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Thermolimit respirometry: an objective assessment of critical thermal maxima in two sympatric desert harvester ants, *Pogonomyrmex rugosus* and *P. californicus*

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Summary

The critical thermal maxima (CT_{max}) of two sympatric, diurnal, thermophilic harvester ants from the Mojave Desert, USA (*Pogonomyrmex rugosus* and *P. californicus*) were measured by ramping their temperature upwards at a rate of 0.25°C min^{-1} during flow-through respirometry with optical activity detection. Rates of CO_2 emission ($\dot{V}_{CO_2}$) increased exponentially to plateau values that were twice as high in *P. californicus* as *P. rugosus* on a mass-specific basis. $\dot{V}_{CO_2}$ then fell sharply, during which gross motor activity (measured optically) and spiracular control (measured from $\dot{V}_{CO_2}$ variation) abruptly ceased, yielding two independent measures of CT_{max}. As determined by loss of muscular coordination, the CT_{max} of *Pogonomyrmex rugosus* was 51.57±0.38°C (mean ± S.D., while that of *Pogonomyrmex californicus* was 51.74±0.25°C. As determined by loss of spiracular control, the CT_{max} of *Pogonomyrmex rugosus* was 51.59±0.35°C, while that of *Pogonomyrmex californicus* was 51.78±0.37°C. In each species a pronounced post-mortal peak of $\dot{V}_{CO_2}$ was observed. The major ecological and behavioral differences of the two species are not reflected in their CT_{max} values, which do not differ significantly. 'Thermolimit respirometry' allows CT_{max} to be estimated objectively with coefficients of variation (S.D./mean) <1%, lending confidence to comparisons between species or treatment groups.

Key words: CT_{max}, temperature, heat shock, thermal stress, *Pogonomyrmex*, thermolimit respirometry.

Introduction

The upper temperature at which an animal loses muscular control is a metric of interest to ecological and evolutionary biologists, not to mention biochemists and other pure and applied scientists (Wehner et al., 1992; Gehring and Wehner, 1995; Denlinger and Yocum, 1998; Berrigan, 2000; Pörtner, 2001; Hoffmann et al., 2003; Stillman, 2003). Interest in thermal stress, which is also relevant to the issue of global warming, has led to a diversity of approaches for quantifying it. Measuring the critical thermal maximum (CT_{max}), especially in small animals, is therefore anything but a standardized exercise, as Lutterschmidt and Hutchison (1997) confirm in their comprehensive review. This methodological diversity complicates inter-study comparisons (see also review by Addo-Bediako et al., 2000), especially because different measures of heat resistance, such as loss of muscular control, may be genetically independent of 'mortality' factors (Berrigan and Hoffmann, 1998), yet those same measures appear ecologically correlated across species (Berrigan, 2000). Given the burgeoning interest in heat stress and related issues (see Feder and Krebs, 1997; Gilchrist et al., 1997; Feder and Hofmann, 1999; Roberts and Feder, 2000; Kregel, 2002, and references therein), which require precise and intercomparable measurements, such a methodological free-for-all in the measurement of CT_{max} circumvents the intention of the term and may also miscue analyses.

What CT_{max} metric is best suited for assessing the whole-organism temperature tolerance of a small animal such as an insect? CT_{max} metrics are reliant on a specific endpoint marker such as the onset of spasms, capsizing or heat paralysis, which are monitored visually. The marker is variably noted as 'knockdown' (Berrigan and Hoffmann, 1998), 'loss of righting response' (LRR), 'onset of spasms' (OS), or other descriptive terms, and is followed finally by heat paralysis (see review by Lutterschmidt and Hutchinson, 1997, and references therein).

Which endpoint marker is most appropriate? Lutterschmidt and Hutchinson (1997) favor OS. LRR or knockdown generally precedes OS and can also be defined as motor coordination failure. It determines ecological perdition – the point beyond which escape from further temperature stress is improbable – as distinguished from OS and heat paralysis, the physiological walls of final mortality [it being understood that perdition is ecological and evolutionary while death is physiological and that perdition, in this case, precedes death], and as such is probably more ecologically and evolutionarily

relevant. But as Lutterschmidt and Hutchison (1997) admit, these observationally determined '*...end points are not definitive, are difficult to determine and are seldom described fully*'.

An objective, operationally defined endpoint that is unambiguous and low in measurement variance (mostly arising from human interpretive error) is desirable. Such an objective endpoint for CT_{max} determination will allow small signals to be teased from measurement noise. Low measurement noise is especially important in evolutionary studies where the noise of genetic variation may be an important part of the signal.

Investigators mostly use two dissimilar techniques for manifesting CT_{max}, however defined, in their experimental organisms (for extended discussions, see the review by Lutterschmidt and Hutchison, 1997). Either method may include induction and acclimation variations. The first technique exposes the organism to an acute temperature. It may employ an LD_{50}-type assessment. The time taken to achieve LRR or OS at a fixed temperature is noted and employed as a metric of thermotolerance. Usually a succession of temperatures is employed (with new subjects for each), and CT_{max} is operationally defined as the temperature at which LRR or OS occur. Lutterschmidt and Hutchison (1997) refer to this as the 'static method'. We refer to this method as the 'total immersion method'.

The total immersion method has the following drawbacks: (a) the organism requires time to thermally equilibrate to the experimental temperature, and this must be measured and accounted for; (b) the choice of test temperature(s) is to a large extent arbitrary, and (c) the duration of normal behavior at any given temperature is thus, necessarily, also an arbitrary metric even if it is replicable. For example, is 54°C the CT_{max} if an insect undergoes LRR or OS at that temperature after 5 or after 2 min, after 1 min, after 0.5 min or after 10 min? Why not 55°C for 2 min rather than 54°C for 3 min? By using a single temperature and comparing times to LRR or OS, comparisons within a group of animals of similar masses can be made. However, the arbitrary nature of the temperature choice and thus of the associated survival time gives this method a clumsy affect and complicates comparisons between studies. In addition, in the field and in the final analysis it may be even more relevant that high resolution of CT_{max} using this method requires a large number of temperatures and animals.

The second technique is to expose the animal to a ramped temperature and note the temperature at which it displays LRR or OS. We refer to this method as the 'temperature ramp method'. Lutterschmidt and Hutchison (1997) refer to it as the 'dynamic method'. The temperature ramp method has the virtue that it yields a hard number for CT_{max}. However, that number's meaning can be difficult to interpret. This is primarily because the temperature ramp method has a strong historical component, easily revealed by conducting a thought experiment. Imagine that the ramping rate is infinitely slow or infinitely high; in either case the temperature ramp method effectively becomes the total immersion method. If the ramp starts out at a low enough temperature, the animal risks dehydration or starvation before it succumbs to the experimental temperature. On the other hand, an infinite temperature ramp rate will cause immediate death. Between these two extremes lies a thermal landscape where the animal at any given moment has endured all of the temperatures from the start of the ramp up to its present temperature. At high temperatures, each preceding temperature has an associated lethal exposure time – yet moment by moment the animal is no longer at that temperature but at a higher one with a shorter lethal exposure time. The slower the ramp, the more extensive will be the animal's exposure to the physiological integral of its previous thermal exposures. Slow ramps may effect stepwise preconditioning and induce heat shock effects (see references in Lutterschmidt and Hutchinson, 1997). But the faster the ramp, the more the animal's body temperature may lag behind that of its environment, yielding misleading overestimates of CT_{max}.

Thus, to sum up, the disadvantages of the temperature ramp technique are (a) that the animal's body temperature will lag behind the ramp temperature if the ramp rate is too rapid, and (b) the temperature at which the animal succumbs to heat stress is a strong function of its recent thermal history, and thus of the rate at which temperature is ramped. Obviously (a) interacts negatively with (b).

We can see from the above that the total immersion method is unsatisfactory because it has a strong arbitrary component. The temperature ramp method is unsatisfactory because it is sensitive to summed historical effects over the ramp duration; and, being thus sensitive to ramping rates, likewise has an arbitrary component. The two techniques are not easily or directly interconvertible and so their results cannot be readily compared. Furthermore, each technique requires skilled visual observation, which may have to be augmented by manipulation that may, in turn, alter the temperature challenging the organism.

Given the choice between two evils, we believe the temperature ramp technique to be the lesser, especially for comparative work involving species within moderate ranges (ca. two- to fourfold) of body mass. This is because the ramping rate can be objectivized by standardizing it to the maximum at which optimal thermal equilibration occurs in any given body mass range. Thus the maximum ramping rate will scale to the –0.33 power of body mass, determined by the ratio of body surface area (L^2) to mass (L^3). It will also vary with body surface conductance, medium conductance (air or water), the animal's radiative environment, the convective characteristics of the medium, and other factors. To some degree ramp rate will depend on the taxonomic or physiological resolution sought by the researcher (see especially Stevenson, 1985). We suggest that the ramp rate appropriate for mass and phylogeny (RRAMP) is therefore usually best determined empirically by trial and if comparative measurements are made, that the ramping rate be constant across groups and determined by adequate equilibration of the largest animals.

In the course of studying the thermal biology of two

sympatric Mojave desert harvester ants, *Pogonomyrmex rugosus* and *Pogonomyrmex californicus*, we have developed a ramped metric that utilizes two independent and objective measures of loss of muscular control, i.e. cessation of coordinated voluntary and involuntary activity. Neither measure is dependent on visual observation, facilitating automated objective analysis of CT_{max}. The technique also yields valuable metabolic data. We refer to this technique as 'thermolimit respirometry'.

Measurements of CT_{max} are particularly relevant to ant biology. As central place foragers (Hölldobler and Wilson, 1990), most diurnal ant species in warm regions are forced by rising temperatures to cease foraging at some point in the day. Because of selective pressures that include maximizing net energy intake to the colony (Lighton and Duncan, 2002 and references therein), and a reduction in competition and predation combined with the presence of heat-stressed prey at high temperatures (Cerdá et al., 1998; Wehner et al., 1992; Marsh, 1985), many ant species (in Rüdiger Wehner's words) '*walk a thermal tightrope*' (ibid.)

Pogonomyrmex californicus and *Pogonomyrmex rugosus* are common sympatric seed-harvesting ants in the Mojave Desert, southwestern USA. *P. californicus* forages at high substrate (though not necessarily ant body) temperatures; up to 53°C (Bernstein, 1974), 54.4°C (Bernstein, 1979) or even, supposedly, 60°C (Whitford et al., 1976). In contrast, *P. rugosus* ceases foraging at approximately 46°C (Bernstein 1974, 1979). Bernstein's estimates are similar to those made at our location (J. Dorn, M. Feder and J.R.B.L., unpublished data). Behavioral observations (J.R.B.L. and R.T.U., unpublished) show that *P. californicus* is a rapidly moving and (in the milieu in which we find them) mostly solitary forager, making extensive use of thermal refuges such as small sticks or stones that allow it to enter a cooler layer of air and dump heat. *P. rugosus* forages more frequently in columns, although it also forages individually (see also Hölldobler and Wilson, 1990, and references therein; Davidson, 1977a,b; Gordon, 1984; Traniello, 1989). Its movements are slower and more directed, and its thermal refuge behavior is less marked. It is reasonable to predict that the CT_{max} of *P. californicus* would be higher than that of *P. rugosus*. We developed the techniques outlined here (see also Lighton and Turner, 2003) to address that question, as well as to characterize metabolic responses to extreme temperature stress. Our null hypothesis was that *P. californicus* would display the same CT_{max} as *P. rugosus*.

Materials and methods

Animals

Individuals of *Pogonomyrmex californicus* Buckley and *Pogonomyrmex rugosus* Emery were collected while they foraged at a field site in the Mojave Desert immediately south-west of Las Vegas, NV, USA, at which all measurements were also made. Substrate temperatures varied from 40°C to 54°C. Ants were stored at an ambient temperature of 25±2°C in polyethylene containers and used within 2–8 h of capture. Water was always available. Ants of both species were seen drinking water, so we considered our animals to be fully hydrated.

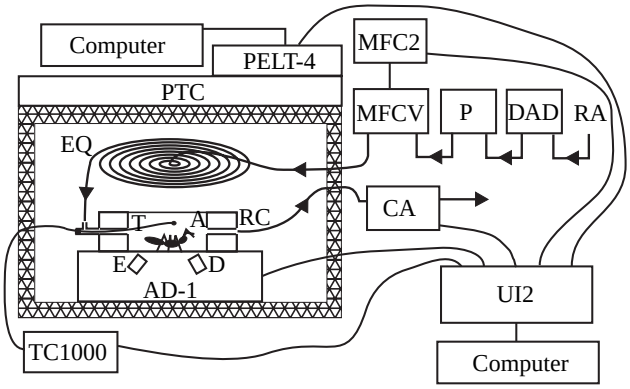

Fig. 1. Thermolimit respirometry diagram. Simplified and not to scale. Gas streams are denoted by thick lines, and their directions by arrowheads. Thin lines are electrical connections. PTC, Peltier-effect temperature control cabinet (controlled area within hatched walls); PELT-4, controller for cabinet, in turn controlled by the adjacent computer; A, ant; AD-1, activity detector with infrared emitter (E) and detector (D); T, thermocouple in chamber, connected to thermocouple meter (TC1000); RC, respirometry chamber; EQ, equilibration coil; CA, CO_2 analyzer; RA, room air; DAD, Drierite/Ascarite/Drierite drying and CO_2 scrubber column; P, pump; MFCV, mass flow control valve; MFC2, mass flow control electronics unit; UI2, 16-bit data acquisition interface attached to the adjacent computer. See text for details.

Respirometry, activity monitoring and temperature control

We used a Sable Systems International (SSI; Las Vegas, NV, USA) TR-2 flow through respirometry system and DATACAN V data acquisition software (www.sablesystems.com), supplemented with activity detection monitoring and temperature measurement and control (Fig. 1). In more detail, each ant we studied was placed in a glass respirometer chamber sealed at each end by an aluminum cap with double Viton O-rings (SSI RC-M). Air scrubbed of CO_2 and H_2O flowed through the chamber *via* 2.5 mm i.d. metal barbs in the end caps. The chamber entry and exit were secured against ant escape with stainless steel mesh (6 mm diameter, <50 μm openings, Dutch weave). At one end the mesh was pushed aside enough to allow a thin (ca. 0.5 mm diameter, type T) thermocouple wire to penetrate about 2 cm into the chamber through the barb.

The thermocouple wire left the chamber *via* the barb, traveled through a 3 cm length of 3 mm i.d. PharMed tubing, and then through the long axis of a HDPE T-adapter inserted into the other end of the tubing. The wire exit was sealed with silicone RTV cement and leak-tested. The flow path entered the respirometer chamber *via* the orthogonal axis of the T-adapter. Finally, the wire left the temperature-controlled chamber and was attached to a SSI TC1000 thermocouple

meter. The accuracy of this meter is better than 0.2°C over the range –75 to +125°C. The 16-bit analog output of the TC1000 was attached to a 16-bit data acquisition interface (SSI UI2, basic accuracy 0.03%), which was connected to a laptop computer. The temperature of the air within the cabinet, as controlled and reported by the controller (see below) was simultaneously monitored.

The respirometer chamber rested on the cradle of an open activity detector (SSI AD-1), which detected the activity of the ant by monitoring fluctuations in reflected infrared light at ca. 900 nm. The infrared radiation was too weak to affect the temperature of nearby objects to any measurable extent. The output of the AD-1 was likewise monitored *via* the UI2.

Incurrent air was pulled from outside the building by a SSI TR-SS1 subsampler pump through a Drierite/Ascarite/Drierite column that scrubbed CO_2 and H_2O from the air. The air was then pushed through a 200 ml min^{-1} mass flow control valve controlled at 50 ml min^{-1} by a SSI MFC-1 mass flow control electronics unit. After entering the controlled-temperature cabinet, but prior to entering the respirometer chamber, the incurrent air flow traveled through 350 mm of coiled 3 mm i.d. aluminum tubing placed next to the respirometer chamber in the temperature cabinet. This ensured that air entering the respirometer chamber had thermally equilibrated with the interior of the controlled-temperature cabinet. Finally, the air entered the respirometer chamber, and then entered the TR-2 CO_2 respirometry system, which was also monitored by the UI2 and data acquisition system. Fig. 1 shows a diagram of the system.

Our experimental design required flexible control of temperature. We used a SSI PTC-1 miniature temperature control cabinet attached to a SSI PELT-4 temperature controller. This cabinet and controller combination regulates temperature with an absolute accuracy of 0.2°C over the range 5–60°C. We attached the PELT-4 controller to a laptop computer running SSI Pelt-C1 temperature control software. We generated a temperature profile that began with 10 min at 45°C (similar to the temperatures at which the ants had been foraging), followed by a ramp at a rate of 0.25°C min^{-1} to a temperature of 55°C. That final temperature was maintained for 10 min. Finally the program reset the cabinet temperature to 45°C. The PELT-4 controller was updated with new values every 5 s during the ramping period. The ramping temperature of 0.25°C min^{-1} was the fastest ramp that did not produce a lag effect (see Discussion).

Equilibration for 10 min probably does not allow for biochemical acclimation to high temperatures, and indeed, this experimental design was intentional. The aim of the 10 min equilibration phase at 45°C was to allow for thermal equilibration, behavioral acclimation, and measurement of initial rate of CO_2 production ($\dot{V}_{CO_2}$) before starting the upward ramp. 10 min of exposure to 45°C is not a challenge for these species. Both species can survive for >90 min at this temperature, which is typical of substrate temperatures while they are actively foraging (J. Dorn, M. Feder, J.R.B.L., M.J.T., unpublished data; see also Bernstein 1974, 1979).

Experimental protocol

The ant was weighed to 0.1 mg on an analytical balance (Mettler AG-245, Columbus, OH, USA). Meanwhile, baseline air (zero CO_2) was pushed through the respirometry system and the recording initiated. After at least 1 min of plateau baseline had been recorded (baseline drift from run to run was <0.5 p.p.m.), the recording was paused, the ant was placed in the respirometry chamber, and the recording was re-started after 2–3 min. Simultaneously, the temperature profile (starting with the 10 min equilibration phase at 45°C) was initiated. CO_2 concentration in p.p.m., temperature in °C within the respirometer chamber, activity (arbitrary units, recorded as volts), and temperature of the air within the temperature-controlled cabinet, were recorded at intervals of 1 s until the recording was manually terminated. The ant was then decanted into a 0.75 ml Eppendorf-type vial, labeled and stored.

Data analysis

Data analysis was performed with a beta release of SSI ExpeData scientific data analysis software. For each recording, data analysis was a two-step process, starting out with transformations of the original data and moving to data reduction. During transformation, the CO_2 trace was baseline corrected and converted to $\dot{V}_{CO_2}$ in µl h^{-1}, then copied to an empty channel. The copy was transformed for later determination of Q_{10} by taking its base-10 logarithm. The original was copied again, into another empty channel, and its absolute difference sum (ADS) was calculated.

The ADS of a given data channel, such as $\dot{V}_{CO_2}$ or activity, is the cumulative sum of the absolute difference between all of that channel's adjacent data points. It is a useful measure of cumulative dynamic variability in a measured variable. Thus, expressed in pseudocode, where ADS is the ADS accumulator, ADSData() is the 0-based ADS destination vector, and the data to be calculated as ADS are in the 0-based vector Data() with Samples being the number of samples, the algorithm is simply:

```
ADS=0
For N=1 to Samples
ADS=ADS + Abs[Data(N) – Data(N–1)]
ADSData(N)=ADS
Next
```

where Abs is the absolute function (|X|). The ADS was originally used as a means of translating bi-directional position measurements into an accumulated displacement vector (Lighton et al., 1993) but has proved to be of broader utility as a measure of the short-term dynamic variability of data. In the case of $\dot{V}_{CO_2}$, the rate of change of the ADS *vs.* time provides an index of short-term spiracular control. Next, the activity channel was converted to its ADS. Because the activity trace contains useful information only in its ADS and its rate of change, the original contents of that channel were overwritten.

The file was then saved under a new name for data reduction. The original data were preserved. All of the transformations listed above were implemented as a macro so that the transformed files could be recreated from the raw data files at any time. The macro could also be edited to add other

transformations, if desired. We now describe the analytical steps in detail to facilitate replication.

For data reduction, the transformed file was loaded and the $\dot{V}_{CO_2}$ and respirometer chamber temperatures were displayed simultaneously. S.D. refers here to standard deviation. (1) The equilibration section was selected, and the identity of the ant, start and end of the selection, mean $\dot{V}_{CO_2}$ and S.D., $\dot{V}_{CO_2}$ ADS rate of change (slope) *vs*. time in min, mean temperature and S.D., and activity ADS slope and S.D. were written to an internal spreadsheet. The reader may wish to consult the Results for explanations of each of the post-equilibration phases that will now be described. (2) Approximately the first half of the temperature ramp was selected. The start and end of the selection, the slope of $\log_{10}$-transformed $\dot{V}_{CO_2}$ *vs.* respirometer temperature, $\dot{V}_{CO_2}$ ADS slope *vs*. time in min, temperature slope *vs.* time in min (= ramping rate), and activity ADS slope *vs.* time in min were written. (3) The pre-mortal plateau $\dot{V}_{CO_2}$ was selected. The start and end of the selection, the mean $\dot{V}_{CO_2}$ and S.D., the slope of $\log_{10}$-transformed $\dot{V}_{CO_2}$ *vs.* respirometer temperature, $\dot{V}_{CO_2}$ ADS slope *vs*. time in min, temperature slope *vs.* time in min (= ramping rate), and activity ADS slope *vs.* time in min were written. (4) The end of the plateau to the lowest post-mortal $\dot{V}_{CO_2}$ ('postmortal valley') was selected. The start and end of the selection, the minimum and maximum $\dot{V}_{CO_2}$, the slope of $\log_{10}$-transformed $\dot{V}_{CO_2}$ *vs.* respirometer temperature, $\dot{V}_{CO_2}$ ADS slope *vs*. time in min, the minimum and maximum respirometer temperature over the selected interval, and activity ADS slope *vs.* time in min were written. (5) The $\dot{V}_{CO_2}$ ADS was viewed and a ca. 5 min interval around its breakpoint selected. The linear regression of $\dot{V}_{CO_2}$ ADS over this interval was obtained and its residuals inspected. The breakpoint of the regression, i.e. the transition from high short-term variability to minimal short-term variability in $\dot{V}_{CO_2}$, was visible as a sharp peak in the residual graph (see Results). The graph of the residuals could act as a selection mechanism by clicking on two points on the graph with a mouse, and then activating the selection. An interval just to either side of the residuals peak was selected. The start and end of the selection, the mean temperature within the respirometer chamber and its S.D., and the mean $\dot{V}_{CO_2}$ and its S.D. were written. Sixth, an exactly analogous technique was used to select the interval immediately around the breakpoint of the activity ADS trace. The start and end of the selection, the mean temperature within the respirometer chamber and its S.D., and the mean $\dot{V}_{CO_2}$ and its S.D. were written. (7) The highest 20 points in the large post-mortal rise in $\dot{V}_{CO_2}$ were found *via* a zenith search algorithm, and the start and end of the selection and the mean $\dot{V}_{CO_2}$ and its S.D. were written. (8) The linear downward slope of the post-mortal-peak $\dot{V}_{CO_2}$ ($\log_{10}$ transformed) *vs*. time in min was written, followed by $\dot{V}_{CO_2}$ ADS slope *vs*. time in min, and activity ADS slope *vs.* time in min. (9) Finally the mass of each ant was determined by scanning the remarks saved with the file for a number followed by 'mg', yielding a final column of data.

Each of the steps outlined above was implemented as a separate macro so that the possibility of mistaken analyses was eliminated after each area was manually selected and the appropriate analysis steps initiated. Each file that was analyzed also yielded a log file that detailed all of the steps taken in analyzing it, allowing the analysis of any file to be reconstructed in full detail. The log files could also be edited to add further analysis steps and played back to automate the re-analysis of any or all of the files, if desired.

Statistics

Means are accompanied by standard deviations (S.D.). Regression analysis is by least squares, with axis transformation where noted. Means are compared using Student's *t*-test, and/or by analysis of variance (ANOVA) where noted. $P<0.05$ was considered significant. Regressions were compared by analysis of covariance (ANCOVA). All statistical tests were performed with RudeStat, a DOS-based statistical package written by J.R.B.L., validated against Systat IV, and available by e-mail from him on request (john@johnlighton.org).

Results

Temporal response to ramped temperatures

An initial study used a ramping rate of 0.5 min^{-1}; however, at this rate, complete thermal equilibration between the ant and its surroundings did not occur. We determined this fact by observing occasional sharp declines of ca. 0.1–0.2°C in the intra-chamber temperature, especially with *P. rugosus*. The ant clinging briefly to the intra-chamber thermocouple caused these declines. Reducing the ramp rate to 0.25°C min^{-1} eliminated this effect.

A typical recording at a ramp rate of 0.25°C min^{-1} is shown in Fig. 2. The two species reacted to a ramp-based temperature challenge almost identically, with seven distinct phases. Following (1) the equilibration phase during which $\dot{V}_{CO_2}$ was constant, (2) ramping began, and the ants' $\dot{V}_{CO_2}$ increased exponentially. The exponential rise in $\dot{V}_{CO_2}$ terminated in (3) a 'premortal plateau' phase, during which $\dot{V}_{CO_2}$ did not increase with temperature. A steep decline in $\dot{V}_{CO_2}$ then occurred (4) during the course of which 'mortal fall' both spiracular control (as measured by $\dot{V}_{CO_2}$ ADS) and activity (as measured by activity ADS) abruptly ceased (which we call the 'theta point', shown in detail in Fig. 3; see Discussion); this was followed by (5) 'postmortal valley', a low point in post-mortal $\dot{V}_{CO_2}$. After this, $\dot{V}_{CO_2}$ rose again, into (6) the 'postmortal peak', before slowly declining (7) with a classic exponential decay which progressed, if the recording was allowed to continue for long enough, back to baseline levels. It is worth re-emphasizing that CT_{max} occurs during phase 4.

The results are summarized in Table 1. Some supplemental information is provided below, divided among the seven phases described above.

Equilibration phase

The nominal temperature during the equilibration phase was 45°C. The actual measured temperature at the ants' location was 44.78±0.14°C, which does not differ significantly from

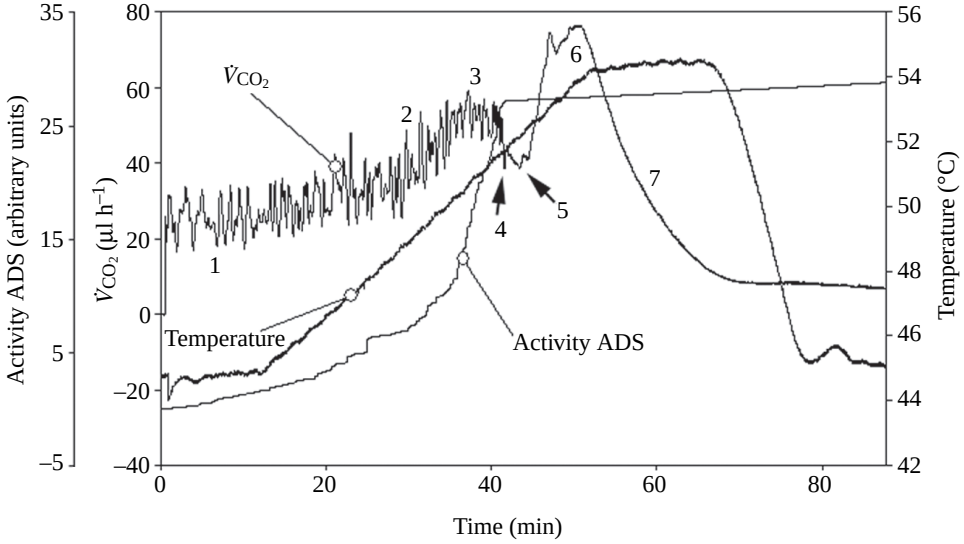

Fig. 2. Thermolimit respirometry on an ant, *Pogonomyrmex rugosus*, mass 10.2 mg (run no. AFPR_011). Clearly visible on the $\dot{V}_{CO_2}$ trace (left scale adjacent to graph; µl h^{-1}) are the seven stages of the ant's response: equilibration (1; ca. 0–10 min), ramping (2; ca. 10–36 min), premortal plateau (3; ca. 36–40 min), mortal fall (4; ca. 40–44 min), postmortal valley (5; ca. 45 min), postmortal peak (6; ca. 50 min) and exponential decay (7; >ca. 50 min) phases. See text for details and definitions. The equilibration temperature (right scale) was 44.84°C. The ramping rate was 0.254°C min^{-1}. The CT_{max} of this ant was 51.87°C (spiracular) and 51.68°C (locomotor), defined as the temperature at the breakpoint of the lag-corrected $\dot{V}_{CO_2}$ ADS trace and the activity ADS trace, respectively. $\dot{V}_{CO_2}$ begins with a brief baseline. The units of the activity ADS scale are arbitrary (left scale); the $\dot{V}_{CO_2}$ ADS trace is similar and is not shown. Note that the absolute value of the ADS trace is shown; where activity is more intense (especially during the premortal plateau) the slope of the ADS trace *vs.* time increases dramatically, only to inflect to near zero at the theta point (see Fig. 3). Only the slopes *vs.* time of the ADS traces, and not their absolute magnitudes, were used for analysis.

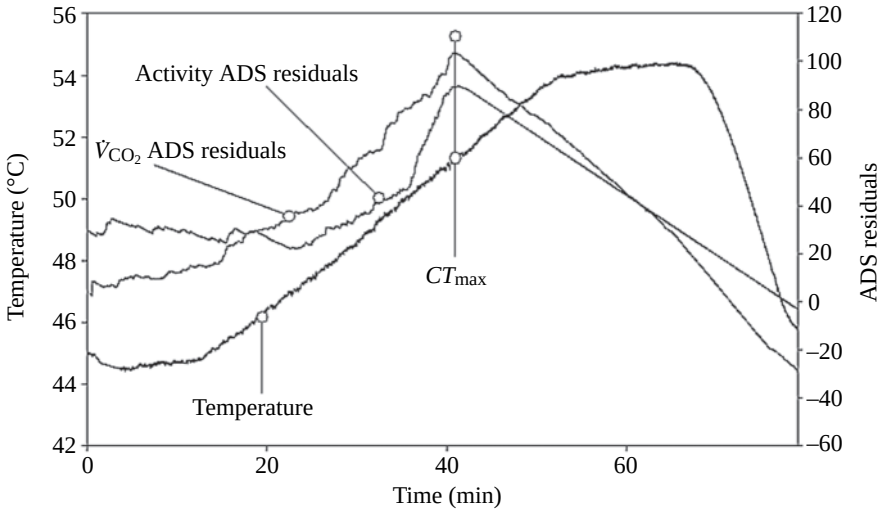

Fig. 3. Objective determination of CT_{max}, using the residuals of the linear regressions *vs.* time of the absolute difference sums (ADS) of $\dot{V}_{CO_2}$ and of activity. Each ADS is a cumulative sum of the absolute differences between adjacent sample points in the respective data channel; the residuals of the linear regressions of each ADS *vs.* time clearly shows an inflection point for each ADS at which spiracular control (in the case of $\dot{V}_{CO_2}$) or motor control (in the case of activity) ceases. See Fig. 2 for reference. We operationally define the inflection point, as measured using our methodology, as the theta point and consider it to be congruent with behavioral measures of CT_{max}. See text for details.

45°C (P>0.12). During equilibration, both species were consistently active, with activity ADS slopes *vs.* time being 9–12× postmortal levels. (Postmortal ADS slopes served as zero-activity controls).

Ramping phase

The nominal ramping rate was 0.25°C min^{-1}. The actual measured ramping rate, measured at the ants' location using a fine-gauge thermocouple, was not significantly different at 0.249±0.003°C min^{-1} (t=0.12; P>0.4). Activity levels, as measured by the slope of the activity ADS *vs.* time, increased relative to equilibration levels. Because of the elevated activity levels, $\dot{V}_{CO_2}$ increased more rapidly with temperature than would be expected from measurements made on inactive ants, which yield a Q_{10} near 2 (see Lighton and Bartholomew, 1988). The slope of $\log_{10}$-transformed $\dot{V}_{CO_2}$ on temperature corresponded to a Q_{10} of 3–4, which did not differ between species (P=0.15).

Premortal plateau phase

Both species reached plateau values of $\dot{V}_{CO_2}$ prior to achieving CT_{max}. During this plateau, $\dot{V}_{CO_2}$ did not vary with temperature. The slope of $\log_{10}$-transformed $\dot{V}_{CO_2}$ *vs.* temperature during the plateau was 0.026±0.055 for *Pogonomyrmex rugosus*, which did not differ significantly from the value for *Pogonomyrmex californicus* of 0.021±0.037 (P>0.4). Neither slope differed significantly from 0 (P>0.4). In terms of temperature, the plateau was nearly 1°C wide in

Table 1. *Thermolimit respirometry on two ant species,* Pogonomyrmex californicus *and* P. rugosus

Parameter (units)	*P. californicus*	*P. rugosus*
Sample size (*N*)	19	20
Body mass (live) (mg)	7.38±0.80	13.6±2.02***
Equilibration $\dot{V}_{CO_2}$ (μl h^{-1})	17.3±4.4	25.4±3.3***
Plateau $\dot{V}_{CO_2}$ (μl h^{-1})	51.8±5.7	53.3±7.8
Plateau entry (°C)	50.38±0.31	50.29±0.42
Plateau exit (°C)	51.32±0.34	51.25±0.39
Activity ADS CT_{max} (°C)	51.74±0.25	51.57±0.38
$\dot{V}_{CO_2}$ ADS (CT_{max}) (°C)	51.78±0.37	51.59±0.35
$\dot{V}_{CO_2}$ at CT_{max} (μl h^{-1})	39.6±9.3	44.1±9.8
Valley $\dot{V}_{CO_2}$ (μl h^{-1})	29.0±6.5	36.7±7.3**
PM Peak $\dot{V}_{CO_2}$ (μl h^{-1})	74.8±9.5	75.9±15.8
Decay TC (min^{-1})	0.0673±0.0099	0.0487±0.0100***

$\dot{V}_{CO_2}$, rate of production per ant. Equilibration $\dot{V}_{CO_2}$ was at 45°C. ADS, absolute difference sum (see text and Fig. 3 for definition and discussion). PM, postmortal (see text); TC, time constant for post-PM peak $\dot{V}_{CO_2}$.

Asterisks indicate significant differences between species: *P<0.05; **P<0.01; ***P<0.001.

both species, and therefore lasted on average for just under 4 min. Even over this rather narrow range of variance in start and end plateau temperatures, the higher the temperature at which an ant entered the plateau phase, the higher the temperature at which it left it. This consistency in the duration of the plateau phase thus shows no evidence for a proscribed or absolute exit temperature. Plateau entry temperature explained 64% of plateau exit temperature variance in a positive direction (Fig. 4). Neither species differed from each other in this relationship (ANCOVA: $F_{[1,35]}$=0.72; $P_{[\text{same slope}]}$=0.4; $F_{[1,36]}$=0.05; $P_{[\text{same intercepts}]}$=0.4).

A huge increase in the activity ADS slope *vs.* time occurred during the premortal plateau phase, elevating activity ADS slopes over 50-fold above inactive control (postmortal) levels. In *P. rugosus* the activity ADS slope was 57.6±29.2, while in *P. californicus* it was 54.6±29.4. These figures do not differ significantly (P>0.3). However, both figures are highly significantly greater than each species' activity ADS slope during the ramp (P<0.0001). The change in spiracular ADS slope, however, was not as marked. In *P. rugosus* spiracular ADS slope was 4.3±1.5, while in *P. californicus* it was 3.9±1, not significantly different (P>0.3). Only in the case of *P. californicus* was the spiracular ADS slope significantly elevated relative to ramp levels (P<0.01).

An unexpected finding was the constant magnitude of $\dot{V}_{CO_2}$ across species during the plateau. Even though *P. rugosus* weighs 84% more than *P. californicus*, and although its equilibrium $\dot{V}_{CO_2}$ at 45°C was significantly higher, the $\dot{V}_{CO_2}$ values of the two species during the plateau were not significantly different (Table 1).

Mortal fall phase

At the termination of the premortal plateau, $\dot{V}_{CO_2}$ fell steeply. Within 2 min, control of the spiracles and voluntary motor control ceased, operationally constituting CT_{max}. Whether measured *via* activity ADS or spiracular ADS, the CT_{max} of *P. californicus* was slightly, but not significantly, higher than that of *P. rugosus*. Because neither the activity nor $\dot{V}_{CO_2}$ ADS estimates of CT_{max} differed significantly between species, it was possible to pool data across species and compare the two independent estimates of CT_{max} directly. In that case the pooled CT_{max} as determined by the activity ADS breakpoint was 51.66±0.33°C. For the spiracular or $\dot{V}_{CO_2}$ ADS breakpoint, the CT_{max} was 51.68±0.37°C. The two means are only 0.02°C apart, an insignificant difference (t=–0.33, P>0.4). To put it another way, the mean difference between activity and $\dot{V}_{CO_2}$-based estimates of CT_{max} was only 0.02±0.24°C, which does not differ significantly from zero (P>0.4). Thus our null hypothesis was not disproved.

In any given ant of either species, even given the restricted range of CT_{max} values, a high activity ADS-derived CT_{max} predicted a high $\dot{V}_{CO_2}$ ADS-derived CT_{max}. By regression analysis, either measure explained 59% of the variance in the other (Fig. 5). The relation between activity ADS-derived CT_{max} and $\dot{V}_{CO_2}$ ADS-derived CT_{max} did not differ between species (ANCOVA: $F_{[1,35]}$=0.48; $P_{[\text{same slope}]}$=0.4; $F_{[1,36]}$=0.40; $P_{[\text{same intercepts}]}$=0.4).

The $\dot{V}_{CO_2}$ at CT_{max} of an ant of either species was 10.5 μl h^{-1} lower than its maximal $\dot{V}_{CO_2}$ (as

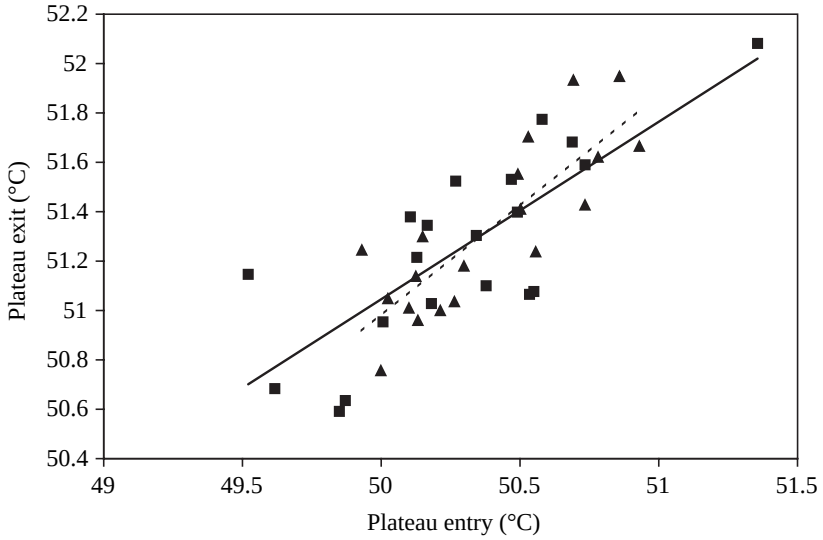

Fig. 4. Entering the premortal plateau section of the ramp response (see text and $\dot{V}_{CO_2}$ trace in Fig. 2) at a higher temperature predicts a higher exit temperature. A type 1 (predictive least squares) regression fit shows that 64% of the variance of exit temperature is explained by entry temperature (line equation: XT=12.1+0.779NT, where XT = exit temperature in °C and NT = entry temperature in °C; P<10^{-6}). Squares, *P. californicus*; triangles, *P. rugosus*; the species do not differ significantly (see text).

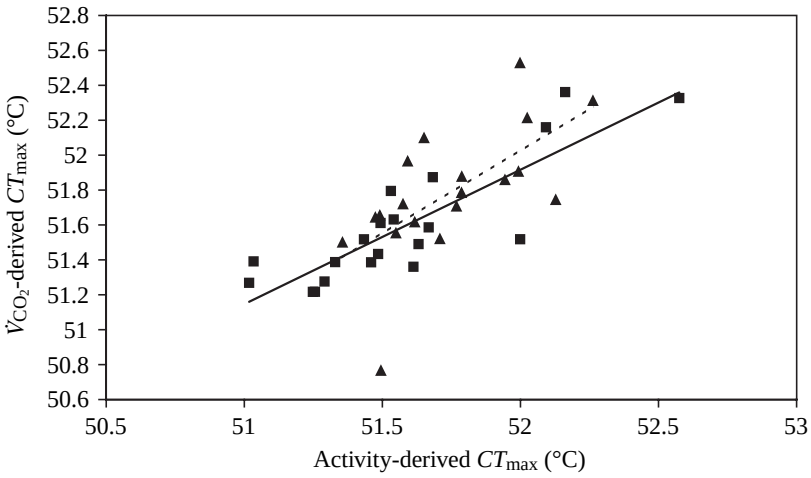

Fig. 5. A high activity ADS-derived CT_{max} predicts a high spiracular control ($\dot{V}_{CO_2}$)-derived CT_{max}. This holds true in spite of the relatively narrow range of CT_{max} values. A type 1 (predictive least squares) regression fit shows that 59% of the variance of $\dot{V}_{CO_2}$ ADS-derived CT_{max} is explained by activity-derived ADS CT_{max} (line equation: VT=8.1+0.845AT, where AT = activity-derived ADS CT_{max} in °C and VT = $\dot{V}_{CO_2}$ ADS-derived CT_{max} in °C; $P<10^{-6}$). Squares, *P. californicus*; triangles, *P. rugosus*; the species do not differ significantly (see text).

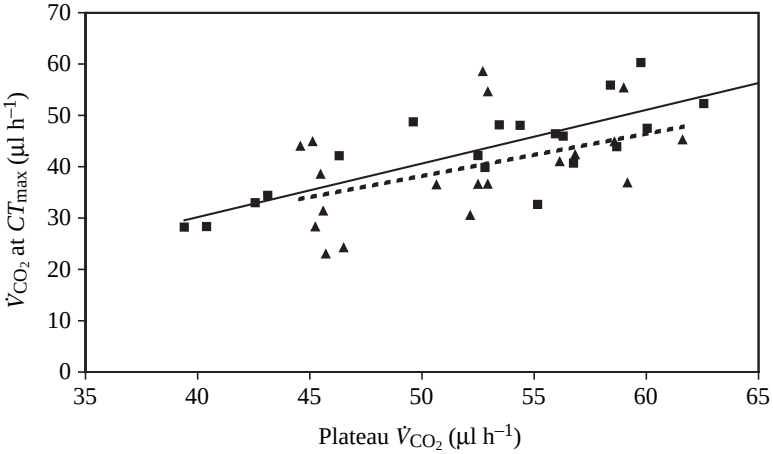

Fig. 6. A high premortal plateau $\dot{V}_{CO_2}$ predicts a high $\dot{V}_{CO_2}$ at CT_{max}. A type 1 (predictive least squares) regression fit shows that 46% of the variance of premortal plateau $\dot{V}_{CO_2}$ is explained by $\dot{V}_{CO_2}$ at CT_{max} (line equation: TV=–10.5+0.998PV, where TV = $\dot{V}_{CO_2}$ at CT_{max} in µl h^{-1} and PV = premortal plateau $\dot{V}_{CO_2}$ in µl h^{-1}; $P<10^{-6}$). The $\dot{V}_{CO_2}$ at CT_{max} is the $\dot{V}_{CO_2}$ at the $\dot{V}_{CO_2}$ ADS breakpoint; the breakpoint is also the marker for CT_{max}. Squares, *P. californicus*; triangles, *P. rugosus*; the species do not differ significantly, in spite of the large difference in body mass between the two species (see text for statistics).

determined by plateau $\dot{V}_{CO_2}$). By regression analysis, plateau $\dot{V}_{CO_2}$ explained 46% of the variance in $\dot{V}_{CO_2}$ at CT_{max} (Fig. 6). The relation between plateau $\dot{V}_{CO_2}$ and CT_{max} did not differ between species (ANCOVA: $F_{[1,35]}$=0.33; $P_{[\text{same slope}]}$=0.4; $F_{[1,36]}$=1.51; $P_{[\text{same intercepts}]}$=0.2). The slope of the consensus relation was 1.0 (0.998±0.177) while the intercept was –10.5.

Valley phase

During this phase, $\dot{V}_{CO_2}$ declined to a minimum in both species (Table 1).

Post-mortal peak phase

After declining to the valley values shown in Table 1, $\dot{V}_{CO_2}$ increased to a well-defined peak (see Fig. 2) that reached its highest value approximately 10 min after CT_{max} (10.4±2.4 min in *P. rugosus* and 10.1±1.4 min in *P. californicus*). As with premortal plateau $\dot{V}_{CO_2}$ values, these $\dot{V}_{CO_2}$ values are not mass dependent. However, an ant that displayed a high premortal plateau $\dot{V}_{CO_2}$ value also tended to display a high postmortal peak $\dot{V}_{CO_2}$ value. The premortal plateau $\dot{V}_{CO_2}$ value explained 28% of the variance of postmortal peak $\dot{V}_{CO_2}$, with the two species having equivalent slopes (ANCOVA; P>0.4; shared slope 1.01) and equivalent intercepts (ANCOVA; P>0.4; shared intercept 22.2 µl h^{-1}).

Exponential decay phase

After reaching a peak of CO_2 output, each ant displayed a classic e^{-kt} decay curve of CO_2 output *vs.* time (Fig. 2; see Table 1). The fit of the experimental data to this model was >95% in all cases. The larger time constant in *P. californicus* is to be expected in view of the smaller mass of *P. californicus*, and thus larger ratio of internal volume to surface area.

Discussion

Terminology

To our knowledge, no one has systematically described the metabolic responses to lethal ramped temperature challenges in an insect or other tracheate arthropod. However, examining the metabolic responses of other organisms to ramped temperatures that meet or exceed the CT_{max} is not new (Pörtner, 2001, 2002, and references therein). Our ants reacted to a ramped thermal challenge in much the same metabolic way as other organisms, as described by Pörtner (*ibid.*). Of course

the methodology, terminology and physiology differ between marine invertebrates and tracheate terrestrial arthropods, which do not rely on indirect oxygen transport *via* a circulatory system and respiratory pigments. This is especially so in the plateau phase of metabolic response, where Pörtner reasonably refers to the peak of oxygen availability as 'optimus'. In our ants the plateau phase of CO_2 output represents an extreme physiological challenge just minutes (or about 1°C) separated from death. And, while the term 'pejus' (getting worse) describes the post-plateau decline in oxygen availability in marine invertebrates, it could be said that the mortal fall in CO_2 output in insects is more terminal than pejorative and, in light of the insect tracheal system, is unlikely to be mediated by tissue-level hypoxia. We have therefore elected to use terms that are more applicable to tracheate, terrestrial organisms.

One area in which we have respectfully parted from standard terminology is to use the term *theta point* (from ΘΑΝΑΤΟΣ, death, brother of sleep) for the operationally defined, objective endpoint that marks CT_{max}. This reflects its distinct methodological origin from conventional endpoints determined by subjective inspection. We nevertheless consider the theta point to be broadly congruent with observationally determined LRR/OS CT_{max}.

Standardization issues

As mentioned in the Results, we found a significant lag between chamber and ant temperatures at a ramping rate of 0.5°C min^{-1}. We are therefore forced to conclude that because of this lag effect, data obtained using fast temperature ramps (e.g. Wehner et al., 1992; 1°C min^{-1}) may overestimate CT_{max}. As noted in the Introduction, ramp rates should be appropriate to mass and phylogeny (RRAMP); phylogenetic considerations are of themselves inclusive of anatomical differences and their thermal qualities. We suggest that ramp rates be standardized to the extent that differing organism masses allow acceptable thermal equilibration to occur without imposing an unacceptable water loss penalty. Of course, this means that the organisms may be exposed to stressful temperatures for a longer time (see Introduction), further reducing measured CT_{max}. Yet this apparent shortcoming may yield useful data if CT_{max} values at different ramp rates are compared within a single study, provided that acceptable thermal equilibration of the experimental organism is achieved. In particular, we anticipate that thermolimit respirometry will be a useful tool for examining the induction of thermoprotective mechanisms.

The post-mortal valley and peak

The post-mortal peak is an unexpected phenomenon. The ants lost spiracular control during the mortal fall (at the theta point = CT_{max}). The spiracles in insects are open by default unless held closed by spiracular closer muscles. It was therefore expected that $\dot{V}_{CO_2}$ should continue to fall after CT_{max} was reached, but unexpectedly it then not only rose, but also slowly increased to levels 50% greater than those maintained at the plateau phase. In other words, after death the ants were capable of a greater effective $\dot{V}_{CO_2}$ than while alive. But from whence did this CO_2 come? The excess CO_2 in the portmortal peak is obviously not from CO_2 stored in the trachea, because such stores would have been released as soon as spiracular control was lost. The two other alternatives are the release of dissolved or bound CO_2 in the hemolymph and tissues, and a short-lived burst of mitochondrial activity. The former alternative at first appears unlikely, because such a release of bound CO_2 would surely have occurred immediately after spiracular control was lost and would (it is reasonable to assume) not have increased in magnitude, but merely fallen gradually after death.

It is likely, however, that intracellular [ATP] plummets in the minutes following the theta point because of ATP-demanding cellular processes. Once [ADP] reaches high enough levels, [ADP]-modulated reactions will accelerate if they are still capable of doing so and if metabolic substrates remain available. If these reactions are primarily anaerobic, a downward shift in hemolymph and/or intracellular pH, as the result of lactate accumulation caused by anaerobic metabolism, could be responsible for 'blowing off' the CO_2 by shifting the bicarbonate/CO_2 equilibrium towards CO_2. (This may also explain the post-mortal valley, during which reserves of ATP may not yet be depleted to the point where [ADP]-modulated reactions begin to increase CO_2 flux.)

Oxygen was freely available through the open spiracles, however, so this anaerobic explanation is only valid if the mitochondria were unable to process the end products of glycolysis (see also Denlinger and Yokum, 1998; Pörtner, 2001, 2002). This raises the second possibility – namely, that mitochondrial respiration is able to take place at a rapid rate in the brief period during which ATP demand is high, respiratory substrates remain available, and the subcellular machinery remains intact. Distinguishing between the anaerobic blowoff and accelerated mitochondrial respiration hypotheses should not be difficult; it merely requires manipulation of external oxygen partial pressure.

Comparative critical thermal maxima

Within animals

The activity ADS and $\dot{V}_{CO_2}$ ADS estimates of CT_{max}, measures of motor control and spiracular control, respectively, gave almost identical results. [Pooled value: CT_{max} ADS activity breakpoint: 51.66±0.33°C; CT_{max} spiracular or $\dot{V}_{CO_2}$ ADS breakpoint: 51.68±0.37°C.] This interchangeability of independent, activity-based and metabolic-based measures shows that our methodology for determining CT_{max} is robust. It might also be mentioned for the ecological context that this CT_{max} is lower than the observed maximal foraging temperatures noted in the Introduction, which are substrate, not animal, temperatures.

The low coefficient of variation (CV) of our observations (<1%) is unusual in thermal biology. For example, Gehring and Wehner (1995), using the ramp method, report CV values of 2–3% in *Cataglyphis bicolor* and *Formica polyctena* (assuming that their unspecified variance statistics are standard deviations; if they are standard errors, the reported CV values

increase to 12–18%). In a comprehensive study of domesticated *vs.* feral honeybees, Atmowidjojo et al. (1997), also using the ramp method, reported CV values ranging from 12–21%. Using the total immersion method, Berrigan and Hoffmann (1998) report CV values of 30% and 28% for *Drosophila birchii* and *D. serrata*, respectively.

A further line of evidence emphasizes the robustness of the 'physiological democracy' correlation between voluntary motor activity and spiracular-control measures of thermal stress. Even though the variability in CT_{max} was minor (CV<1%), activity and $\dot{V}_{CO_2}$ ADS values were strongly correlated (Fig. 4). Thus ants in either species with high CT_{max} values in one measure tended to have high CT_{max} values in the other (for a different example, see also Berrigan, 2000).

The almost complete congruence between CT_{max} values derived from locomotor and spiracular effectors suggests that control of the two disparate effector systems is lost *via* the failure of a common heat-sensitive mechanism in the CNS. It is worth noting that although the spiracular closer muscles may be inactivated directly by hypercapnia, they are under direct CNS control as well (see reviews by Kestler, 1985; Lighton, 1996, and references therein).

It follows that a sub-set of thermolimit respirometry, i.e. simple optical activity detection along with temperature ramping, may yield CT_{max} values equivalent to those obtained when using flow-through respirometry in addition or by itself. Therefore, unless gas exchange data are required, investigators may obtain equivalent data using a simpler 'thermolimit activity' system. That being said, obtaining two independent measures of CT_{max} does add confidence to the result.

Between species

The only significant differences found in this study between these two sympatric and congeneric harvester ant species were, for *P. californicus*, (a) much smaller mass, (b) higher mass-specific rate of CO_2 emission as measured during the premortal plateau and (c) greater degree of spiracular control (as measured by $\dot{V}_{CO_2}$ ADS slope) during the premortal plateau; it may also exhibit greater variability in its plateau entry/exit point temperatures. The more thermophilic behavior of *P. californicus* relative to *P. rugosus* is thus not explained by CT_{max}. It is, however, supported at least in part by the above physiological adaptations and/or exaptations. The biophysical criteria separating the two congeners facilitate the observed behavioral divergence in the field by allowing more rapid dumping of heat during thermal refuge behavior (a), and by allowing higher mass-specific rates of metabolic flux (b, c) thus permitting the more intense motor activity required to exploit thermal refuges, especially when laden. Phenomena such as biochemical induction of thermotolerance or behavioral frequency of achieving thermal refuges should be considered in further studies.

To summarize, the mass-specific maximum aerobic capacity of *P. californicus* is higher than that of *P. rugosus*, serving its ability to seek out thermal refuges under heat-stressed conditions, while its lower mass allows it to dump heat more rapidly. The result is a suite of characteristics that raise its maximum foraging temperature by about 7–8°C relative to *P. rugosus* in spite of an equivalent CT_{max} under the conditions of our experiments. Our null hypothesis that *P. californicus* would display the same CT_{max} as *P. rugosus* was not disproved.

Was the higher aerobic capacity of *P. californicus* an exaptation (along with smaller body size) that facilitated solitary foraging and the athletic exploitation of thermal refuges, or did that higher aerobic capacity (and smaller body size) evolve in response to niche separation pressures and accompanying behavioral selection? We are aware that assigning a direction to the arrow of causation is problematic and rife with potential phylogenetic complications. Our study reminds us again (see Huey and Stevenson, 1979) that ecological and evolutionary divergences do not always have simple biophysical explanations.

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