it is possible that this value (breakpoint) will also depend on other factors (acclimation, age, sex, diet, etc.)(compare *Fundulus* and *Drosophila*, Fig. 7B). Accordingly, extrapolation beyond the parameterized time-temperature domain of the TTL should be met with great caution for instance when modelling over diurnal temperature fluctuations as this likely includes temperatures below the incipient temperature.

The role of repair and acclimation. From the present study and historical data [9,10,12,21,24] it is clear that damage attained within the boundaries of the TTL is additive and that this model can be used to assess heat injury accumulation during fluctuations. Additivity is, however, only empirically validated within the boundaries of the TTL model (i.e. above T_c; Fig. 7B), and at temperatures below T_c it is likely that heat injury can be repaired (Fig. 7C). A study using split-dose heat exposures interspaced by benign temperature

exposure found that breaks (> 6 hours) between heat exposure disrupted additivity, suggesting that injury is repaired at benign temperature [46]. Injury repair rate is largely understudied but repair rate is generally increasing with temperature [47–49]. It is therefore an intriguing and promising idea to include a temperature-dependent repair function in more advanced modelling of heat injury. Until such repair processes are introduced in the model, we recommend that additivity of heat injury is evaluated critically if it involves periods at temperatures both above and below T_c (i.e. over consecutive days, see also Fry et al. [9]). An alternative, but not mutually excluding, explanation of increased heat resilience in split-dose experiments relates to the contribution of heat hardening as it is likely that the first heat exposure in a series can induce hardening responses that increase resilience (and thus change the TTL parameters) when a second heat exposure occurs. Such issues of repeated thermal stress have been discussed previously [50] but for the purpose of the present study the main conclusion is that simple TTL modelling is not applicable to fluctuations bracketing T_c unless this is empirically validated. Future studies should address this issue as inclusion of repair functions would add further promise to the use of TTL in modelling of the impacts of temperature fluctuations.

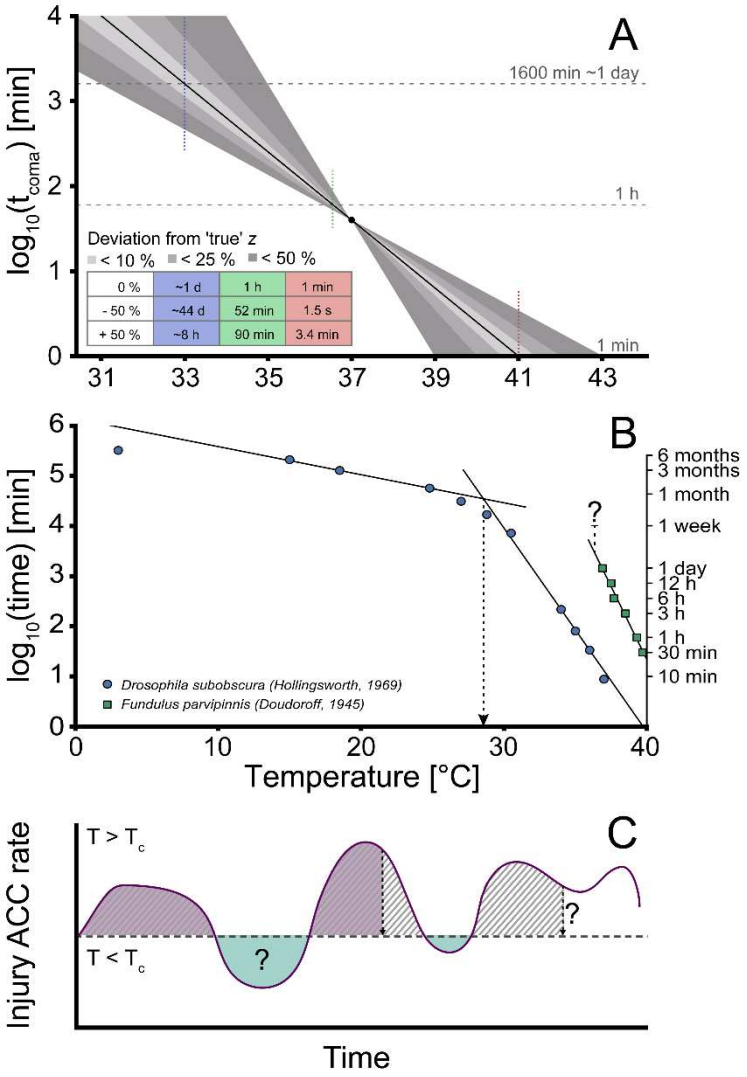


Fig. 7 Potential pitfalls of extrapolation and the ambiguity of heat damage repair and hardening. A) A theoretical TTL created from a single point (37°C, 40 min) with an assumed 'true' value of *z* (black line). Grey areas show the TTLs produced from the same point with deviations from the 'true' *z* of ±10-50%. Horizontal lines are used to compare estimates of sCT_{max} for 1 min, 1 h and 1600 min, while the vertical coloured lines are used to compare time estimates for the temperature of the sCT_{max} for the 'true' TTL (calculated times in table) **B)** The linearity of TTLs should only be assumed within the time-temperature domain where it is parameterized, and it may vary in temperature and time between species. Data and TTL estimates for *D. subobscura* from Hollingsworth [21] and *F. parvipinnis* from Doudoroff [24]. The dashed line for *Fundulus* represents the temperature where with no mortality within the tested time domain (≤1 week). The dashed arrow indicates the breakpoint temperature found by Hollingsworth. **C)** Hypothetical fluctuating temperature profile where temperature (and accordingly the injury accumulation (ACC) rate) fluctuate around the incipient lethal temperature T_c (the temperature where injury accumulation rate surpasses injury repair rate, i.e. net injury accumulation). The purple area indicates the part of the temperature profile that would attain the critical amount of injury, under the assumption that no repair or hardening (i.e. processes counteracting injury accumulation) takes place in the green shaded areas. However, when little is known about the processes counteracting injury accumulation and their relation to temperature, it is difficult to predict when coma onset occurs (hatched area).

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Author contributions

JO, HM and LBJ conceived the ideas and designed the study; HM and MØ formulated the mathematical model and prepared *R* scripts, NAK and LBJ collected the data; LBJ, JO, HM, NAK and MØ analysed the data; LBJ and JO led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Additional information

Data availability

Competing interests

The authors declare no competing interest.

Figures

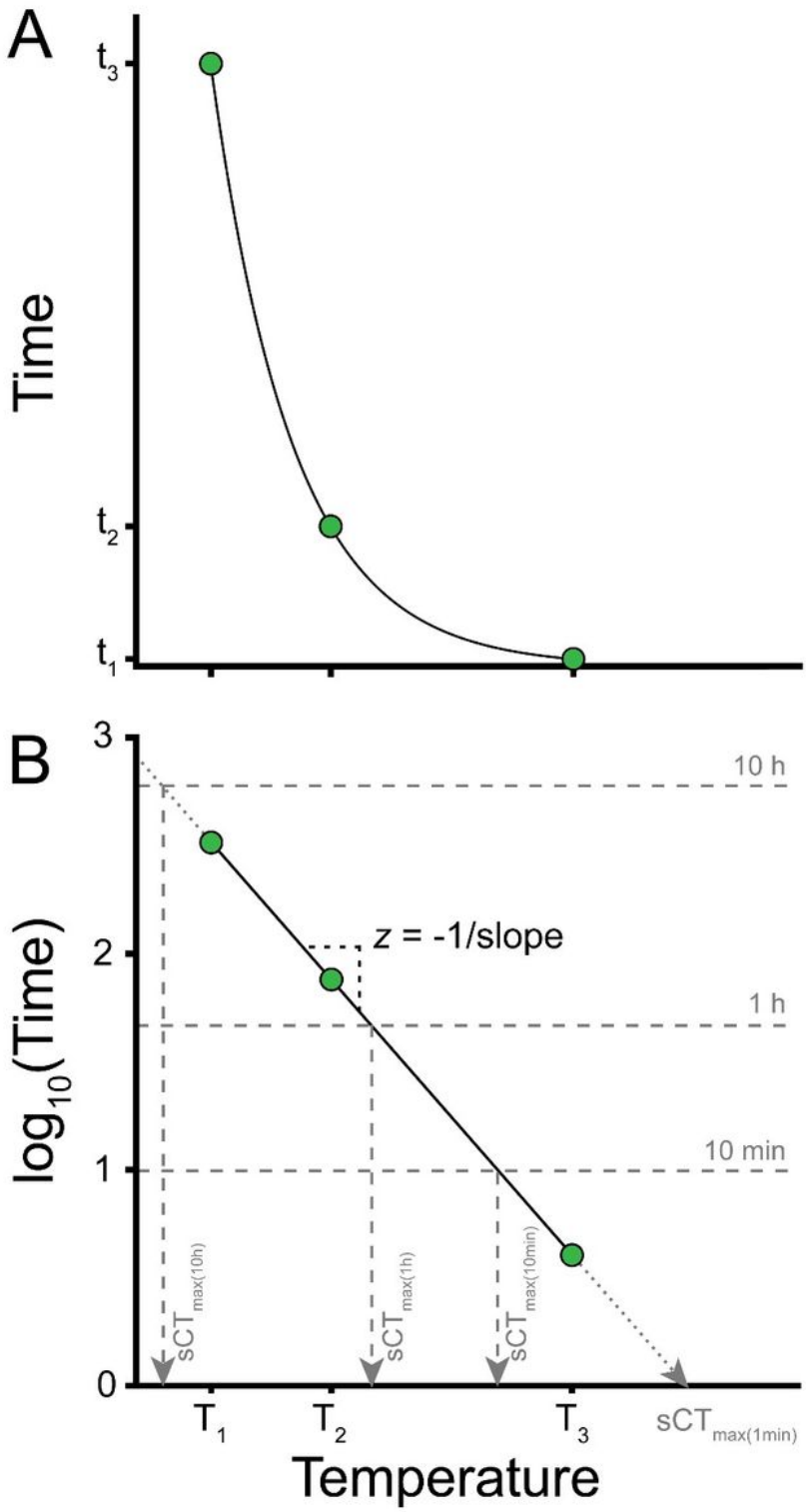

Figure 1

Exponential relation between temperature and tcoma presented as a thermal tolerance landscape (TTL). A) Time to coma (tcoma) decreases exponentially with temperature. B) Observations from (A) presented as a TTL where the linearity of log10-transformed tcoma vs. temperature indicates the exponential

relation (full line). The negative inverse slope of the TTL is used as the thermal sensitivity parameter z (z= the change in temperature required to change tcoma by an order of magnitude). Intersections between the TTL and horizontal lines show the temperature (sCTmax) at which coma is expected to occur after a fixed time (e.g. 1 h, sCTmax (1h)). Extrapolations in either end of the TTL (dotted lines) are tempting but should be met with caution as the relationship is only linear in a certain time-temperature interval (see discussion).

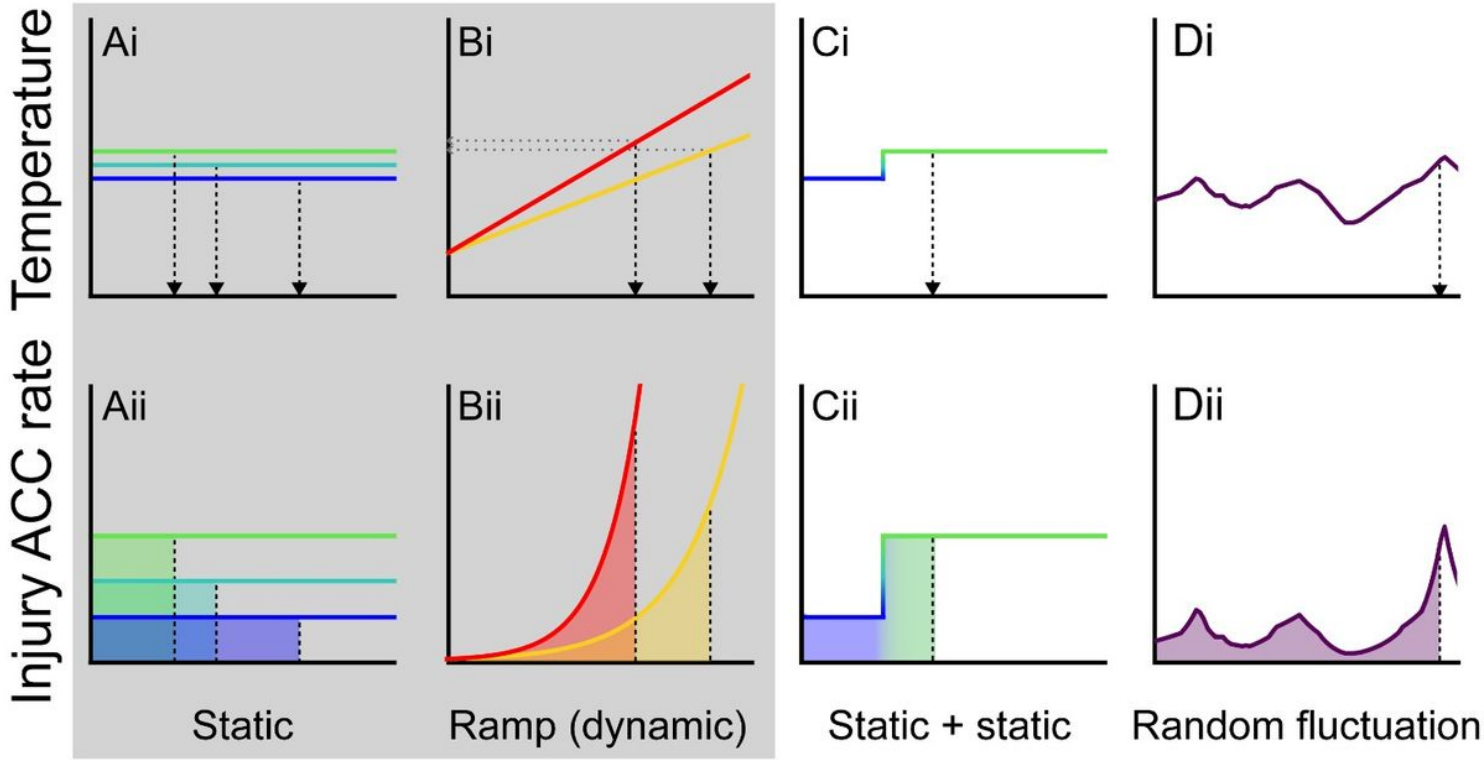

Figure 2

Temperature profiles of heat tolerance experiments and their corresponding temperature-dependent injury accumulation (ACC) rates based on parameters from the thermal tolerance landscape. x-axes represent a time scale. Shaded areas under the curves represent identical amounts of accumulated injury that result in heat failure (lethal dose, Aii-Dii). The exposure duration resulting in accumulation of the lethal dose is reported as tcoma (static) or CTmax (dynamic), marked by vertical and horizontal arrows, respectively, in Ai-Di. The grey box highlights the two classical assay types (static and dynamic). Ai-Aii) Exposure to a static temperature yields a constant temperature-dependent injury accumulation rate. Bi-Bii) Exposure to a dynamic ramp where temperature changes by a fixed rate resulting in an exponentially increasing injury accumulation rate with time. The fast ramping rate (red) results in a shorter exposure before CTmax is observed than the slow rate (yellow), but as the injury accumulation rate increases more slowly for the low ramping rate, the final CTmax is lower. Ci-Cii) During initial exposure to a static temperature (blue), some injury is accumulated, but not sufficient to trigger coma onset. Successive exposure to another static temperature (green) yields the critical amount of injury, but as some injury had already accumulated, the exposure to the second temperature is shorter than if only this temperature had been

used (compare with Aii). The prediction of the model will only hold true if injury is additive at the two static temperatures. Di-Dii) Varying (natural or laboratory generated) temperature changes that cannot be described by a simple fixed ramping rate. However, knowing the temperature-time profile permits calculations of the injury accumulation at any time and thus estimation of tcoma.

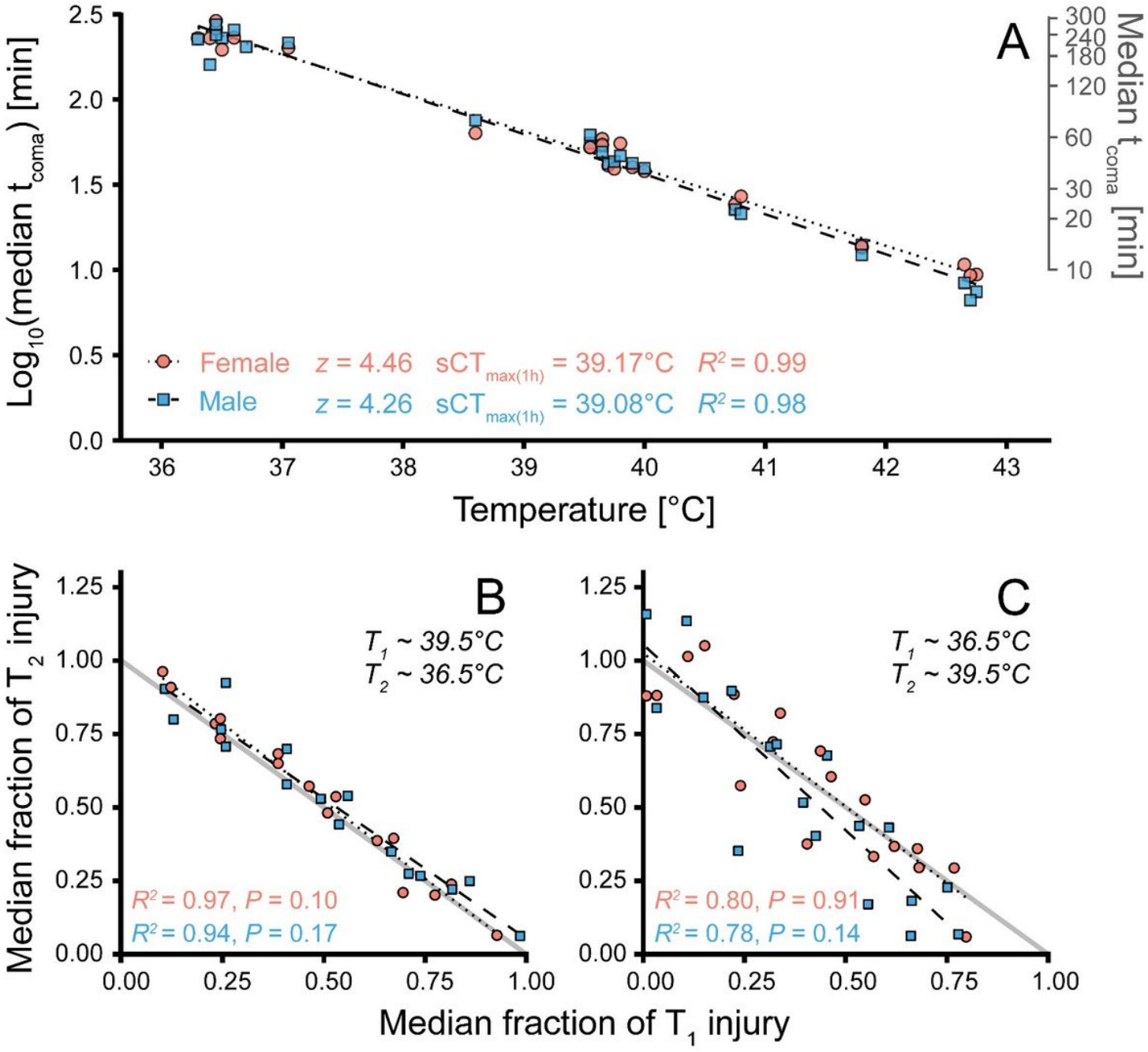

Figure 3

Thermal tolerance landscapes and test of injury additivity between two temperatures. A) Flies were exposed to a static stressful temperature until coma onset (tcoma). Each point is the median tcoma of a group (n=2-10) and TTLs are made for each sex by linear regressions of median log10(tcoma) vs. assay temperature. B) Groups of flies (n=5-6 per sex) were exposed to a high temperature (≈39.5°C) and then, after different durations, transferred to a lower temperature (≈36.5°C) where median tcoma was recorded.

The exposures are shown as fractions of injury resulting in coma at T1 (x-axis) and T2 (y-axis), where small and large fractions indicate brief and long exposurs, respectively (i.e. brief exposure results in small accumulation of injury relative to the critical amount of injury). If injury acquired at two different temperatures is additive, the two fractions should sum to 1, i.e. follow the (full) line of additivity. C) A similar experiment as (B), but with initial exposure to 36.5°C and the subsequent exposure to 39.5°C.

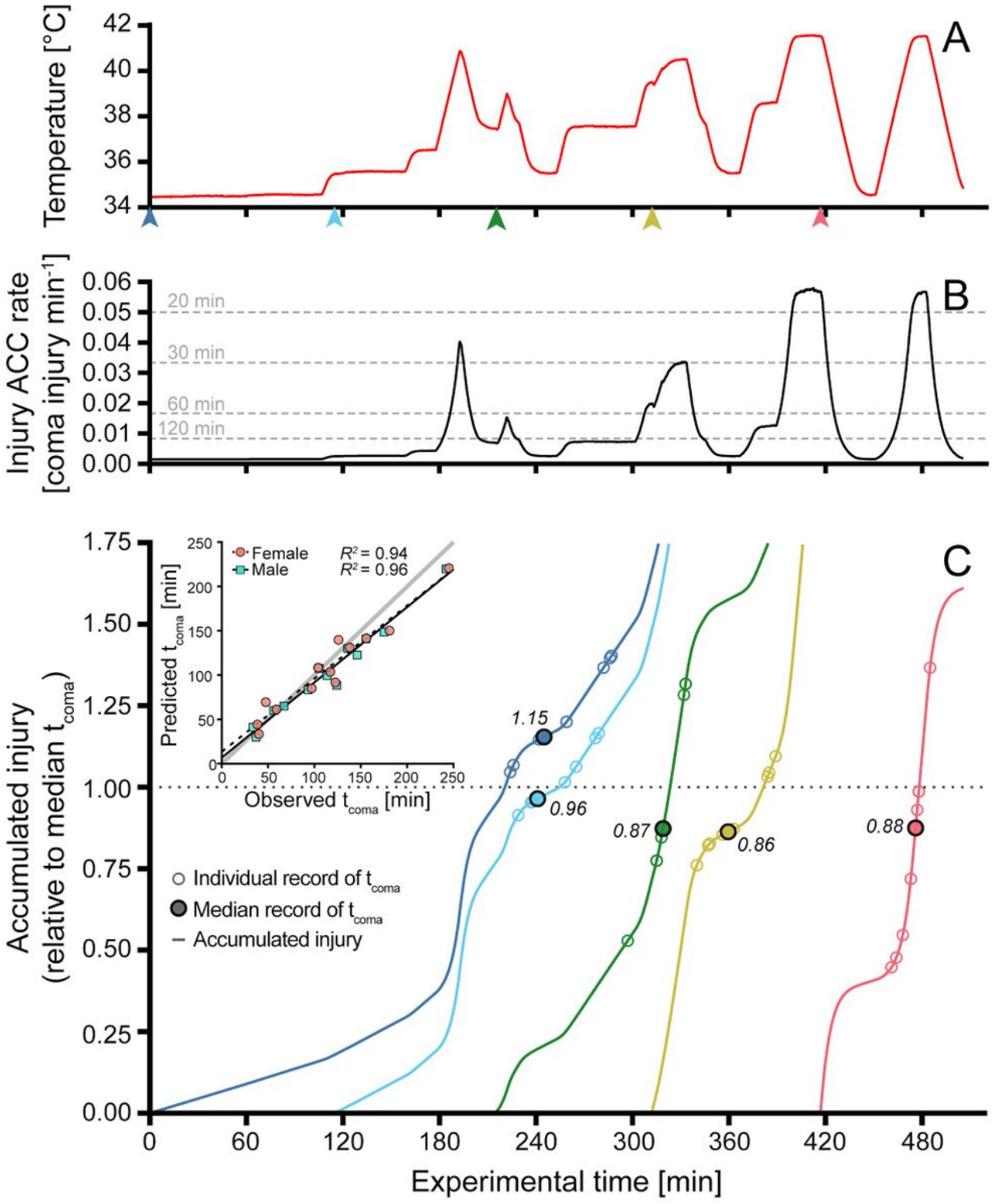

Figure 4

Test of injury additivity during random temperature fluctuations. A) Fluctuating temperature profile from one of the experiments. B) The corresponding temperature specific injury accumulation (ACC) rates for each 10-second interval calculated from the TTL (Fig. 3A). Rates are presented as a fraction of coma injury min-1 (i.e. 0.01 corresponds to an injury rate that would cause coma in 100 min), and dashed lines show the injury accumulation rate that, if held constant, would result in coma after 20, 30, 60 and 120 min. C) Groups of flies (n=8-9) were introduced at different times during the assay (arrowheads in (A)), and thus each group experienced a unique sequence of fluctuating temperatures. The temperature-specific injury accumulation rates were used to predict the gradual injury accumulation (coloured lines), and accordingly, when each experimental group should have accumulated the critical amount of injury according to the TTL (fraction = 1, dotted line). Numbers accompanying median points are the calculated amount of injury accumulated at that specific time for this group. Inset) Observed vs predicted tcoma from all experiments (grey line of unity).

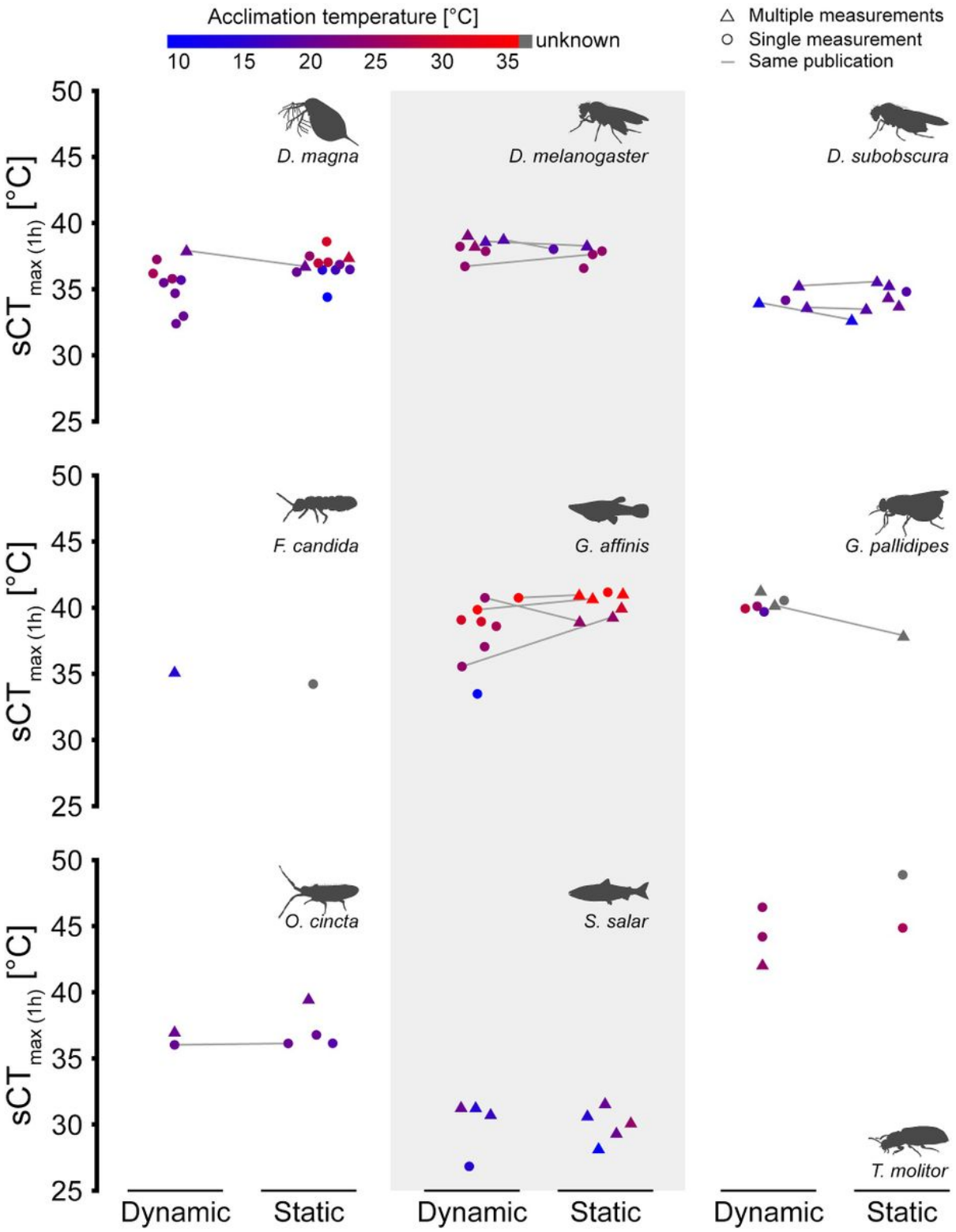

Figure 5

Heat tolerance measures from the literature re-calculated to the temperature resulting in heat failure after a 1-hour exposure (sCTmax (1h)). Heat tolerance measurements from both dynamic and static assays were obtained from the literature for nine ectothermic species and used as input for the associated R-scripts. If multiple measures of the same assay type were available in a publication (triangles), these measurements were used for TTL parameterization and then calculate sCTmax (1h). If only a single

measurement was available (circle) an estimated value of z was supplied to create a TTL for calculation of sCTmax (1h) (see main text). Connected points represent publications that provided both dynamic and static measures and colour indicates acclimation temperature.

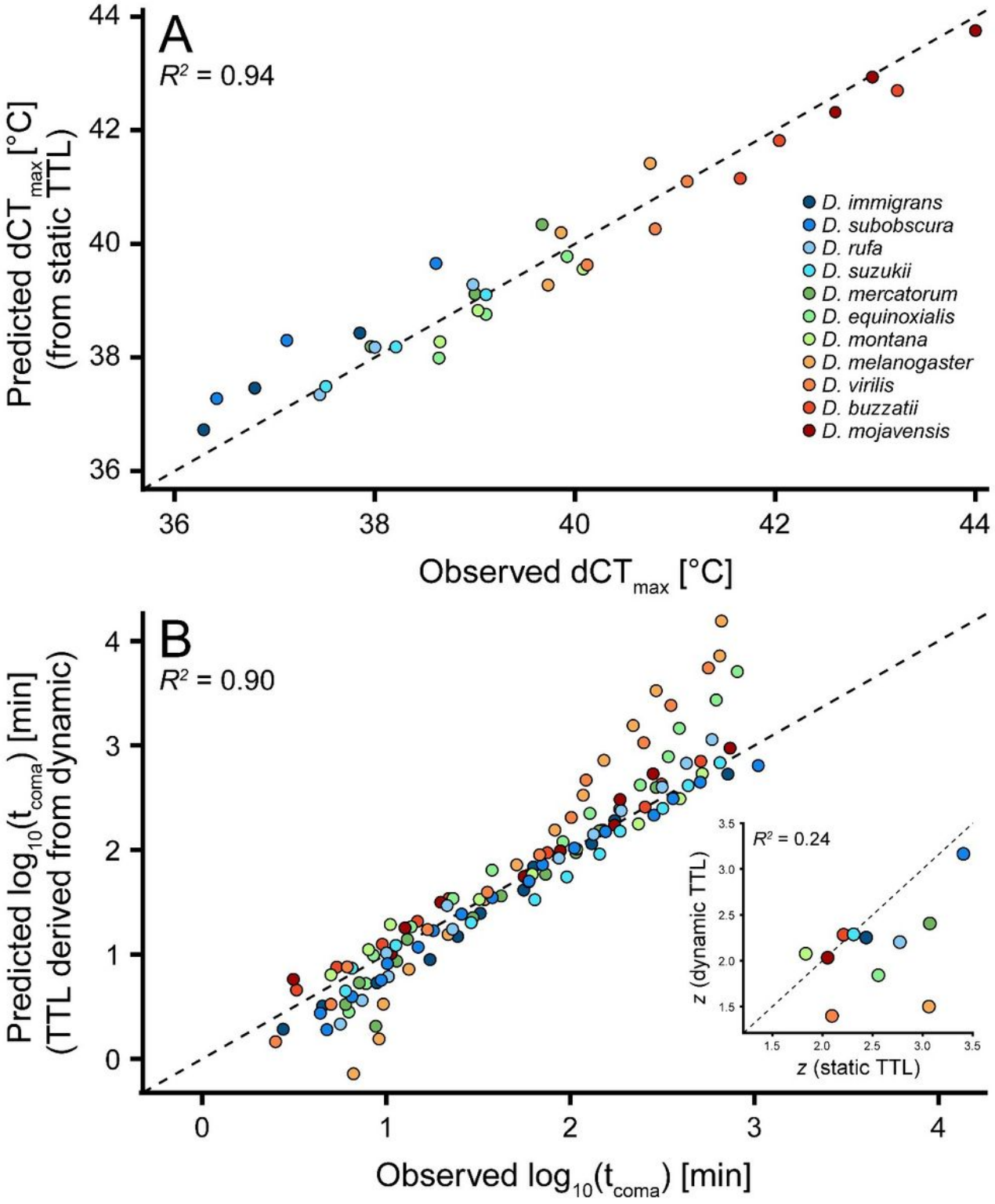

Figure 6

Conversion of heat tolerance measures between static and dynamic assays in Drosophila. Data from Jørgensen et al. 40. A) Heat tolerance (dCTmax, d for dynamic assays) plotted against predicted dCTmax

derived from species-specific TTLs created from multiple static assays. Data are presented for three different ramping rates (0.05, 0.1 and 0.25°C min-1). Note that this graph is adapted from Fig. 4b in Jørgensen et al. 11. B) TTL parameters based on dCTmax from three dynamic tests were used to predict tcoma in static assays. Each point represents an observed vs. predicted value of species- and temperature-specific log10(tcoma). Inset) Species values of the thermal sensitivity parameter z parameterized from TTLs based on static assays (x-axis) and from TTLs based on dynamic assays (y-axis). The dashed line represents the line of unity in all three panels.

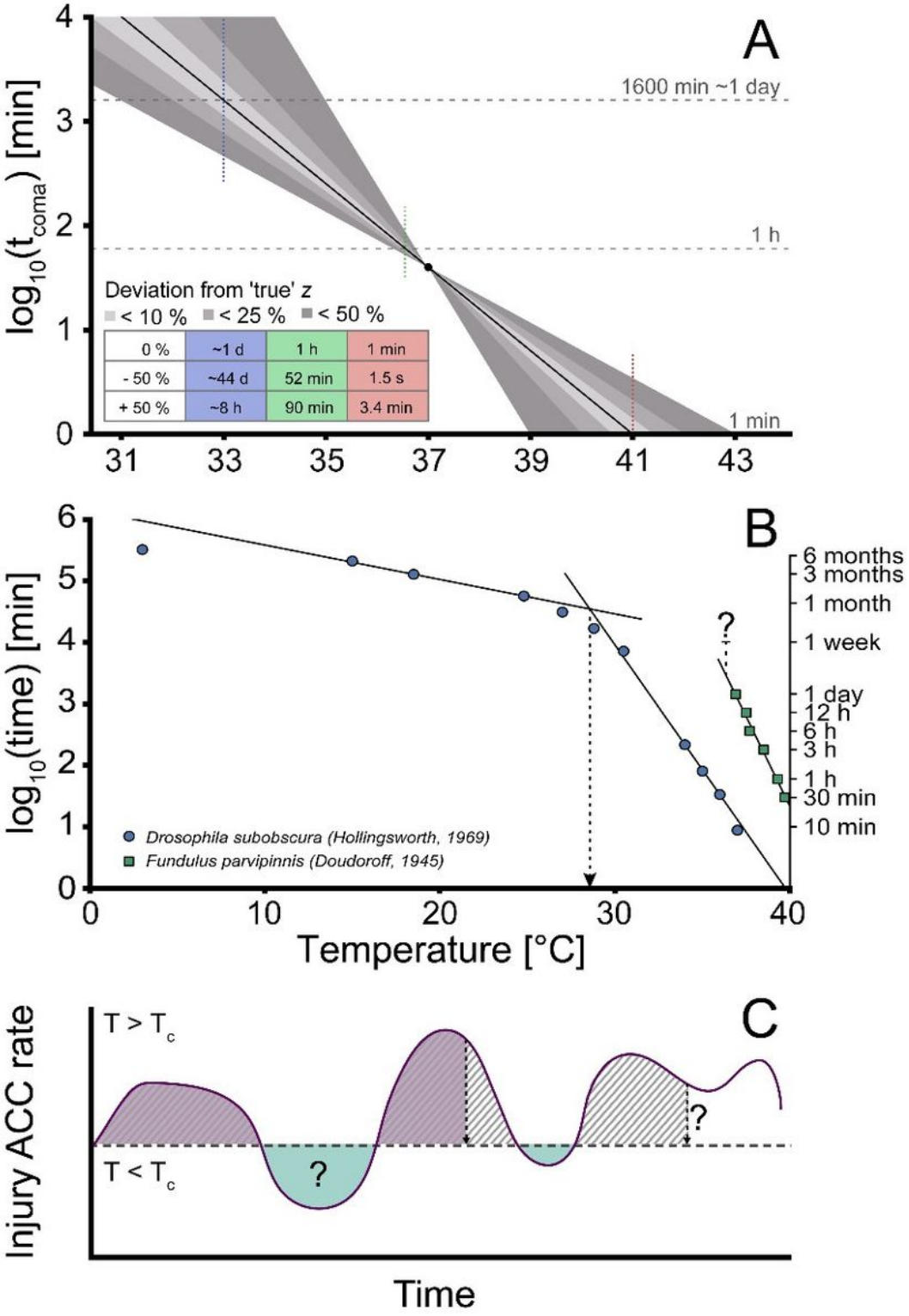

Figure 7

Potential pitfalls of extrapolation and the ambiguity of heat damage repair and hardening. A) A theoretical TTL created from a single point (37°C, 40 min) with an assumed 'true' value of z (black line). Grey areas show the TTLs produced from the same point with deviations from the 'true' z of ±10-50%. Horizontal lines are used to compare estimates of sCTmax for 1 min, 1 h and 1600 min, while the vertical coloured lines are used to compare time estimates for the temperature of the sCTmax for the 'true' TTL (calculated times in table) B) The linearity of TTLs should only be assumed within the time-temperature domain where it is parameterized, and it may vary in temperature and time between species. Data and TTL estimates for D. subobscura from Hollingsworth 21 and F. parvipinnis from Doudoroff 24. The dashed line for Fundulus represents the temperature where with no mortality within the tested time domain (≤1 week). The dashed arrow indicates the breakpoint temperature found by Hollingsworth. C) Hypothetical fluctuating temperature profile where temperature (and accordingly the injury accumulation (ACC) rate) fluctuate around the incipient lethal temperature Tc (the temperature where injury accumulation rate surpasses injury repair rate, i.e. net injury accumulation). The purple area indicates the part of the temperature profile that would attain the critical amount of injury, under the assumption that no repair or hardening (i.e. processes counteracting injury accumulation) takes place in the green shaded areas. However, when little is known about the processes counteracting injury accumulation and their relation to temperature, it is difficult to predict when coma onset occurs (hatched area).

Supplementary Files

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