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A simple method to account for thermal boundary layers during the estimation of CTmax in small ectotherms

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ABSTRACT

As temperatures rise, understanding how ectotherms will become impacted by thermal stress is of critical importance. In this context, many researchers quantify critical temperatures – these are the upper (CTmax) and lower (CTmin) thermal limits at which organisms can no longer function. Most studies estimate CTs using bath-based methods where organisms are submerged within a set thermal environment. Plate-based methods (i.e. hot plates), however, offer huge opportunity for automation and are readily available in many lab settings. Plates, however, generate a unidirectional thermal boundary layer above their surface which means that the temperatures experienced by organisms of different sizes is different. This boundary layer effect can bias estimates of critical temperatures. Here, we test the hypothesis that biases in critical temperature estimation on hot plates are driven by organism height. We also quantify the composition of the boundary layer in order to correct for these biases. We assayed four differently sized species of UK ants for their CTmax in dry baths (with no boundary layer) and on hot plates (with a boundary layer). We found that hot plates overestimated the CTmax values of the different ants, and that this overestimate was larger for taller species. By statistically modelling the thickness of the thermal boundary layer, and combining with estimates of species height, we were able to correct this overestimation and eliminate methodological differences. Our study provides two main findings. First, we provide evidence that organism height is positively related to the bias present in plate-based estimates of CTmax. Second, we show that a relatively simple statistical model can correct for this bias. By using simple corrections for boundary layer effects, as we have done here, researchers could open up a new possibility space in the design and implementation of thermal tolerance assays using plates rather than restrictive dry or water baths.

1. Introduction

Global surface temperatures have risen by approximately 1.07 °C since the middle of the 20th century – largely as a result of anthropogenic activity (IPCC, 2022). This warming is having major impacts on ecosystems worldwide by causing shifts in the spatial and temporal distribution of species (Pecl et al., 2017), and through being linked to a suite of population declines and extinctions (Cahill et al., 2013; Habibullah et al., 2022). The impacts of climate warming are of particular concern for ectotherms as they largely lack the physiological mechanisms required to regulate their internal body temperatures (Harrison et al., 2012; Heinrich, 1996). Consequently, the physiological functions, activity patterns, and potential role of ectotherms in ecosystems are all strongly influenced by the temperature of their surrounding environment (Gunderson and Leal, 2016; Harrison et al., 2012). As estimates now suggest that temperatures are likely to exceed 1.5 °C above pre-industrial levels by 2030 (IPCC, 2022), it is imperative that we understand how vulnerable ectothermic species are to thermal stress (Sunday et al., 2014). Not only do ectotherms constitute over 90% of all organisms, but they underpin the structural integrity of a plethora of ecological networks and ecosystem functions (Del Toro et al., 2012; Griffiths et al., 2021; Metcalfe et al., 2014).

Within this context, physiological thermal tolerance is often studied to understand and predict the response of ectotherms to both natural and anthropogenically induced thermal gradients (Kellermann and van Heerwaarden, 2019). In particular, many studies quantify critical temperatures (CTs). These represent the highest (CTmax) or lowest (CTmin) temperatures at which a given individual, population, or species can maintain activity and physiological functioning (Terblanche et al., 2011). As measures of lethal thermal tolerance, CTs can help us to

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understand the spatial or temporal distribution of a species (Bishop et al., 2017; Boyle et al., 2021; Diamond and Chick, 2018; Sunday et al., 2014), predict potential trophic cascades (da Silva et al., 2023), and inform on where climate warming may have the most acute impacts (Deutsch et al., 2008; Diamond et al., 2012). Recent work has highlighted how an emphasis on these lethal limits, at the expense of sublethal thermal performance, may be underestimating the biotic response to climate warming and to thermal gradients in general (Braschler et al., 2020; Gunderson and Leal, 2016; Guo et al., 2020; Parratt et al., 2021). Even against this backdrop, however, CTs remain a useful, accessible, and comparable physiological metric to estimate from organisms (Bennett et al., 2018; Clusella-Trullas et al., 2021; Terblanche et al., 2011).

There is huge diversity in the methodological approaches and protocols used to estimate critical temperatures. This diversity has perhaps arisen due to the relative ease with which CTs (and related thermal tolerance measures) can be estimated for a range of organisms and experimental contexts. For example, there is ongoing debate in the literature as to the relative merits of dynamic vs static tolerance assays (Jørgensen et al., 2019; Terblanche et al., 2011), the appropriate ramping rates to use in dynamic assays (Chown et al., 2009; Kingsolver and Umbanhowar, 2018; Leong et al., 2022; Terblanche et al., 2011), and whether organisms should be acclimated in controlled conditions prior to testing (Hoffmann and Sgrò, 2018; Maclean et al., 2018). These are all important issues, and often centre around what experimental treatment is most ecologically "realistic" for organisms (Terblanche et al., 2011). Less well explored, however, is the impact that different pieces of temperature controlling equipment have on the estimation of CTs and of thermal tolerance metrics more broadly.

Today, when estimating CTs, researchers most often use a water bath or dry bath to manipulate temperature (Boyle et al., 2021; Liu et al., 2020; Roeder et al., 2021; Woon et al., 2022) – sometimes via circulating water through an exposed stage (Ritchie et al., 2021). In these experiments, individual organisms are placed within small vials which are then submerged within the heated or cooled substrate of the bath (i.e. within the water itself or the aluminium blocks of a dry bath). In a dynamic CT assay, temperature is then ramped up or down and the loss of a righting response in the target organisms is looked for to indicate that CTmin or CTmax has been reached. An alternative approach, however, is to use hot or cold plates (Bujan et al., 2022; Cerdá and Retana, 2000; DeVries et al., 2016; Porras et al., 2021). These work in the same way as baths except the target organism is placed on top of the temperature-changing plate rather than being fully submerged in a bath. Plate-based methods are not as widespread as bath-based methods (Roeder et al., 2021). We argue, however, that plates could offer advantages over baths in a world where there is an urgent need to physiologically phenotype a diversity of individuals, populations, and species at large scales (Chown, 2022). For instance, plates are more amenable to automation and adaption for high-throughput as cameras could be used to film thermal tolerance assays of many individuals at a time (Kaspari et al., 2016; Werkhoven et al., 2019). This kind of automation and high-throughput is not possible with bath-based methods. In addition, plate-based methods do not require the constant removal and replacement of individuals from the apparatus (and subsequent thermal relief, even if small, Fig. S1) in order to check whether they have reached their CTs. Despite these potential advantages, key biases presented by plate-based methods remain underexplored.

The key difference between bath and plate-based methods is that baths fully "submerge" organisms within the target temperature (Fig. 1). In bath-based methods, the desired temperature is conducting and radiating toward the organism from nearly all directions. Consequently, as long as any lag between the set temperature and that within the holding vials is accounted for, the set temperature on the equipment matches the temperature experienced by the test organism. This is not always the case with plate-based methods. Hot or cold plates generate a unidirectional thermal boundary layer from the bottom up. As a result, organisms sitting within this boundary layer may be experiencing the set temperature of the equipment, but those standing above it are likely experiencing cooler (if the plate is warmer than the ambient room temperature) or warmer (if the plate is cooler than ambient) temperatures. Some hybrid plate systems that likely reduce the boundary layer are possible, however (Leclerc et al., 2022). This boundary layer effect could lead to bias in the estimation of thermal tolerance metrics such as CTs. Indeed, this effect has been illustrated elegantly by Kaspari et al. (2015) in tropical canopy ants. In their study, Kaspari and colleagues

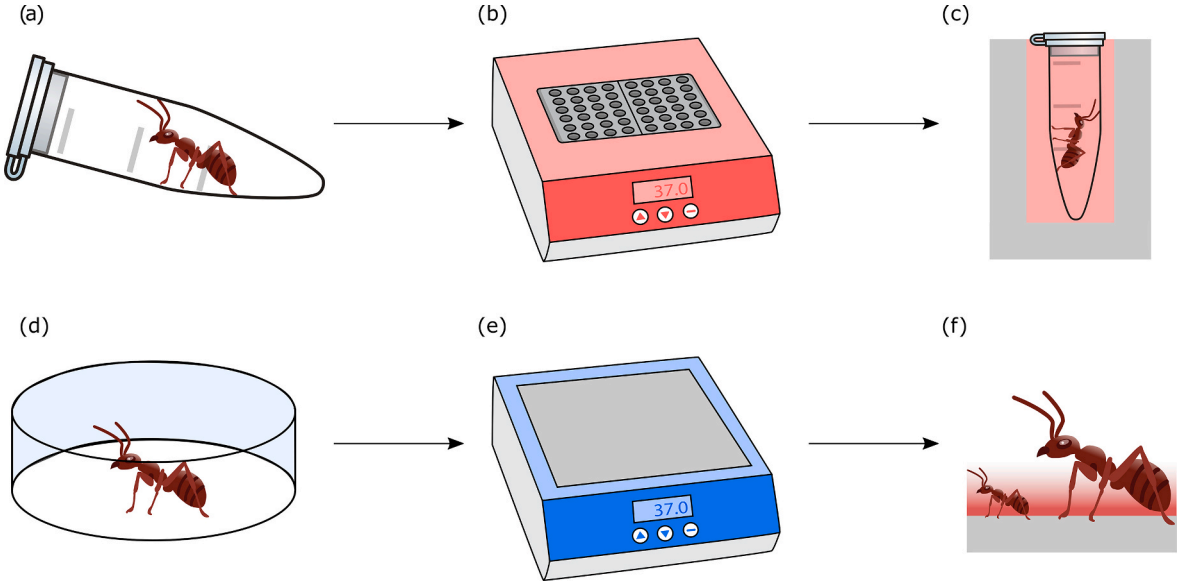

Fig. 1. Conceptual figures showing the key difference between bath and plate methods. In bath methods, individuals are kept in small tubes (a) which are inserted into a dry or water bath (b). Tubes within the bath are "submerged" in a set temperature, with the walls of the dry block, or the water itself, radiating heat toward the target organism from all directions (c). This is represented by a cutaway diagram in (c) where uniform temperature is represented by a red shading within the grey walls of the dry block. In plate methods, individuals are kept in closed top containers with open bottoms (d) which are placed onto the hot plate (e). In (e), the hot plate generates a boundary layer whereby temperatures are hottest (darkest shade of red) close to the plate and gradually get cooler with height above the plate. The larger individual in (e) consequently experiences a lower temperature than the smaller individual despite the hot plate being at the same temperature.

showed that species tended to have a higher CTmax when assayed via a hot plate compared to a dry bath, and that a positive body size-CTmax relationship was only present in hot plate derived data. The authors concluded that larger ant species were able to effectively "stilt" above the superheated boundary layer generated by the hot plate and experience lower temperatures than advertised by the equipment. By doing so, these larger species reached their thermal limits at a later point in the experiment, which translated to an apparently higher CTmax.

Here, we aim to understand the relationship between the boundary layer generated above a hot plate and an organisms size. In doing so, we attempt to show that biased CT estimates from hot plates can be easily corrected to better reflect actual organism body temperatures and thermal performances. To do this, we quantify the CTmax of four differently sized UK ant species in a dry bath (with no boundary layer) and on a hot plate (with a boundary layer). Ant thermal tolerance is well-studied and they make for an ideal taxon to represent a generic small-bodied ectotherm (Nascimento et al., 2022; Roeder et al., 2021). We expect that the larger species will display larger differences between bath and plate CTmax estimates, but that differences will be erased following a statistical correction based on thermal boundary layer composition and species size.

2. Material and methods

2.1. Ant collection

We collected individual worker ants of four different species from across South Wales, UK, during June–September 2022. We targeted four common ant species: *Lasius niger*, *Lasius flavus*, *Myrmica rubra*, and *Formica rufa*. Individual workers of *Lasius* and *Myrmica* were collected using an aspirator following active searching for colonies in urban parks and green spaces within the city of Cardiff. Workers of *Formica* were collected from Wentwood Forest (latitude: 51.65, longitude −2.84) by gently scraping workers and a small amount of their nesting material into 25 mL falcon tubes. This procedure avoided the aggressive defence response exhibited by *Formica* when aspirated. We sampled 20 worker ants each from 12 colonies for each species, except *Myrmica* for which we sampled 13 colonies. Crucially, we sampled colonies for each species at the same time: *Lasius niger* in June, *Formica rufa* in July, *Lasius flavus* and *Myrmica rubra* in August and September. Consequently, we do not expect any seasonal plasticity in thermal tolerance to influence our analyses (Bujan et al., 2020). This is because our research goals are focussed on intraspecific variation, not interspecific variation. Given our temporally staggered species sampling, potential seasonal plasticity may alter the differences in CTmax between species, but not the intraspecific variation across different methods that we are interested in.

Once collected, we transported worker ants back to the laboratory in Cardiff University and placed them in plastic containers lined with Fluon® (Blades Biological, Kent, UK) to prevent escape. Ants were provided with 40% sugar solution via a liquid feeder or a damp piece of cotton wool and laboratory conditions were kept at 20 °C in a temperature control room with a 12:12 light-dark cycle (Forder and Marsh, 1989).

2.2. Thermal tolerance assays

We used dynamic ramping experiments (Terblanche et al., 2011) to assay individual worker ants for their CTmax within 5 days of returning to the lab. For *Lasius* and *Myrmica* species, thermal tolerance assays usually took place within 2–5 h of collection. For *Formica rufa*, assays occurred later due to logistical issues with accessing the sampling site and running the assays within the same day (max two days). We assayed 10 individuals from each colony from each species in the dry bath, and 10 on the hot plate (n = 120 per method per species, except *Myrmica rubra* for which n = 130 as we sampled an additional colony).

2.2.1. Dry bath assays

We used a BIOER HB-202 Thermocell dry bath (Hangzhou, China, advertised accuracy = ± 0.5 °C) and assayed 10 ants from each colony of each species as follows: Individual ants were placed within 1.5 mL Eppendorf tubes (Fig. 1) which were then sealed with tissue paper to eliminate the presence of a thermal refuge at the top of the tube (Oberg et al., 2012). Tubes were placed into the dry block which was preheated to 25 °C. Individuals were maintained at 25 °C for 10 min before raising the temperature at 0.5 °C min-1 until all individuals lost their righting response. We chose this ramping rate as it is commonly used in the literature and allowed tubes to equilibrate with the programmed temperatures (see bath lag section below). Eppendorf tubes were briefly removed from the dry block and lightly tapped and rotated to check for the loss of a righting response every 2 min before being placed back into the dry block (Bishop et al., 2017; Lutterschmidt and Hutchison, 1997). The temperature of the dry bath at which an individual lost its righting response was recorded and considered as that individuals' CTmax. Ten individual ants were assayed during a single run of the protocol.

2.2.2. Hot plate assays

We used a ThermoElectric AHP-1800CPV hot/cold plate (TECA, United States). Individual ants were placed on the plate which was preheated to 25 °C and kept within close-topped, transparent plastic containers (Figs. 1 and 5.3 cm wide X 3.8 cm high). Each container was lined with Fluon® to ensure the ants would remain on the surface of the plate instead of climbing up the sides of the container. Once in place, the ants were kept at 25 °C for 10 min before experiencing a temperature ramp of 0.5 °C min-1. We visually assessed individuals for the loss of a righting response and sometimes tapped the lid of the plastic container to alert the individuals if they were not already moving. Again, we recorded the temperature of the hot plate at which individuals lost their righting response, stopped the ramp once all individuals had reached this point, and assayed 10 individuals at a time using this method.

2.2.3. Methodological differences

We manually and repeatedly adjusted the temperature of the dry bath to generate the ramping protocol (i.e. 1 °C increase every 2 min). The hot plate, however, was programmed via a laptop and so did not require any manual input beyond the initiation of the 0.5 °C min-1 ramping protocol. Consequently, while the temperature ramps experienced by ants in the dry bath or on the hot plate were the same (Fig. S1), the hot plate procedure was a much easier process to manage from a practical perspective as we could focus on the ants' behaviour without having to repeatedly alter the temperature of the equipment.

2.3. Estimating experienced temperature

2.3.1. Dry bath temperature lag

While in the dry bath, temperatures within the Eppendorf tubes can lag behind the advertised temperature of the bath (Kaspari et al., 2015). To understand and correct this lag we inserted a type K thermocouple attached to a FLIR TG56 infrared thermometer (Teledyne FLIR, United States, advertised accuracy ±1 °C) within an Eppendorf tube and sealed the tube with tissue paper. We then performed the dynamic ramping procedure as above and recorded the temperature of the dry bath and the temperature of the thermocouple every 60 s after setting a new temperature.

We used linear regression to relate thermocouple temperature (dependent variable) to dry bath temperature (independent variable). We use the intercept and slope parameters of this relationship to correct the CTmax estimates of bath-tested individuals (see below and Kaspari et al., 2015).

2.3.2. Hot plate temperature boundary layer

On the hot plate, we threaded a type K thermocouple through a hole drilled into one the closed-top containers that we used in the ant thermal

tolerance assays. We then used modelling clay to position the thermocouple at either 1, 2, 5, or 10 mm above the surface of the plate. Consequently, the thermocouple was experiencing the same thermal conditions that individual ants were during the thermal tolerance assays. We then set the plate to either 20, 30, 40, 50, or 60 °C and recorded the thermocouple temperature at each of these set points. We also used a spot IR thermometer (FLIR TG56) to estimate the temperature of the surface of the hot plate at each of these temperature set points. The IR thermometer data represented the temperature at 0 mm above the plate (i.e., the surface of the plate). We took 10 separate thermocouple and IR readings at each set temperature at each height.

We calculated deviation in temperature as surface temperature subtracted from air temperature at each of the five heights (0, 1, 2, 5, and 10 mm) to characterise the composition of the boundary layer above the hot plate. Negative deviations would mean that the air was cooler than the hot plate, positive deviations would mean that the air was warmer than the hot plate. For heights of 0 mm, the temperature deviation was always 0 °C. We modelled the relationship between temperature deviation, height, and surface temperature using the following equation:

$$Deviation = 0 + height^2 \times surface\ temperature$$

where the constant of 0 forced the model to fit an intercept of 0 (i.e. zero deviation at a height of 0 mm), height was represented as a squared term to capture curvature in the relationship, and height and surface temperature were interacting and dependent on each other. We use the parameters of this modelled relationship to adjust the CTmax estimates of individuals assayed on the hot plate with respect to their height (see below).

2.3.3. Ant height

We placed individuals of each species on the surface of the hot plate and cooled to between 10 and 15 °C to limit their movement (low temperatures generally slow ectotherms down, allowing us to more easily measure height). We then placed a ruler behind them and estimated height of the ant mesosoma by eye. We consider height of the mesosoma a reasonable proxy of body position in relation to thermal boundary layers as the mesosoma is typically the largest body segment with, consequently, the most thermal inertia. The mesosoma represents the middle segment of ant bodies although is not termed the thorax (as in other insects) because it is actually composed of both thoracic and abdominal body segments (Hölldobler and Wilson, 1990). We estimated the height of 10–20 individuals per species from a range of different colonies. We took a species-level mean to represent height for each species as a whole.

2.4. Statistical analysis

All data manipulation and statistical analysis took place within the R version 4.1.2 statistical programming environment (R Core Team, 2021).

2.4.1. Adjusting CTmax estimates

We used the following formula, calculated from a linear regression model (described above), to adjust bath-estimated CTmax values and account for a lag between the temperature within the Eppendorf tubes and that advertised by the dry bath unit:

$$Adjusted\ bath\ CTmax = 0.99 + (0.96 \times raw\ bath\ CTmax)$$

where raw bath CTmax values were in °C. We used the following equation, calculated from a linear regression model (described above) to adjust plate-estimated CTmax values and account for the different temperatures that differently sized ants would experience while standing on the hot plate:

$$\begin{aligned} Adjusted\ plate\ CTmax = {} & plate\ CTmax + (height \times 1.47) + \left(height^2 \times -0.08\right) \\ & + (height \times plate\ CTmax \times -0.06) \\ & + \left(height^2 \times plate\ CTmax \times 0.003\right) \end{aligned}$$

where height was in mm and plate CTmax was in °C. This relationship described the boundary layer composition at various heights above the hot plate at different plate temperatures (Fig. 2b). Thus, we can use it to estimate the body temperature that an ant was actually experiencing given that it was standing at a certain height above the plate, and that it's CTmax was initially recorded as the plate temperature. The average species heights input into this equation for each species were (mean ± SD): *Lasius niger* = 0.95 ± 0.37 mm, *Lasius flavus* = 1 ± 0.12 mm, *Formica rufa* = 3.75 ± 0.26 mm, *Myrmica rubra* = 1.5 ± 0.13 mm. Note, only mean values were used in the correction of plate CTmax estimates, standard deviations are given here for context. Example R code for these corrections is presented in the Supporting Information.

2.4.2. Comparing assay methods

We used the median CTmax value within a given colony/species/method combination to represent colony-level CTmax for our statistical analyses. This represents the temperature at which at least 50% of the worker individuals reached their CTmax (Kaspari et al., 2015). Consequently, our dataset contained 98 datapoints (12 colonies X 2 methods X 4 species = 96, plus one extra colony per method for *Myrmica rubra* = 98). We summarise and analyse our data at the colony level as colonies represent true biological replicates in ants, whereas individual workers do not (Gotelli et al., 2011).

We used ANOVA to compare the CTmax estimates from bath and plate-assayed ants from the four species. Specifically, we used two different ANOVA analyses to investigate the effect of controlling for the thermal boundary layers present on the hot plate or not. The first analysis used bath-adjusted CTmax estimates and raw plate estimates and represented the difference between the two methods without controlling for the boundary layer. The second analysis used bath-adjusted and plate-adjusted CTmax estimates and represents the difference between the two methods while controlling for the boundary layer effect. In each ANOVA, CTmax was the dependent variable while species identity and method type were interacting explanatory factors. We used Tukey honest significant differences (Tukey HSD) post-hoc tests to investigate which species-method combinations were significantly different from each other.

2.4.3. Linking methodological differences to height

Finally, we explicitly tested the link between species height and the methodological differences found in CTmax. We subtracted raw colony-level bath CTmax from raw colony-level plate CTmax and used this as the dependent variable in a linear regression. We used average species height as the explanatory variable.

3. Results

3.1. Experienced temperatures

The set temperature of the dry bath explained 99% of the variation in the actual temperature within the Eppendorf tubes (Fig. 2a). The intercept of this relationship was 0.99, and the slope was 0.96, meaning that temperatures within the Eppendorf tubes were slightly lower than advertised by the dry bath unit at high temperatures, and slightly higher than advertised at low temperatures (Fig. 2a).

The deviation between the surface temperatures of the hot plate and the air above it were also well predicted by height and surface temperature (R2 = 0.98, Fig. 2b). Deviations from the surface temperature were larger (i.e. more negative) with greater heights and with greater surface temperatures. This pattern fits the predicted composition of a

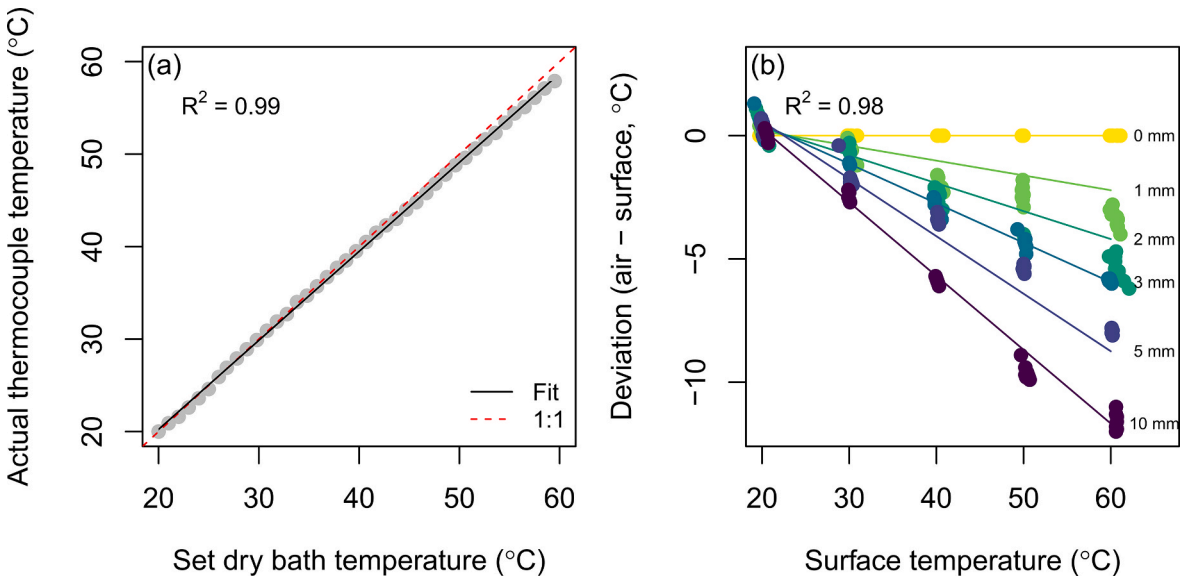

Fig. 2. (Aa) Plot showing the relationship between the set temperature of the dry bath and the temperature that ants experience within individual Eppendorf tubes as measured by a type K thermocouple. Thermocouple temperatures lag slightly behind set temperatures at high temperature values. Black line indicates fitted regression line, red dashed line indicates 1:1 relationship. (b) Plot showing the deviation in temperature (the difference between air and surface hot plate temperature) at different heights above the hot plate and at different set temperatures. At 0 mm above the hot plate temperatures match those of the plate. At 10 mm above the hot plate, air temperatures are ~12 °C cooler than the plate when it is set at 60 °C. Different colours represent different heights above the plate (pale colours = low, dark colours = high). Points represent raw data and lines represent modelled relationships.

thermal boundary layer: air temperatures were increasingly different form the plate with increasing height, and these differences were greater still when the plate was much hotter than the ambient room temperature (20 °C).

3.2. Comparing assay temperatures

The first ANOVA analysis, using bath-adjusted by plate-unadjusted CTmax values, revealed a range of differences between methods across the four species. Species ($df = 3$, $F = 499.9$, $p < 0.01$), method ($df = 1$, $F = 370.58$, $p < 0.01$) and their interaction ($df = 3$, $F = 88.62$, $p < 0.01$) all influenced estimated CTmax. Post-hoc Tukey HSD tests revealed significant differences between the CTmax values estimated from the dry bath and the hot plate for each species except *Lasius flavus* (Fig. 3a, Table 1). For all four species, CTmax was higher when estimated on the hot plate compared to when estimated in the dry bath.

The second ANOVA analysis controlled for the effect of the boundary layer by using both bath-adjusted and plate-unadjusted CTmax values. Species ($df = 3$, $F = 251.6$, $p < 0.01$) and the interaction between species and method ($df = 3$, $F = 3.82$, $p = 0.013$) influenced estimated CTmax. The main effect of method did not ($df = 1$, $F = 0.5$, $p = 0.47$). Post-hoc Tukey HSD tests revealed that there were no significant differences between the CTmax values estimated from the dry bath and the hot plate for any of the four species (Fig. 3b, Table 1).

Full ANOVA tables are presented in Table S1 and details for all post-hoc Tukey HSD comparisons are presented in Table S2.

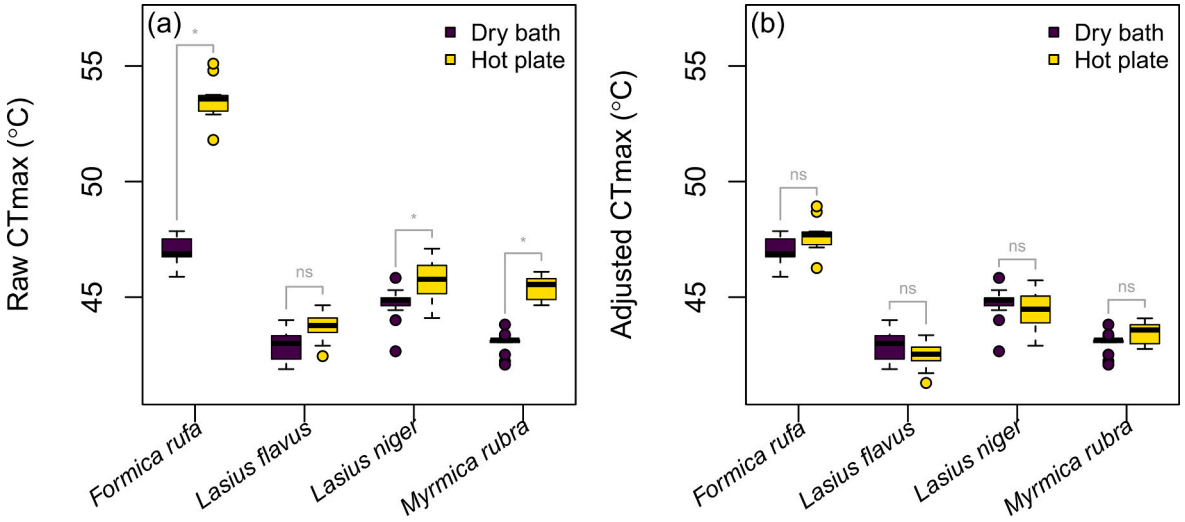

Fig. 3. Boxplots showing the estimated CTmax values for each of the four species with plate CTmax values left (a) unadjusted or (b) adjusted for the boundary layer effect. In each, dark (purple) boxes represent CTmax estimates from the dry bath and pale (yellow) boxes represent estimates from the hot plate. Grey lines indicate comparisons within species but across methods and highlight significant (*) or non-significant (ns) differences according to Tukey HSD tests.

Table 1
Table showing Tukey's honest significant differences between bath and plate assay methods for each species. Comparisons presented using unadjusted plate CTmax data (top) and adjusted CTmax data (bottom).

Comparison	CTmax difference	Lower estimate of difference	Upper estimate of difference	Adjusted p value
Unadjusted plate CTmax				
Formica rufa	6.49	5.62	7.36	0.00
Lasius flavus	0.84	−0.03	1.71	0.07
Lasius niger	0.98	0.11	1.85	0.02
Myrmica rubra	2.38	1.55	3.22	0.00
Adjusted plate CTmax				
Formica rufa	0.62	−0.20	1.44	0.28
Lasius flavus	−0.40	−1.22	0.42	0.80
Lasius niger	−0.31	−1.13	0.51	0.94
Myrmica rubra	0.43	−0.36	1.22	0.69

3.3. Linking methodological differences to height

There was a significant positive relationship between average species height and the difference in CTmax estimated from the dry bath compared to the hot plate (linear regression, a = −0.89, b = 1.98, t = 18.14, p < 0.01, R2 = 0.87, Fig. 4). The difference between plate-estimated and bath-estimated CTmax increased by 1.98 °C per mm of height.

4. Discussion

Our data show that estimates of ectotherm CTmax are typically higher when using hot plates compared to using dry baths. These differences are related to an interaction between the thermal boundary layers that are generated above hot plates and the height of the target organism. By using a simple statistical model to control for the thermal boundary layer effect, however, these methodological differences can be corrected. By doing so, we provide an easy method to estimate metrics of thermal tolerance relative to body temperature regardless of the specific method and equipment used. We anticipate that the simplicity of this correction will prove useful for researchers planning on using hot plates to estimate the thermal performance of small ectothermic organisms – hot plates are readily available and can offer advantages over bath-based methods as they could be upgraded and automated for high-throughput data collection. In a warming world, it is crucial that we characterise the upper thermal tolerances of as many individuals, populations, and species as possible. This will allow us to fully understand and predict the temperature-dependent shifts that ecological communities will be subjected to. Accurately estimating thermal tolerances with respect to organism body temperature is the first step toward doing this.

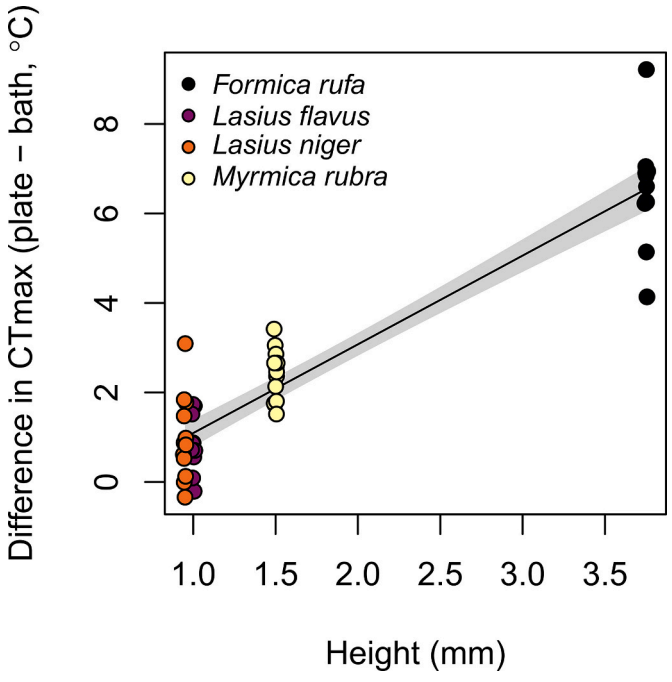

Fig. 4. a) Plot showing the relationship between height and the difference in CTmax estimated from the dry bath compared to the hot plate. Bigger differences indicate that hot plate CTmax estimates were higher than bath estimates. Each ant species is colour coded according to the legend. The black line represents the regression line, the grey polygon represents the 95% confidence intervals and each point represents a different ant colony (n = 12 except for Myrmica rubra where n = 13. Points given a small jitter in the x-axis as a visual aid.

Our data provide two key results. The first, is that we make an explicit link between organism height and the degree to which the hot plate overestimates their CTmax (Fig. 4). In our data, the tallest species had the largest CTmax overestimate (*Formica rufa*, 3.65 mm tall, ~6 °C overestimate) and the shortest species had the smallest (*Lasius niger*, 0.95 mm, ~1 °C overestimate). This finding underpins a key assumption about how small ectotherms are influenced by different methods: hot plates generate a unidirectional boundary layer (Fig. 2b) which taller species are able to walk above (Kaspari et al., 2015). As a result, the body temperatures of these larger species is cooler than what the set temperature is on the hot plate and CTmax is erroneously recorded as occurring at a higher temperature. This finding is in agreement with previous work highlighting how the body height of small insects is the most important determinant of body temperature (Pincebourde et al., 2021). Of course, in studies on a single species or size class of organisms, this bias may be safely ignored (Villalta et al., 2020). Most studies on thermal tolerance, however, are multispecies and macroecological in nature (Bishop et al., 2017; Sunday et al., 2019) and so controlling for the boundary layer effect when hot plates are used will be critical.

The second key result is that the bias introduced by the boundary layer effect can be easily corrected. Without correcting for the boundary layer and the height of the organism, we found significant differences between plate-estimated and bath-estimated CTmax values for three of the four species we tested (Fig. 3a, Table 1). Plate-estimated CTmax values were always higher on average (Fig. 3a). After characterising the boundary layer (Fig. 2b) and combining with estimates of ant height, these methodological differences in CTmax were eliminated (Fig. 3b, Table 1). This result is important because it shows that, in principle, an understanding of the boundary layer and how organisms interact with it is enough to correct the bias found using the hot plate method. In practice, however, we argue that our finding is even more useful. We did not quantify the thermal composition of the boundary layer in particularly high resolution or describe it with physical models or thermography (Pincebourde et al., 2021; Stevenson, 1985). Neither did we use complicated equipment to estimate the average heights of the species we assayed. Instead, we used a series of thermocouple measurements, an easy (if tedious) estimate of ant height, and a simple statistical model to combine and understand these variables. Despite this simplicity, the corrected hot plate CTmax estimates were statistically indistinguishable from those estimated from the dry bath. If boundary layer-free bath techniques are seen as the "gold standard" in ectotherm thermal tolerance work, then our accessible method will allow researchers to reach this standard from an alternative direction using hot plate apparatus. Researchers will simply need to capture the composition of their boundary layers using thermocouples, estimate the height of their target organisms, and construct linear models to perform the correction.

Our findings feed into a broader conversation concerning how researchers working at the interface of physiological and climate ecology measure relevant temperatures. For instance, within climate ecology, it is now well recognised that macroclimate (i.e., as measured by weather

stations) can be largely decoupled from the microclimate that organisms actually experience (including boundary layer microclimates) (Maclean et al., 2021; Zellweger et al., 2020). As a result, assessing the physiological tolerances of organisms in relation to the macroclimate may be entirely misleading (Caillon et al., 2014; Duffy et al., 2015; Pincebourde et al., 2021). Physiological ecology has been grappling with conceptually similar problems: what methodological details best capture the conditions that organisms actually experience (Leong et al., 2022; Maclean et al., 2018; Terblanche et al., 2011)? In this vein, it is crucial that physiological ecologists estimate the body temperatures at which various lethal or sublethal effects take place (Angilletta, 2009; Clarke, 2017). Body temperature provides an understandable and comparable measure that can be integrated with a range of other data sources on life history, physiology, morphology, and microclimate to predict the activity and distribution of species (Briscoe et al., 2022; Malishev et al., 2018). Our work here provides a simple way by which researchers working on small ectotherms can estimate body temperatures from plate-based equipment.

There are a number of caveats to our work, however. The first is that we did not estimate the time it took for ant bodies to equilibrate with their thermal environments. Larger individuals may display an artificially high CTmax as it takes longer for their bodies to equilibrate to their thermal environments. However, the ants used in this study weighed between 1 and 10 mg and data from (Kaspari et al., 2015) indicate that ants of this size would equilibrate with their surroundings within 10–20 s. Consequently, we think it unlikely that long equilibration times would inflate our CTmax estimates given the 0.5 °C min-1 ramping rate. Secondly, we made the simplifying assumption that the primary physical process influencing ant body temperature was convection. Our results support this assumption, as does other work (Pincebourde et al., 2021), but we note that in reality it was not the only process influencing ant body temperatures. Ants were likely conducting heat directly from the hot plate via their tarsi and legs and in nature would also be subject to heat gain via radiation (Clarke, 2017). Thirdly, we were unable to disentangle the relationship between thermal tolerance and desiccation in our experimental setup. Given the different volumes that the ants were confined to in the dry bath vs the hot plate, it is likely that desiccation risk was higher in the hot plate assays. This may have had the effect of lowering species CTmax in the hot plate assays. Finally, we were unable to test our statistical correction for CTmin. However, given that boundary layers are generated in a similar (yet reversed) way when a plate is colder than the ambient air temperature, we anticipate that our method works similarly for CTmin estimates. We expect that uncorrected plate-estimated CTmin will be lower than the body temperature at which the target organism entered a chill coma.

Moving forward, we see huge potential in the use of hot plates in thermal ecology. For instance, hot plates could allow us to automate thermal physiology assays and massively increase their throughput using video cameras. Such techniques have been used before but only at a relatively small scale (Kaspari et al., 2016). We argue that there is scope for high throughput thermal assays using camera, tracking software, and hot plates similar to what researchers have been developing in behavioural ecology and evolution (Geissmann et al., 2017; Werkhoven et al., 2019). Bath-based methods, however, prevent these kinds of automations taking place. If thermal ecologists, or physiological ecologists interested in other environmental variables want to phenotype many organisms at once then a shift toward hot plates or other “open” apparatus may be the answer. Crucially, we now know that the thermal boundary layers produced by hot plates can be simply and easily corrected for.

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Data accessibility statement

All datasets and scripts are available from doi:10.17632/fshhh8r5rb.1.

CRediT authorship contribution statement

Rebecca B. Corley: Conceptualization, Methodology, Investigation, Writing – original draft. **Will Dawson:** Investigation. **Tom R. Bishop:** Conceptualization, Methodology, Software, Formal analysis, Resources, Data curation, Writing – review and editing, Visualization, Supervision, Funding acquisition.

Declaration of competing interest

There are no competing interests to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2023.103673.

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