## Applying Multivariate Modelling Approaches

## Box 1 – Comparing the utility of TDT Curves to other measures of heat tolerance within a comparative framework

Parameters from Thermal Death Time (TDT) curves provide a powerful tool for understanding the thermal sensitivity of organisms. But there are drawbacks to estimating TDT curves in comparison to other lethal (e.g. ) and sublethal (CFL) measures. TDT curves are logistically challenging requiring more individuals for reliable estimates than other measures like and CFL’s. TDT curves (and ) also cannot be used for all organisms due to ethical and conservation considerations. Ethical issues could be alleviated by the use of sublethal measures of thermal tolerance like CFL’s1, but we do not know how well lethal (TDT curves/) and sublethal (CFL’s) measures capture thermal sensitivities/vulnerabilities. Identifying whether a correlation exists between lethal and sub-lethal limits will help us understand the mechanisms of vulnerability to heat stress that might lead to population extinction well before mortality is identified. Because of these limitations data on thermal sensitivity/heat vulnerability is missing for many species, creating significant knowledge gaps across the tree of life.

Phylogenetic comparative approaches could be leveraged to help fill these knowledge gaps because closely related species are often more likely to share similar traits owing to their shared evolutionary history (phylogenetic niche conservatism)2. If heat tolerance is linked to the phylogeny, shared characteristics that make species more or less heat tolerant will not be randomly distributed across the phylogeny but biased by species groups3. We can use evolutionary relatedness to predict species thermal sensitivities and such insights can be used to inform policy and conservation related decisions making for species with little to no data.

#### Application of Multivariate Phylogenetic Comparative Approaches using Thermal Tolerance Measures: A case study using *Drosophilids*

Here we use powerful multivariate hierachical models (i.e., multi-response models) that are capable of dealing with missing data, and provide a means by which multiple physiological thermal tolerance measures can be modelled simultaneously4,5. When used in conjunction with an understanding of the phylogenetic relationships among species and/or populations, such modelling approaches can provide informed estimates for species missing data and explicitly estimate the covariance between tolerance measures while accounting for their uncertainty.

We integrated static lethal limits () collected by Heerwaarden & Sgrò6 and Kellerman *et al*7 with dynamic lethal limit measures from TDT curces [i.e., slope () and intercept ()] estimated by Jorgensen *et al*8 and Rezende *et al*9. We also collected data to estimate TDT curve parameters for eight additonal species following protocols outlined in Jorgensen *et al*10 and Rezende *et al*9 (See *Supplementary Materials*). Critical fertility limits for *Drosophilids* were compiled from11 and6. These data were integrated with data on lethal limits at the species-level. Overall, we obtained data for 117 species ( = 117 species; = 51 species and = 51 species). When the same species were sampled multiple times we retained within species variation in models. We then fit the following model in R (vers. 4.2.3)12 with the package *MCMCglmm* (vers. 2.34), which uses data augmentation during model fittng to estimate missing data13:

where is the overall mean (intercept) for each trait for each observation, *i*, for species, *k*; and are phylogenetic (evolutionary) and species effects (i.e., ecological species effects) (assumed to be sampled from a normal distribution) with the evolutionary relatedness being structured according to the phylogenetic relatedness matrix () as opposed to an identity matrix (). Heat tolerance measures are modelled according to a multivariate normal distribution. Covariances between thermal tolerance measures at each random effect level are also estimated.

##### Evolutionary signatures of thermal tolerance in Drosophilids

Models including phylogeny were better supported than models without phylogeny ( = 17.94), with all traits exhibiting a moderate to strong phylogenetic signal indicating evolutionary conservatism in heat tolerance (Phylogenetic : – 0.25, 95% CI: 0.08 to 0.49; – 0.51, 95% CI: 0.23 to 0.76; CFLs – 0.53, 95% CI: 0.12 to 0.76).

##### Evolutionary relationships can help understand thermal tolerance in species lacking data

The model predicted known data reasonably well for most traits (correlation between known values and values estimated from the model: – 0.94, 95% CI: 0.86 to 0.98, p < 0.001, [Figure 1](#fig-treesub); – 0.69, 95% CI: 0.58 to 0.78, p < 0.001; CFLs – 0.91, 95% CI: 0.85 to 0.95, p < 0.001), suggesting that even this basic model could be used to provide sensible quantitative measures for *Drosophila* species without heat tolerance estimates. While modelling approaches such as these are not a replacement for actual empirical data, they could be used to provide informed judgements on thermal tolerance in the absence of such information, but should be tested (using cross-validation) and refined when necessary.

##### Static lethal limits are evolutionarily correlated with sub-lethal limits in Drosophilids

Interestingly, there was a reasonably strong phylogenetic correlation between and critical fertility limits in *Drosophilids* (r = 0.75, 95% CI: 0.04 to 0.94, [Figure 1](#fig-treesub)), but not between , CFLs and the slope from TDT curves (cor(, ) = -0.07, 95% CI: -0.7 to 0.57; cor(CFLs, ) = -0.06, 95% CI: -0.71 to 0.58). These results suggest that sub-lethal measures, such as CFLs strongly coincide with a species’ upper lethal limit. It would be interesting to understand if this is a general phenomena across more taxonomic groups.

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| Figure 1- Species included in the dataset and their phylogenetic relationships. |

## Supplementary Methods and Results – Box 1

### Quantifying Thermal Death Time Curves in eight new species of *Drosophila*

#### Methods

Thermal death time curves were estimated in eight species of Drosophila: *Drosophila bipectinata*, *Drosophila birchii*, *Drosophila hydei*, *Drosophila kikkawaii*, *Drosophila pseudoananassae*, *Drosophila simulans* and *Drosophila sulfurigaster*. All species were collected from the field between 2021 and 2022 by sweep netting over rotten bananas. Flies were taken back to the laboratory with isofemale lines established from field inseminated females. Mass bred populations were established by combining 20 isofemale lines together, with mass bred population sizes maintained at 20°C 12/12hr light at an approximate population size of 750 individuals. Prior to initiation of the experiment flies were placed onto laying pots and 30-40 eggs were collected into each 10-15 replicate vials to control for density. On emergence flies were maintained for 4-5 days prior to separating the sexes via CO2 anesthesia. Flies were given two days recovery prior to estimation of the thermal death time curves, with TDT curves estimated on on female flies.

For each species thermal death time curves were estimated at five temperatures (35, 36, 37.5, 39 and 40°C), with 11-20 replicates per temperature. Flies were placed into a sealed 5mL glass vial and placed into a water set to one of the above temperatures. Flies were then monitored continuously and the time at which they knocked down from heat stress and ceased all movement was recorded. For two species *D. simulans* and *D. hydei* we were not able to get all the knockdown time data at 35 °C due to the length of time of the experiment. When the experiment was terminated around 50% of *D. simulans* had knockdown while not a single individual of *D. hydei* had knockdown (although flies were beginning to show signs of heat stress). After considering the data with and without this temperature (individuals not knockdown were scored as the time at which the experiment was terminated) we decided to include it. By including the data we are likely biasing towards a shallower slope but excluding the data we believe would create a stronger bias in the opposite direction.

#### Results

We analysed the thermal death time curves for 8 new species of *Drosophila* using the same methods as8, using linear models with log 10 coma time as the response and assay temperature as the predictor ([Figure 2](#fig-tdt)). We then used the model to estimate the slope and intercept () for each species (Table 1).

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| Figure 2- TDT curves for each species. The x-axis is temperature and the y-axis is the log10 of the time to coma. |

**Table** **1:** Estimated parameters for each of the 8 new species.

| **Species** | **Slope** | **CTMax** |
| --- | --- | --- |
| bip | 2.5 | 41 |
| bir | 3.1 | 42 |
| hydei | 2.9 | 42 |
| kik | 3.3 | 42 |
| ps | 2.6 | 41 |
| rub | 2.0 | 39 |
| sim | 2.5 | 41 |
| sulf | 2.0 | 39 |

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