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Abstract

The thermal-death-time (TDT) model has proven highly consistent in describing upper thermal limits in ectotherms through space and time. TDT model parameterization could thus yield new insights into the associations between heat tolerance, acclimation strategies, and species distribution in a range of animal models, including ants. In this study, we first demonstrate that TDT parameterization represents a strong conceptual model to describe upper thermal limits in a multispecies comparison of European ant species. In addition to accurately predicting heat tolerance from dynamic and static assays, TDT models further provide species-specific coefficients of thermal sensitivity (z) that are largely uncorrelated with absolute thermal limits. Second, using these validated parameters, we show that neither heat tolerance nor coefficients of heat sensitivity are responsive to adult acclimation and, using a recently released database of soil temperatures, show that soil surface temperatures are poor predictors of species' heat tolerance. These results highlight that TDT models offer strong conceptual advantages to unify heat-tolerance metrics resulting from various methodologies, but also that most of the interspecific information on heat tolerance is already captured in the simpler, more commonly used dynamic assays. In addition, the lack of clear association between thermal limits, thermal sensitivity, and ground temperatures lends further support to the suggestion that the evolution of heat tolerance in ants is driven by temperature variations at the microclimatic scale, behavior, and phylogenetic history.

Key words: Hymenoptera, Formicidae, heat tolerance, CTmax, acclimation, biogeography, phylogeny, thermal sensitivity.

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Introduction

Temperature is a key factor governing insect physiology (CHOWN & NICOLSON 2004, GONZÁLEZ-TOKMAN & al. 2020), and limits of thermal tolerance often correlate with the geographic distribution of insect species (MITCHELL & al. 2010, KELLERMANN & al. 2012, HALSCH & al. 2021). The association between thermal tolerance and species distribution has also been investigated in ants, which constitute one of the largest insect families in terms of species number (BOLTON 2022), abundance, and ecosystem services (DEL TORO & al. 2012). Ants are found in virtually all terrestrial ecosystems ranging from warm deserts to subarctic tundra (HÖLLDOBLER & WILSON 1990), and spurred by the projected problems associated with climate change, evolutionary and eco-physiological correlates of ant heat tolerance are now receiving increasing attention (see PEREZ & ARON 2020 and ROEDER & al. 2021 for reviews). Hymenopteran heat tolerance typically ranks high within insects; the range of recorded values for Formicidae species mostly fall within 40 - 51 °C (DIAMOND & CHICK 2018). As a comparison, the range of thermal limits for most *Drosophila* species remains within 35 - 43 °C (KELLERMANN & al. 2012). Interestingly, findings suggest that interspecific variance in ant heat tolerance is only weakly associated with environmental temperatures and distribution (DIAMOND & CHICK 2018, NOWROUZI & al. 2018, BUJAN & al. 2020a, 2022) and appears driven more by habitat parameters at the microclimatic scale. For example, species occupying habitats exposed to large variations in environmental temperatures, such as canopies or open areas, often show increased heat tolerance (WIESCHER & al. 2012, KASPARI & al. 2014, BOULAY & al. 2017, BAUDIER & al. 2018, BUJAN & al. 2020a). This trend seems to carry over at the population level as well (VILLALTA & al. 2020). The lack of a clear biogeographic pattern

in heat tolerance could also suggest that the evolution of high temperature tolerance in ants is phylogenetically constrained (Araújo & al. 2013, Hoffmann & al. 2013, Diamond & Chick 2018, Bujan & al. 2022). Furthermore, thermal acclimation may affect thermal tolerance within and among species, reducing the reproducibility of heat-tolerance assays performed in wild-caught workers. There is strong evidence, in ants, for increased tolerance associated with short term heat-hardening of workers (i.e., shorter exposure to potentially lethal temperatures; Kay & Whitford 1978, Willot & al. 2017, Perez & al. 2021), while adult acclimation (i.e., exposure of adults to warmer but non-damaging temperatures for extended periods) in laboratory conditions yielded ambiguous results (Nelson & al. 2017, Coulin & al. 2019). Finally, it is also possible that suboptimal tolerance assays have been used to assess relevant aspects of thermal tolerance, or that this lack of biogeographical association is linked to the difficulties of estimating operative temperatures experienced by small ectothermic animals (i.e., the maximal steady-state body temperature experienced under a given microclimate, after accounting for all routes of heat transfer; Sunday & al. 2014). Thus, despite the copious amount of information available, further exploration of the relationship between thermal tolerance, phylogeny, physiology, and biogeography in ants remains of continued interest. To this end, an in-depth characterization and homogenization of the metrics used to define their upper thermal limits is paramount.

The choice of adequate metrics to quantify tolerance traits underpins our ability to predict shifts in species' geographical ranges and ecosystem services (Andersen & al. 2015, Sinclair & al. 2016, Bates & Morley 2020). Historically, static (i.e., exposure to constant temperatures until knockdown) or dynamic assays (i.e., exposition to ramping temperatures until knockdown) have been used to quantify insect heat tolerance (Bak & al. 2020), resulting in a debate on the ecological relevance and the optimal conditions to conduct such assays (i.e., different starting temperatures, ramping rates, etc.) (Terblanche & al. 2007, Mitchell & Hoffmann, 2010, Santos & al. 2011). More recently, the parameterization of thermal-death-time (TDT) curves has been reintroduced as a methodological approach to alleviate these discrepancies and unify findings obtained from static and dynamic assays by treating these under a common mathematical framework (Rezende & al. 2014, Jørgensen & al. 2019, 2021). According to this model, the accumulation rate of thermal injury increases exponentially with temperature, and heat knockdown is observed once a fixed amount of injury has accumulated, regardless of whether heat stress is experienced during static or dynamic assays (Jørgensen & al. 2021). TDT parameters have thus accurately been able to predict three important metrics (Jørgensen & al. 2021): (i) static CT_{max} values at any given time (sCT_{max}, the constant temperature causing 50% knockdown in individuals), (ii) dynamic CT_{max} values at any ramping rate (dCT_{max}, often defined as the temperature at which indi-

viduals lose control of motor functions during exposure to ramping temperatures), and (iii) coefficients of thermal sensitivity (z), which describe the relative sensitivity of species to augmentation of temperatures (i.e., z denotes the temperature change needed to alter tolerance duration by a factor 10). Therefore, deep investigation of insects' upper thermal limits can be performed through TDT parameterization, the use of which had never yet been formalized on ants.

The objectives of the present study were threefold. First, we aimed at validating the use of TDT curves as a unifying method to characterize upper thermal limits in 13 phylogenetically distinct European ant species covering a wide range of heat tolerance and latitudinal distributions (Fig. 1). Second, we compared the use of TDT parameters (sCT_{max} and coefficients of thermal sensitivity z) and dCT_{max} to examine the potential for plasticity of thermal tolerance through adult acclimation in workers. Finally, we used the recently released global map of soil temperatures (Lembrechts & al. 2022), that likely offers enhanced resolution to track environmental variables for ground-dwelling insects (Pincebourde & Salle 2020), to revisit the potential association between species' heat tolerance and generalized climatic variables or latitude.

Materials and methods

Animal model system, field sampling, and laboratory rearing

Thirteen ant species selected broadly from the phylogeny of common genera in western Europe were collected to represent a wide diversity in heat tolerance and latitudinal distribution (Fig. 1). Five colonies of *Lasius niger*, *Lasius flavus*, *Formica fusca*, *Myrmica rubra,* and *Leptothorax acervorum* were collected near Aarhus, Denmark (56° 9' 36" N, 10° 11' 60" E), which is characterized by a hemiboreal climate (Köppen classification: Dfb; Köppen 1936). Three to five colonies of all other species (*Lasius cinereus, Lasius grandis, Lasius emarginatus, Lasius myops, Aphaenogaster senilis, Aphaenogaster gibbosa, Aphaenogaster subterranea, Cataglyphis piliscapa*) were collected around Collioure, France (42° 31' 12" N, 3° 4' 48" E), which is characterized by a Mediterranean climate (Köppen classification: Csa; Köppen 1936). Identification of workers was carried out following the relevant regional identification keys and / or taxonomic revisions (Collingwood 1979, Agosti 1990, Boer 2013, Seifert 2020). Colonies were kept in 30 × 40 × 10 mm plastic boxes with Polytetrafluoro-ethylene (PTFE)-coated sides (Sigma-Aldrich Chemie, GmbH) and a thin layer of clean sand on the bottom. The colonies were provided with 16 × 150 mm plastic test tubes for nesting. Each tube had a water-filled section in the bottom separated with a moist cotton plug that allowed ants access to drinking water. Colonies were reared under a 12:12 light:dark cycle at constant 26 °C, and were provided with honey water and sliced meal-
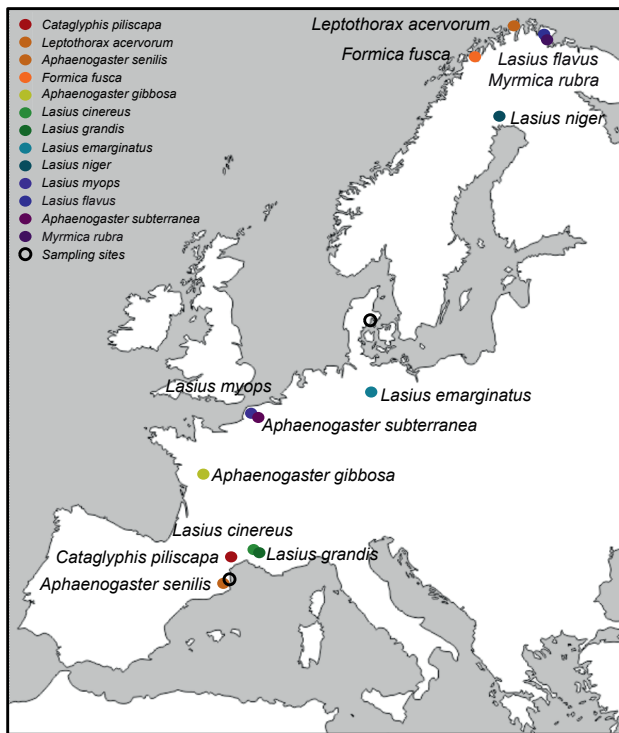
Fig. 1: Representation of northernmost records of species distribution used in our model system within continental western Europe (available from AntMaps, Janicki & al. 2016). Species were chosen to reflect a wide range of latitudinal distributions, being either Ibero-Mediterranean (*Aphaenogaster senilis, Cataglyphis piliscapa, Lasius cinereus, Lasius grandis*) or extending their range towards central (*Aphaenogaster gibbosa, Aphaenogaster subterranea, Lasius myops, Lasius emarginatus*) and northern (*Lasius niger, Lasius flavus, Formica fusca, Myrmica rubra, Leptothorax acervorum*) Europe. Species are color-coded from higher (red) to lower (dark purple) heat tolerance (Fig. 2, Tab. 1). Sampling sites are represented by black circles.

worms twice a week. All colonies were kept under these laboratory conditions for at least two months prior to experimentation.

Static heat-tolerance assays and thermal-death-time (TDT) curves

Knockdown times were assayed at six static temperatures per species chosen to induce knockdowns at regular intervals between 5 min and 480 min (Fig. 2). Experiments were conducted on workers collected foraging outside of the nesting tubes (10 workers from a single nest per species per temperature treatment, six temperature treatments per TDT curves, 60 workers from a single nest total per TDT curve) placed individually into 5 ml closed glass vials containing a droplet of 2% agar / 5% sucrose solution as a source of water and food. Glass vials were mounted to a rack and submerged into a transparent water-filled tank heated to the desired experimental temperature with a programmable water bath (LAUDA-Brinkmann, Delran, NJ, USA). The temperature was constantly monitored during experiments with a reference thermometer set up inside an empty control vial. Vial temperature typically equilibrated with the water temperature within 3 minutes after submersion. During the tolerance tests, the workers were checked continuously (short assays, < 60 minutes) or intermittently (long assays, > 60 minutes) for movements, with observation frequency increasing when muscular coordination started to decline. Recordings of knockdown in individual workers were defined as the exposure time resulting in the total absence of movement, even after external stimulation (gentle vial shaking), and for each temperature species treatment, knockdown time was recorded as the median of 10 workers (see Jørgensen & al. 2019 for discussion on median vs. average knockdown time). Six experimental temperatures were used for each species to expose all species to temperatures resulting in median heat knockdown times ranging from 5 to 500 minutes. Given the exponential relationship between temperature and knockdown time, it is possible to create species-specific TDT curves from the multiple static experiments (Rezende & al. 2014, Jørgensen & al. 2021). TDT curves are typically created from linear regression of $\log_{10}$ (knockdown time) against temperature, and this analysis effectively captures the relation between tolerance time and temperature with only two parameters: a point on the curve (e.g., temperature that causes knockdown after 1 hour) and a coefficient of thermal sensitivity (z) which is derived from the slope of this relationship (z = -1 / slope; Rezende & al. 2014).

Dynamic heat-tolerance assays

The experimental approach to record dynamic thermal knockdowns was generally the same as in the static assay except workers were exposed to gradually increasing temperatures. Briefly, workers were loaded into vials and left to settle for 15 minutes in the water bath set at 20 °C. The temperature was then gradually increased at a rate of either 0.1 or 0.25 °C / min until knockdown. Ramping rates were chosen to reflect both acute (0.25 °C / min) and more chronic (0.1 °C / min) heat stress. Dynamic CT_{max} (dCT_{max}) was recorded as the mean knockdown temperature (N = 10 workers from a single nest) for each species at each ramping rate.

Acclimation effects on heat tolerance

A subset of six species within our model system (*Aphaenogaster senilis, Aphaenogaster subterranea, Lasius niger, Lasius emarginatus, Lasius flavus, Lasius myops*) was used to investigate the impact of acclimation temperature on thermal-tolerance metrics. Approximately 200 workers from each species were transferred from standard rearing conditions (26 °C) and acclimated for 7 to 10 days at either 17 or 30 °C. Static assays, TDT curve parametrization, and dynamic assays at 0.1 °C / min were performed for each acclimation condition as described above (with the exception of mean dCT_{max} values being calculated from 6 to 10, on average 8, workers).

Tab. 1: Coefficients of determination (R²) and their linked p-values for species-specific thermal-death-time (TDT) curves (Fig. 2). The thermal sensitivity coefficient (z) defined as -1 / slope of the species-specific TDT curves and static CT_{max} ($sCT_{max\,(60min)}$) values were derived from the regressions. 95% confidence (CI) limits of z and $sCT_{maxes\,(60min)}$ values are provided.

| Species	Regression parameters			sCT_{max_60min} (°C)	
	R²	p value	z (± 95% confidence limits)	Values	95% CI
Cataglyphis piliscapa	0.987	$<1*10^{-4}$	2.543 ± 0.404	48.0	47.7 - 48.2
Leptothorax acervorum	0.994	$<1*10^{-4}$	2.265 ± 0.237	45.1	45.0 - 45.3
Aphaenogaster senilis	0.995	$<1*10^{-4}$	3.000 ± 0.296	44.5	44.3 - 44.6
Formica fusca	0.995	$<1*10^{-4}$	2.439 ± 0.238	44.0	43.9 - 44.2
Aphaenogaster gibbosa	0.996	$<1*10^{-4}$	2.567 ± 0.215	43.0	42.9 - 43.1
Lasius cinereus	0.999	$<1*10^{-4}$	2.816 ± 0.114	42.4	42.3 - 42.5
Lasius emarginatus	0.958	$7*10^{-4}$	2.570 ± 0.746	42.3	41.9 - 42.8
Lasius niger	0.994	$<1*10^{-4}$	2.545 ± 0.272	42.2	42.0 - 42.4
Lasius grandis	0.997	$<1*10^{-4}$	2.733 ± 0.198	42.1	42.0 - 42.3
Lasius myops	0.958	$7*10^{-4}$	2.996 ± 0.867	41.8	41.2 - 42.3
Lasius flavus	0.994	$<1*10^{-4}$	3.031 ± 0.322	41.7	41.5 - 41.9
Aphaenogaster subterranea	0.996	$<1*10^{-4}$	2.230 ± 0.185	41.2	41.1 - 41.3
Myrmica rubra	0.995	$<1*10^{-4}$	4.078 ± 0.390	39.0	38.7 - 39.3

Phylogenetic signal testing

To test for a phylogenetic signal, a genus-level ant phylogeny (MOREAU & BELL 2013) was used and pruned to the six genera in our model system. For *Lasius* species, first, a time-calibrated phylogeny of *Lasius* (BOUDINOT & al. 2022) was compiled to reconstruct the phylogeny of four species included in our dataset (*L. niger, L. emarginatus, L. flavus, L. myops*). Then, *Lasius cinereus* and *Lasius grandis* were added manually at their right relative position within the genus (BLATRIX & al. 2020). Similarly, within the *Aphaenogaster* genus, species were placed at their right relative position based on GÓMEZ & al. (2018). Then Pagel's λ (PAGEL 1999) was used to test for phylogenetic signal in $dCT_{max\,(0.1\,°C\,/\,min)}$, $sCT_{max\,(60\,min)}$, and z using the R packages ape (PARADIS & al. 2004) and phytools (REVELL 2011).

Biogeography

Species distribution data were extracted from the Global Ant Biodiversity (GABI) database (GUÉNARD & al. 2017), which includes occurrence records of ants from publications, museum collections, and citizen science projects. Only georeferenced records were used and were restricted from longitude 30° W to 145° E and latitude 30° N to 72° N, since the species considered in this study are endemic to the palearctic ecozone. To match each species' distribution with climatic and distribution variables, soil temperature bioclimatic variables were used from recent maps of global soil temperature at 0 - 5 cm depth (LEMBRECHTS & al. 2022), precipitation variables from the WorldClim v2.1 database (FICK & HIJMANS 2017), and limits of distribution based on data from AntMaps (JANICKI & al. 2016) (Tab. 2). All bioclimatic variables were used in a 30-arc second resolution (approx. 1 × 1 km at

the equator). These maps were projected to a Behrmann's cylindrical equal-area projection with true scale along latitude 50° N. At each occurrence record, values for all bioclimatic variables were extracted. To account for sampling bias, these values were aggregated to a 20-arc min
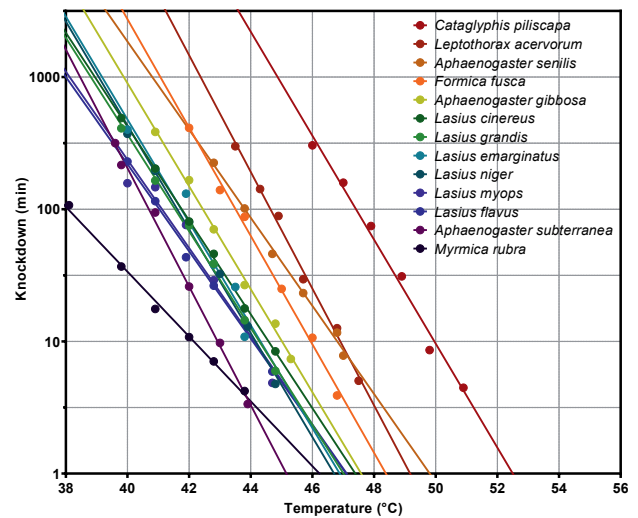
Fig. 2: Thermal-death-time curves represented by the regressions of $\log_{10}$-transformed median knockdown time against tested temperature for 13 ant species. For each species, median knockdown times were recorded experimentally at six temperatures in static assays. Linear regression lines were well fitted (R² = 0.958 to 0.999, Tab. 1), and the thermal sensitivity coefficient (z) was derived from -1 / slope of the regression. Calculated values of static CT_{max} ($sCT_{max\,(60min)}$) and the coefficient of thermal sensitivity (z) for each species are reported in Table 1. Species are color-coded from higher (red) to lower (dark purple) heat tolerance.

Tab. 2: Correlation coefficients (R) of the regressions between climatic variables averaged over species distribution, dynamic CT_{max} ($dCT_{max\ (0.1°C\ /\ min)}$), static CT_{max} ($sCT_{max\ (60min)}$), and the coefficient of thermal sensitivity (z) for the 13 species included in the dataset. Distribution data were extracted from the GABI database (Guénard & al. 2017), and matched with the global map of soil temperature at a 0 - 5 cm depth (for temperature variables, Lembrechts & al. 2022) or the WordClim database (for precipitation variables, Fick & Hijmans 2017). After correction for sampling bias, $dCT_{max\ (0.1°C\ /\ min)}$, $sCT_{max\ (60min)}$, and z were regressed against extremes of latitudinal distribution as well as average values for soil and precipitation variables for each species considering the phylogenetic non-independence of the data. Overall, climatic variables correlated moderately to poorly with heat-tolerance metrics, with absolute R values peaking at 0.534. Values are color-coded in shades of red (R) or blue (p) according to the absolute strength of the correlation and its statistical significance, respectively. No correlation reached statistical significance at the p ≤ 0.05 threshold.

	$dCT_{max\ (0.1°C/min)}$		$sCT_{max\ (60min)}$		z	
Climatic variables	**R**	***p***	**R**	***p***	**R**	***p***
Annual Precipitation	-0.534	0.060	-0.400	0.174	0.270	0.370
Max soil temperature	0.512	0.073	0.440	0.131	-0.304	0.308
Mean soil temperature diurnal range	0.497	0.084	0.383	0.194	-0.334	0.263
Precipitation seasonality	0.493	0.087	0.431	0.141	-0.314	0.292
Annual mean soil temperature	0.423	0.149	0.378	0.202	-0.212	0.483
Soil isothermality	0.334	0.264	0.204	0.501	0.228	0.451
Northernmost latitude of occurrence	-0.310	0.248	-0.264	0.379	0.164	0.591
Soil temperature seasonality	0.260	0.390	0.252	0.401	-0.054	0.842
Southernmost latitude of occurrence	-0.147	0.632	-0.221	0.467	0.151	0.618
Min soil temperature	0.124	0.123	0.109	0.711	-0.268	0.373

resolution (approx. 40 × 40 km), that is, all 1 × 1 km observations within the same cell were averaged. This yielded between seven (*Cataglyphis piliscapa*) and 347 (*Lasius niger*) values for each bioclimatic variable per species (more widespread species had more records of occurrence far apart, and thus a greater number of cells attributed). Then, a Brownian motion co-variance linear model was used accounting for phylogenetic signals using the R-package Caper (Orme & al. 2018) to regress values of $dCT_{max\ (0.1°C\ /\ min)}$, $sCT_{max\ (60\ min)}$, and z against soil and precipitation climatic variables.

Data analysis

Phylogenetic signal testing as well as regression / acclimation and biogeography analyses were performed in R version 4.1.2 (R Core Team 2017). For clarity, the detailed analysis concerning the TDT parameterization through linear regression and predictions between static and dynamic CT_{max} are provided in the corresponding results section. Graphs were drafted using Graphpad Prism version 9.3.0 (GraphPad Software, San Diego, CA, USA).

Results

Thermal-death-time model as a tool to characterize upper thermal limits in ants

We used log_{10}-transformed values of median knockdown time at six static temperatures to regress TDT curves for the 13 species included in our analysis (Fig. 2). Linear regression of transformed values reported high coefficients of determination (R^2) ranging from $R^2 = 0.958$ to 0.999

(Tab. 1). The parameters from the TDT regression allowed for a simple calculation to assess the temperature needed to cause 50% knockdown after a specific time (i.e., 10 min, 1 hour or 5 hours, etc). Here, we used the temperature causing static CT_{max} after 1 hour ($sCT_{max\ (60\ min)}$) in the further analysis. As seen from Figure 2 and Table 1, there was considerable interspecific variance in upper thermal limits of our 13 species extrapolated from regression lines, with $sCT_{max\ (60\ min)}$ values ranging from 39.0 °C (*Myrmica rubra*) to 48.0 °C (*Cataglyphis piliscapa*). The TDT analysis also provided a coefficient of thermal sensitivity z (calculated as -1 / slope of the regression line), which reports thermal sensitivity of heat knockdown as the change in temperature required for a 10-fold change in knockdown time. z values ranged from 2.23 (*Aphaenogaster subterranea*) to 4.08 (*Myrmica rubra*, Tab. 1, also see values with 95% confidence limits in Fig. S1, as digital supplementary material to this article, at the journal's web pages), with lower z values indicative of greater thermal sensitivity (i.e., a larger change in knockdown proportion with a change in exposure temperature). A weak but significant negative association between $sCT_{max\ (60\ min)}$ and thermal sensitivity (z) was present ($R^2 = 0.308$, p = 0.048, Fig. S2).

Thermal-death-time curve parameters can accurately bridge static and dynamic heat-tolerance assays

We used two R-scripts (Jørgensen & al. 2021, Ørsted 2021) to derive TDT curve parameters for each species using either six static knockdown experiments or two dynamic experiments as input data. Once the TDT parame-
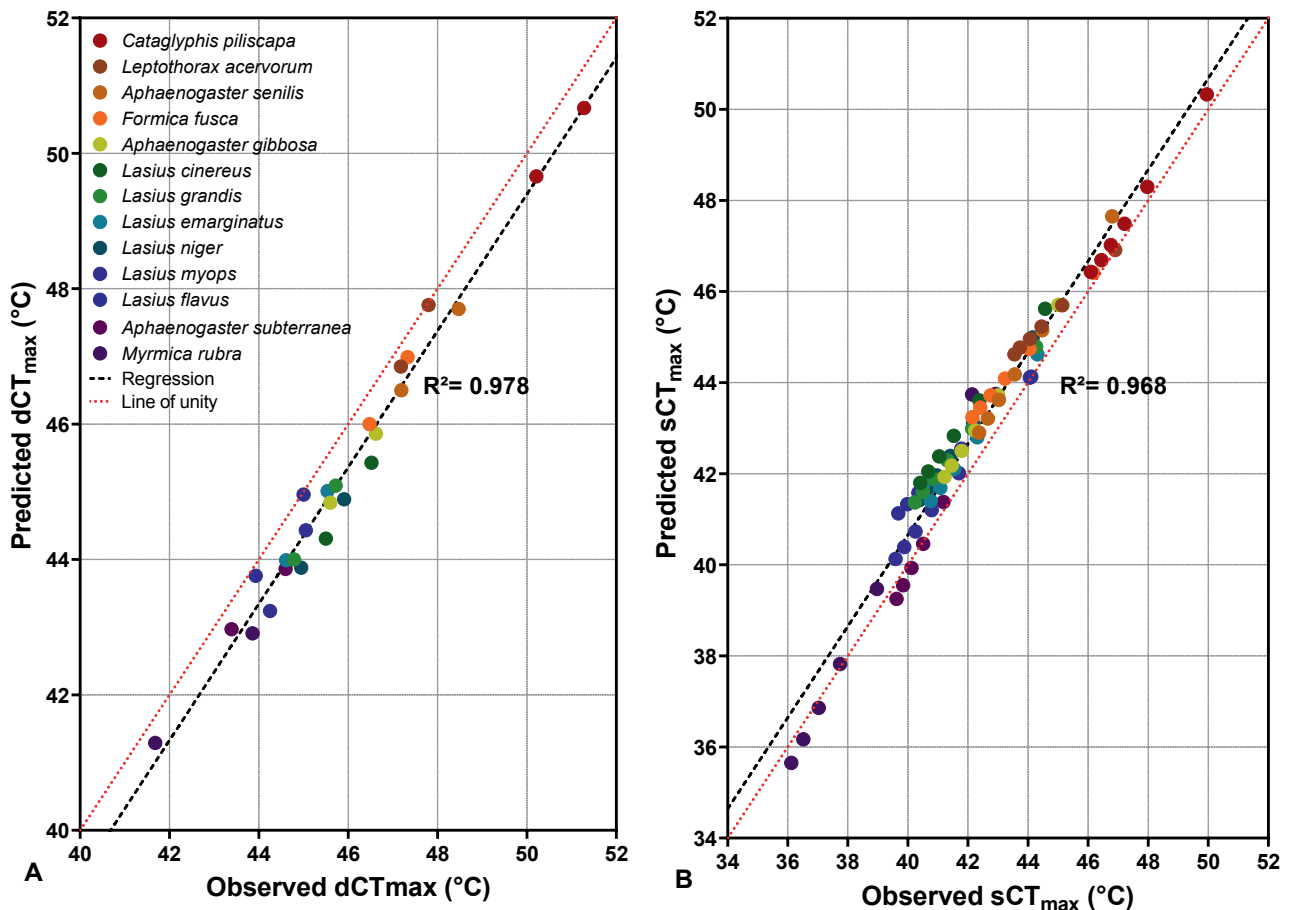
Fig. 3: Reciprocal prediction of static and dynamic metrics of heat tolerance based on thermal-death-time (TDT) parameters in 13 species of ants. A: Model predictions of dynamic CT_{max} (dCT_{max}) derived from TDT parameters obtained from static experiments, and plotted against the observed dCT_{max} values (empirically estimated at ramping rates of 0.1 and 0.25 °C / min). B: Model predictions of static CT_{max} ($sCT_{max (60min)}$) derived from TDT parameters based on dynamic experiments, and plotted against observed sCT_{max} values (estimated at six experimental temperatures per species). The red dashed line represents the line of unity. A linear regression represented a fit in both cases (black dashed lines, R^2 = 0.978 and 0.968), however model predictions underestimated dCT_{max} on average by 0.8 °C (A) and overestimated sCT_{max} on average by 0.5 °C (B, see the departure of regression lines from lines of unity). Species are color-coded from higher (red) to lower (dark purple) heat tolerance.

ters are known it is possible to calculate a predicted dCT_{max} at any given ramping rate (Tab. S1) and likewise calculate sCT_{max} at any given exposure duration. Thus, to validate this versatile use of TDT parameters and examine if the information gained from static and dynamic experiments is comparable, we contrasted the model predicted estimates of heat tolerance with empirical observation of knockdown in ramp tests. Specifically, we regress experimental dCT_{max} values against model predicted dCT_{max} derived from TDT parameters obtained from static experiments (Fig. 3A, Tab. S1). Likewise, we regress experimental sCT_{max} values against predicted sCT_{max} derived from TDT parameters obtained from dynamic experiments (Fig. 3B). In both cases, we found the predicted and observed values to be highly correlated (Fig. 3, Tab. S1). The use of TDT parameters thus allowed for accurate back and forth predictions between static (sCT_{max}) and dynamic (dCT_{max}) metrics of heat tolerance. Interestingly we found that for our comparative ant system, there was good equivalence between

the temperatures modelled to result in knockdown after 10 min ($sCT_{max(10 min)}$) and the CT_{max} recorded during ramping at a rate of 0.1 °C / min ($dCT_{max (0.1 °C / min)}$, Fig. S3, Tab. S2).

Limited plasticity for upper thermal tolerance through worker acclimation

To examine the influence of adult acclimation in CT_{max}, we used a subset of six species from our model system where workers were kept either at 26 °C (control) or acclimated for 7 to 10 days at 17 or 30 °C. We then tested the plasticity of the upper thermal limit using both TDT curves (Fig. 4) and dynamic assays (Fig. S4). First, TDT curves of all acclimation groups were characterized by high coefficients of determination (R^2 = 0.915 to 0.995 across the dataset, Tab. S3). We found no effects of acclimation on time to knockdown due to treatment in the species tested (Fig. 4, Tab. S3), with TDT curves having similar slopes and intercepts. Second, we found no evidence for an effect of
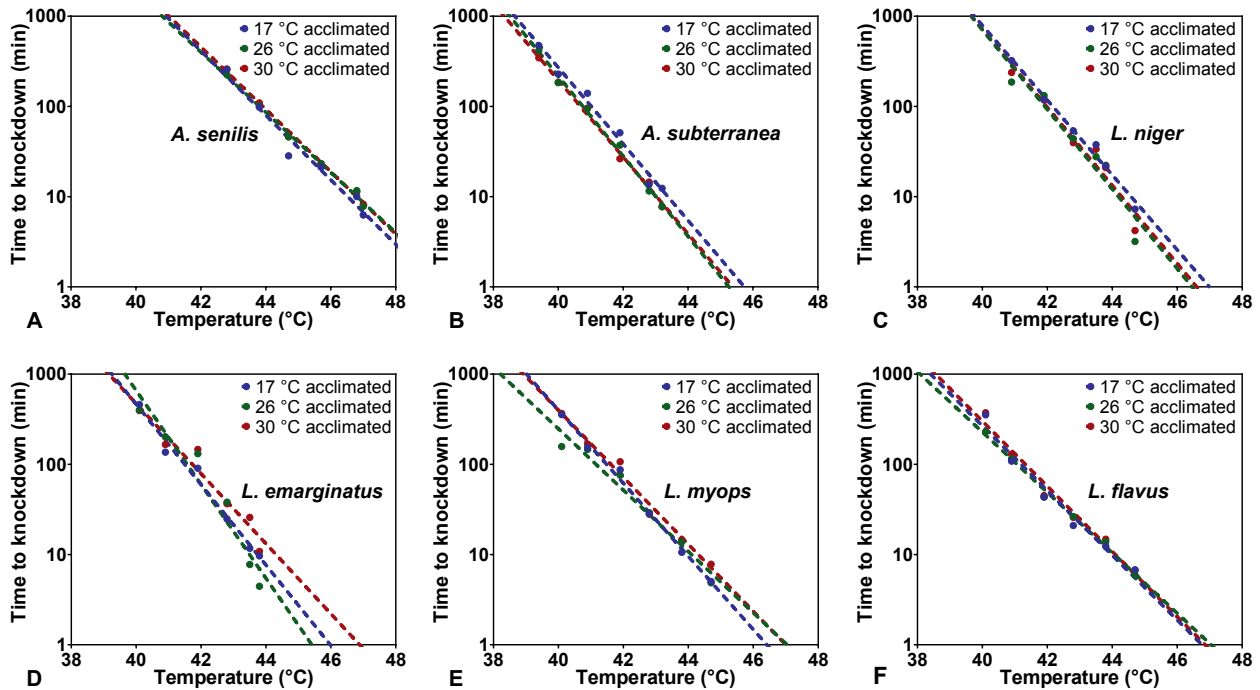
Fig. 4: Thermal-death-time (TDT) curves for $\log_{10}$-transformed median knockdown times against exposure temperature for six ant species collected from three acclimation regimes (17, 26, and 30 °C, color-coded in blue, green, and red, respectively). A*: Aphaenogaster senilis.* B: *Aphaenogaster subterranea.* C: *Lasius niger.* D: *Lasius emarginatus.* E: *Lasius myops.* F: *Lasius flavus.* For each species, median knockdown times at six temperatures were measured in static assays to derive TDT parameters. Regression lines were well fitted across the dataset (R^2 = 0.915 to 0.995, Tab. S3). We found no statistical evidence of acclimation influencing either curve intercepts or slopes, except for the intercept of *A. subterranea* being slightly higher when acclimated at 17 °C (p = 0.006, B).

acclimation at either 17 or 30 °C on dCT_{max} values (2-way analysis of variance, p = 0.11, Fig. S4, Tab. S4). Overall, we found no clear acclimation response on heat tolerance in worker ants for both TDT parameterization and ramping assays.

**Associations between heat tolerance and biogeography of ant species**

Information on the geographical distribution of the 13 ant species included in this study was extracted from the GABI database (GUÉNARD & al. 2017) and corrected for sampling bias. Putative correlations between three metrics of heat tolerance ($dCT_{max\ (0.1\ °C\ /\ min)}$, $sCT_{max\ (60min)}$, and z) were examined against northern / southern extremes of latitudinal distribution as well as several soil (for temperatures) and air (for precipitations) climatic variables averaged over species distribution (Tab. 2). We considered phylogeny since a strong phylogenetic signal in all metrics of heat tolerance tested was observed (Fig. 5): dCT_{max} (λ = 1.034, p = 0.054), sCT_{max} (λ = 1.038, p = 0.002), and z (λ = 1.037, p = 0.002). Thus, this analysis explores the correlations between estimates of heat tolerance against climatic and geographical information while correcting for the phylogenetic non-independence of the data. Overall, we found no significant association between heat tolerance and any tested variables (Tab. 2). Limits of latitudinal dis-

tribution (Fig. 1, Tab. 2) as well as soil-surface temperature and climatic precipitations were moderate to poor predictors of either $dCT_{max\ (0.1\ °C\ /\ min)}$, $sCT_{max\ (60\ min)}$, or z, with absolute coefficients of correlation (R) peaking at 0.534 (Tab. 2). The best predictors of species $dCT_{max\ (0.1\ °C\ /\ min)}$ were annual precipitation (R = -0.534) and max soil temperature of the warmest month (R = 0.512). The best predictors of species' $sCT_{max\ (60\ min)}$ were max soil temperature of the warmest month (R = 0.440) and precipitation seasonality (R = 0.431). Finally, the best predictors of z were mean soil diurnal range (R = -0.334) and seasonality of precipitation (R = -0.314), but none of these climatic variables reached the level of statistical significance (p < 0.05, Tab. 2).

Discussion

The description of heat tolerance using TDT parameters has strong conceptual advantages as it provides information across a wide range of temperatures, bridges static and dynamic assays of heat tolerance (Fig. 3), and further provides coefficients of thermal sensitivity (REZENDE & al. 2014, JØRGENSEN & al. 2019, 2021). The coefficient of thermal sensitivity describes the relative sensitivity of species to temperature augmentation. Within our model system, z values vary from 2.04 to 4.08 (Tab. 1), an interval that falls well within the range of other ectothermic
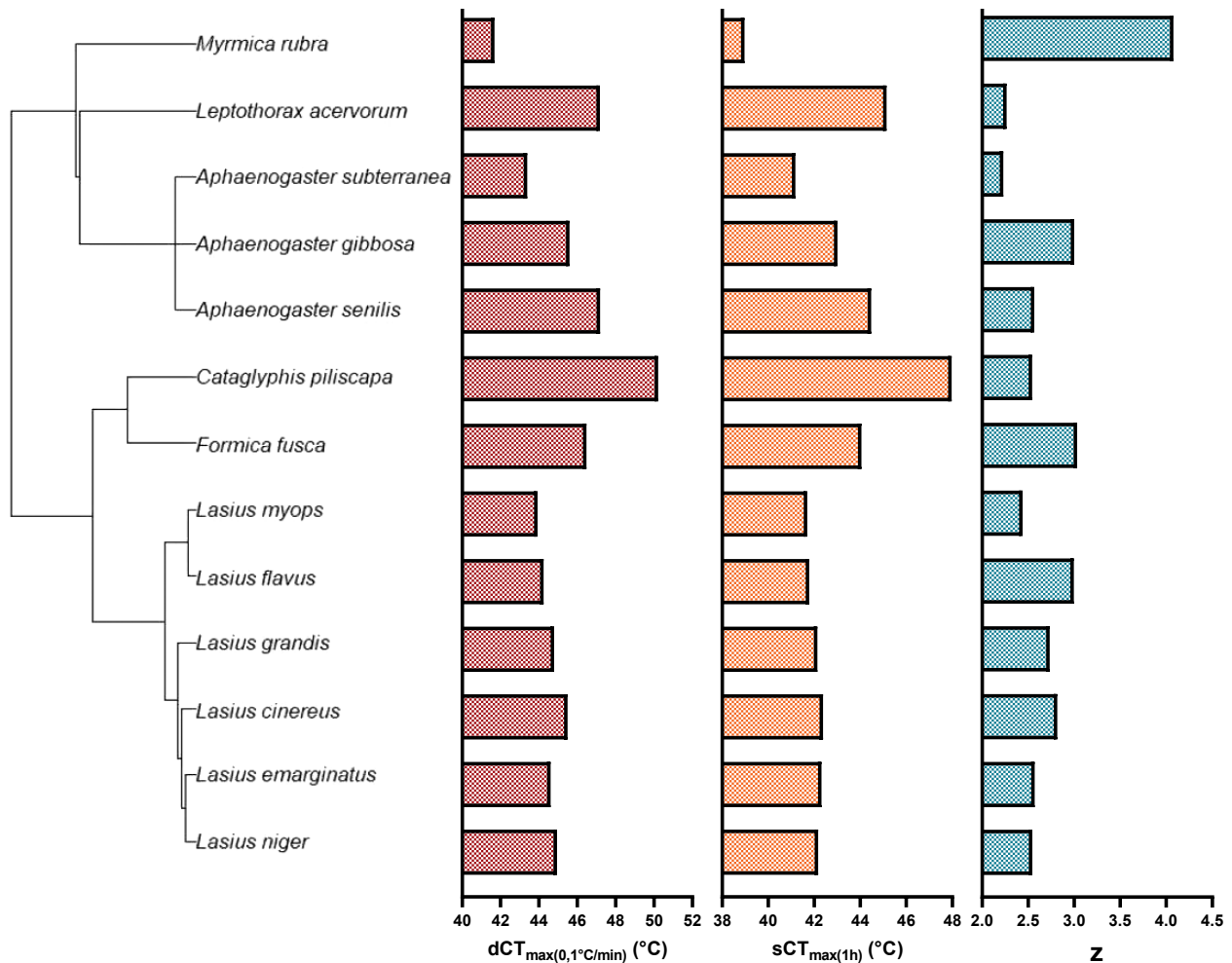
Fig. 5: Graphical representation of the values for heat-tolerance parameters ($dCT_{max\,(0.1\,°C\,/\,min)}$, $sCT_{max\,(60\,min)}$) and the coefficient of thermal sensitivity (z) mapped across the phylogeny of the 13 species of ants included in our analysis. All parameters showed a strong phylogenetic signal ($\lambda > 1$), indicating that values were markedly impacted by the evolutive history of species. Heat tolerance was consistent between dynamic ($dCT_{max\,(0.1\,°C\,/\,min)}$, red) and static ($sCT_{max\,(60min)}$, orange) metrics. The coefficient of thermal sensitivity (z, blue) showed a weak negative correlation with $sCT_{max\,(60min)}$ ($R^2 = 309$, $p = 0.048$, Fig. S2).

animals (REZENDE & al. 2014). In accordance with previous findings in *Drosophila* (JØRGENSEN & al. 2019), we only found a weak negative empirical correlation between z and absolute heat tolerance (Fig. S2). The practical implication of inter-specific variation in heat sensitivity might be best illustrated within our dataset by the TDT curve of *Aphaenogaster subterranea* and *Myrmica rubra* (Fig. 2). These species exhibit high and low extremes in z values, and for each increase of temperature unit *A. subterranea* will experience a proportionally greater decrease in knockdown time than *M. rubra* will, despite both displaying lower levels of interspecific tolerance to acute heat-stress (Fig. 2, Tab. 1). Heat tolerance (dCT_{max} and sCT_{max}) and coefficients of thermal sensitivity (z) therefore describe complementary aspects of upper thermal limits, and these parameters have been argued relevant to include when exploring the various aspects of thermal biology in insects and other ectotherms (SCHMIDT-NIELSEN 1997, JØRGENSEN & al. 2019, 2021). The methodological validation of TDT parameterization to describe upper thermal limits in

ants thus opened two interesting perspectives. First, TDT parameters enabled us to convert and predict static and dynamic metrics of heat tolerance (Fig. 3, Tab. S1). Since TDT parameters can be extrapolated from as little as two dCT_{max} at different ramping rates, or two static knockdown times (sCT_{max}) at different temperatures (although a minimum of three would be recommended), they provide a quick, accessible methodological framework to compile and compare large amounts of data, considering the diverse methodologies previously used in ants (see ROEDER & al. 2021). Second, data from this model allowed us to explore if / how heat tolerance and especially the coefficient of thermal sensitivity is associated with acclimation plasticity, phylogeny, and biogeography.

The ability to adjust thermal tolerance through acclimation has been suggested to represent an ecologically relevant and species-specific strategy to cope with fluctuations of temperatures (SGRÒ & al. 2016, KELLERMANN & VAN HEERWAARDEN 2019, TERBLANCHE & HOFFMANN 2020). In insects, the plasticity of upper-thermal limits

resulting from acclimation is typically more limited than the plasticity of lower ones (Gunderson & Stillman 2015, Sørensen & al. 2016). The effect of the different forms of acclimations (i.e., developmental or adult acclimation or heat-hardening) on heat tolerance has received relatively little attention in ants but has yielded contrasting results. Heat tolerance increased by about 2 °C maximum as a result of larval developmental acclimation to higher temperatures in *Aphaenogaster senilis* (Oms & al. 2017). Heat-hardening significantly delayed knockdown during static assays in some, but not all, *Cataglyphis* species (Willot & al. 2017, Perez & al. 2021). Finally, a shorter 3-hour adult heat-acclimation period had no effect on the heat tolerance of *Ectatomma ruidum* workers, while a longer 10-day period shifted up the thermal limits of *Wasmannia auropunctata* workers of about 2 °C maximum (Nelson & al. 2017, Coulin & al. 2019). In the present study, TDT parameterization allowed for a thorough investigation of acclimation capabilities. Horizontal displacements of TDT curves reflect general changes in heat tolerance, while changes in slopes attest to alteration of heat sensitivity (z). Overall, our experiments on six different ant species did not reveal any consistent effect of a 10-day adult acclimation period at either 17, 26, or 30 °C (Fig. 4, Tab. S3), which was further confirmed through a dynamic assay showing no difference in associated dCT_{max} (Fig. S4, Tab. S4). Our results thus contradict previous findings at similar acclimation periods. This also supports that, in ants, adult acclimation appears to have lesser effects on thermal limits than developmental acclimation and / or adult heat-hardening (Oms & al. 2017, Willot & al. 2017, Perez & al. 2021). Surprisingly, this absence of adult acclimation responses also somewhat contrasts the marked seasonality of heat tolerance found in wild-caught foragers of several species (Coulin & al. 2019, Bujan & al. 2020b). In line with the previous point, these discrepancies could indeed be linked to larger effects of developmental acclimation in field-caught animals, and / or especially a larger contribution from short-term heat-hardening, as operative temperatures experienced by foragers in temperate habitats during summer are supposedly close to their own thermal limits (Sunday & al. 2014). Given the diversity of thermal niches occupied by ants, it remains overall likely for various species to exploit different acclimation strategies (i.e., different combinations of development acclimation, adult acclimation, and heat-hardening) that contribute to seasonal changes in heat tolerance in a species-specific manner. Future studies using TDT analysis could reveal if such strategies also include changes associated with coefficients of thermal sensitivity.

Another important aspect to consider while exploring heat tolerance in insects is phylogeny (Kellermann & al. 2012). In ants, upper thermal limits exhibit a significant phylogenetic signal (Diamond & Chick 2018, Bujan & al. 2020a, 2022), which could indicate that evolution of heat tolerance is phylogenetically constrained (i.e., closely related species are more likely to display similar dCT_{max}).

Building on these premises, we confirm the presence of a strong signal on both dCT_{max} and sCT_{max} in our dataset, underpinning that such constraint consistently carries over regardless of the metric of heat tolerance recorded (Fig. 5). Likewise, we show that the coefficient of thermal sensitivity (z) also appears to display a strong phylogenetical signal (Fig. 5), indicative of more similar z values among close relatives. This highlights the need to consider phylogenetic non-independence of the data when using coefficients of thermal sensitivity within multi-species comparisons.

Finally, considering the important roles that ants play in terrestrial ecosystems, it is no surprise that the ongoing changes in global temperatures have sparked a surge of interest in the association between ant thermal biology, distribution, and overall performances (Diamond & Chick 2018, Bujan & al. 2020a, Guo & al. 2020, Roeder & al. 2021, Parr & Bishop 2022). In this regard, multi-species studies analyzing eco-physiological associations of heat tolerance and distributions have found that upper physiological limits show poor associations with distribution, environmental temperature variables, and realized thermal niches in Formicidae (Diamond & Chick 2018, Nowrouzi & al. 2018, Bujan & al. 2020a, 2022). This finding is well-aligned with a more generalized trend in insects where upper thermal limits, as opposed to lower (cold) ones, often weakly correlate with latitudinal distribution and biogeographical sourced information on climate variables (Mitchell & al. 2010, Sunday & al. 2011, Kellermann & al. 2012, Jørgensen & al. 2019, Bujan & al. 2020a). The absence of robust correlations likely stems from several factors: For example, the maximal operative temperature that insects can experience in open habitats often exceeds both the air temperature of their environment and their own thermal limits (Sunday & al. 2014, Clusella-Trullas & al. 2021). Insects must therefore intermittently rely on behavioral mitigation strategies to avoid overheating. In the case of ants, most species are able of mitigating exposure to above-optimal temperatures by retreating inside their nests and / or modifying nest architecture (Yela & al. 2020, Sankovitz & Purcell 2021). Behavioral plasticity in foraging (e.g., nocturnal foraging pattern) can also allow species to persist in warmer habitats despite comparatively lower physiological heat tolerance (Yela & al. 2020), and it appears that limits of foraging temperature can thus return as better predictors of species distribution than physiological heat tolerance (Guo & al. 2020). In line with this, the availability of moist and cool microhabitats is probably of considerable importance to protect individuals from excessive heat, and it is often found that thermal limits in smaller insects are negatively correlated with precipitation parameters (Kellermann & al. 2012, Jørgensen & al. 2019). Thus, rather than mean thermal conditions, it has been argued that upper thermal limits in ectotherms are closely related to both precipitations and variations of temperature at the microclimatic scale (Clusella-Trullas & al. 2011, Kellermann & al. 2012, Baudier & al. 2018, Villalta & al. 2020), that current

resolutions in climatic databases such as WordClim (Fick & Hijmans 2017) or SoilTemp used in the present study (Lembrechts & al. 2022) fail to capture (Potter & al. 2013). Our results are somewhat supportive of these associations: First, annual precipitations ranked as the strongest predictor of $dCT_{max\,(0.1\,°C\,/\,min)}$ and third-best predictor of $sCT_{max\,(60min)}$ (albeit both correlations were weak and did not reach high statistical significance, Tab. 2). Second, species supposedly exposed to the greatest variation in temperatures due to their nesting or foraging behavior (e.g., *Cataglyphis piliscapa*, *Leptothorax acervorum*, *Aphaenogaster senilis*) are characterized by much higher heat tolerance than species typically nesting in moister habitats or displaying subterranean foraging tendencies (*Myrmica rubra*, *Aphaenogaster subterranea*, *Lasius flavus*, *Lasius myops*). Finally, most of the literature addressing biogeographical patterns of physiological heat tolerance in ants sampled several populations of larger number of species through the use of the simpler dCT_{max} as thermal-tolerance metrics (Diamond & Chick 2018, Bujan & al. 2020a). We adopted a different approach: Our analysis was based on a more limited number of species and populations (13 species, one population per species) that allowed for in depth TDT parameterization and subsequent analysis including the coefficient of thermal sensitivity z. While this fails to account for potential intraspecific variations in thermal tolerance between populations, either due to non-plastic divergences or to developmental acclimation, our analysis yields complementary results to the current literature that remain supportive of the ongoing consensus. Taken together, they suggest that the evolution of heat tolerance in ants, including the coefficient of thermal sensitivity, is likely indeed driven by the interaction between experienced operative temperatures, behavior, and phylogeny.

In conclusion, our results first methodologically validate TDT-curve model parameterization in ants as a consistent framework capable of unifying static and dynamic assays to rank species according to their respective heat tolerance. While TDT parameterization offers clear conceptual advantages to compare metrics of heat tolerance across methodologies, we also show that most of the associate interspecific variation can already be captured through the simpler dynamic assays (ramping assay). Second, using the TDT model, we highlight an absence of plasticity in both heat tolerance and coefficient of thermal sensitivity through adult acclimation in workers. Finally, we find that both heat tolerance and the coefficient of thermal sensitivity display strong phylogenetic signals but have weak or absent associations with climatic variables characterizing species distributions. This result remains consistent, even when considering soil-surface temperatures that could be regarded as more relevant to the ecology of ground-dwelling models. Thus, we ultimately lend support to the suggestion that the evolution of heat tolerance in ants is both phylogenetically constrained and primarily driven by the interaction between microclimatic conditions and behavior.

Data availability

All data are included in the manuscript and supporting information, as digital supplementary material to this article, at the journal's web pages.

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