The Journal of Experimental Biology 215, 2319-2326

doi:10.1242/jeb.070342

RESEARCH ARTICLE

Differences in critical thermal maxima and mortality across life stages of the mealworm beetle *Tenebrio molitor*

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SUMMARY

Thermal limits to activity profoundly affect the abundance and distribution of ectothermic animals. Upper thermal limits to activity are typically reported as the critical thermal maximum (CT_{max}), the temperature at which activity becomes uncontrolled. Thermolimit respirometry is a new technique that allows CT_{max} to be quantified in small animals, such as insects, as the point of spiracular failure by measuring CO_2 release from the animal as temperature increases. Although prior studies have reported a characteristic pattern of CO_2 release for insects during thermolimit respirometry trials, no studies have been carried out to determine the universality of this pattern across development, or at what point death occurs along this pattern. Here, we compared the CT_{max} and patterns of CO_2 release among three life stages of a beetle species, *Tenebrio molitor*, and mapped heat death onto these patterns. Our study is the first to report distinct patterns of CO_2 release in different life stages of an insect species during thermolimit respirometry. Our results show that CT_{max} was significantly higher in adult beetles than in either larvae or pupae ($P<0.001$) and, similarly, death occurred at higher temperatures in adults than in larvae and pupae. We also found that death during heating closely follows CT_{max} in these animals, which confirms that measuring the loss of spiracular control with thermolimit respirometry successfully identifies the point of physiological limitation during heat stress.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/215/13/2319/DC1

Key words: thermolimit respirometry, critical thermal maximum, life stage, spiracular activity.

Received 17 January 2012; Accepted 13 March 2012

INTRODUCTION

Thermal limits to activity are profoundly important for determining the abundance and geographic distribution of ectothermic animals. Understanding thermal limitation is a topic of growing interest among biologists, primarily because of concerns regarding the effects of global climate change on biodiversity (Deutsch et al., 2008). Our ability to identify the physiological factors that underlie temperature-related distribution patterns is highly important for making predictions about where species will occur and how they will perform in warmer climates (Somero, 2010). Thermal limits to activity are often determined experimentally as the critical thermal maximum (CT_{max}) and minimum (CT_{min}) (Klok and Chown, 2003; Folk et al., 2007; Terblanche et al., 2007; Nyamukondiwa and Terblanche, 2009), which are the temperatures at which activity becomes uncontrolled (see Cowles and Bogart, 1944). Although critical thermal limits are widely measured, methods for identifying CT_{max} and CT_{min} are highly varied. To determine upper thermal limits, CT_{max} is commonly measured by identifying the temperature at which behavioral endpoints (loss of righting response, onset of muscle spasms, knock down) occur as temperature increases (reviewed by Lutterschmidt and Hutchison, 1997). As behavioral responses must be identified visually by the experimenter, these measurements of CT_{max} strongly depend on both the visual clarity of the animal subject and the skill and vigilance of the observer.

Prior work has shown that CT_{max} measurements depend on a variety of factors, especially those that influence the thermal history of the animal. These include season (Terblanche et al., 2006), hardening and acclimation (Jumbam et al., 2009; Nyamukondiwa and Terblanche, 2010), and aspects of the experimental design such as the rate of temperature increase (Terblanche et al., 2007; Chown et al., 2009). Terblanche and colleagues demonstrated that both the rate of temperature change and the starting temperature significantly impact upper and lower critical limits in tsetse flies (Terblanche et al., 2007). For example, they showed that using faster rates of change and starting at temperatures closer to the thermal limit both result in higher estimates of CT_{max} and lower estimates of CT_{min} (Terblanche et al., 2007). The results of that study highlight the importance of experimental design on the outcome of CT_{max} experiments, and the need to clearly define which experimental conditions are used.

Recently, a technique termed ‘thermolimit respirometry’ was developed (Lighton and Turner, 2004) that allows CT_{max} to be identified as the point of spiracular and/or motor failure. The technique employs flow-through respirometry to measure CO_2 output from the animal during increasing temperature, typically with simultaneous infrared activity detection to monitor locomotor activity (Lighton and Turner, 2004; Klok et al., 2004; Folk et al., 2007; Lighton, 2007; Stevens et al., 2010). Prior studies have shown that these two independent measures of thermal limitation, spiracular and motor failure, occur simultaneously in adult insects tested under normoxic conditions, and thus represent statistically equivalent estimates of CT_{max} (Lighton and Turner, 2004; Klok et al., 2004; Folk et al., 2007; Stevens et al., 2010). The technique has several advantages. First, it allows CT_{max} to be identified directly from respiratory and/or activity data, rather than by visual inspection alone. Additionally, it permits CT_{max} to be determined without

disturbing the animal. This is an important advantage over techniques that require repeated disturbances during the experiment (i.e. assessments of righting response), given that these methods have been shown to produce significantly higher CT_{max} values compared with assessments of undisturbed animals (Stevens et al., 2010). Because of these advantages, thermolimit respirometry continues to gain popularity as a technique among investigators measuring CT_{max}, particularly in arthropods.

Prior studies using thermolimit respirometry have reported that tracheated arthropods (i.e. most insects) produce a characteristic pattern of CO_2 output during increasing temperature, which includes seven phases: (1) $\dot{V}_{CO_2}$ remains constant while temperature is maintained at the start temperature (equilibration), (2) $\dot{V}_{CO_2}$ increases exponentially as temperature increases (ramping), (3) $\dot{V}_{CO_2}$ reaches a plateau, (4) $\dot{V}_{CO_2}$ drops abruptly and spiracular activity ceases (mortal fall), (5) $\dot{V}_{CO_2}$ declines to a low point (valley), (6) $\dot{V}_{CO_2}$ rises to form a large peak (post-mortal peak) and, finally, (7) $\dot{V}_{CO_2}$ decays exponentially after death (Lighton and Turner, 2004) (see also Fig. 1). The dramatic reduction in $\dot{V}_{CO_2}$ variance that occurs during the mortal fall phase signifies the loss of spiracular control, and therefore CT_{max}. This seven-phase pattern has been described in a variety of insects, including harvester ants (Lighton and Turner, 2004), drosophilid flies (Folk et al., 2007; Lighton, 2007) and tenebrionid beetles (Klok et al., 2004; Stevens et al., 2010). Only isopods have shown a different pattern of CO_2 release, which may result from differences in respiratory morphology between isopods and insects (Klok et al., 2004; Stevens et al., 2010). However, the seven-phase pattern shows little variation in those insects with tracheated respiratory systems gated by spiracular closure muscles (Lighton and Turner, 2004; Klok et al., 2004; Folk et al., 2007; Lighton, 2007; Stevens et al., 2010).

Because this $\dot{V}_{CO_2}$ pattern is a fundamental component of thermolimit respirometry, accurate determination of CT_{max} using this technique requires that the phases of the pattern be both well understood and highly repeatable. However, to date there has been no formal investigation of the variability in the $\dot{V}_{CO_2}$ pattern in the different developmental stages of an insect. Specifically, work is needed to determine the universality of the CO_2 release patterns across development. Additionally, it is not known at what point during this response death occurs under sustained heating, or how lethal temperatures relate to CT_{max}. Unlike sub-lethal assessments of CT_{max} (such as knock-down experiments), thermolimit respirometry requires the animal to be exposed to a sustained thermal, which continues even after CT_{max} is reached. As temperature increases, CT_{max} will occur first during the ramp, and if heating is sustained beyond this, the animal will later die. Although it is known that death occurs at some point following CT_{max} under sustained heating, it is not known how closely lethal temperatures and CT_{max} are related, or which phase of the CO_2 release pattern corresponds to death. We have used thermolimit respirometry to investigate these responses in three life stages of *Tenebrio molitor* beetles during increasing temperature. Our objectives were (i) to determine the extent to which patterns of CO_2 release during heating are consistent across life stages of *T. molitor*, (ii) to compare three different methods of analysis for determining CT_{max}, (iii) to compare CT_{max} across life stages, and (iv) to map lethal temperatures onto the pattern of CO_2 release for each life stage. The results of this study provide the first comparison of CO_2 release patterns during heating across the developmental stages of an insect, and the first experimental link between mortality and the CO_2 release pattern produced by insects during thermolimit respirometry.

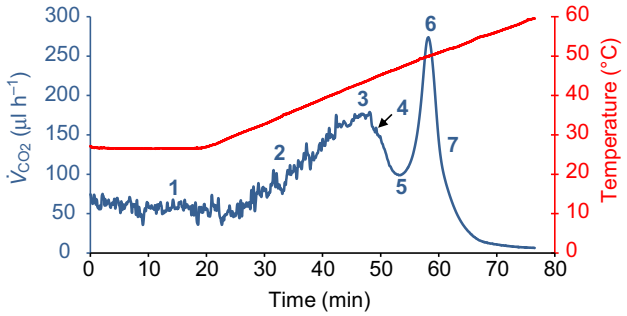

Fig. 1. A representative trace from a thermolimit respirometry trial in an adult *Tenebrio molitor* beetle. $\dot{V}_{CO_2}$ (blue) is shown over time as temperature (red) increased. (1) $\dot{V}_{CO_2}$ remained stable during the equilibration phase, (2) increased as temperature was increased at 0.5°C min^{-1}, (3) reached a plateau at maximal $\dot{V}_{CO_2}$, (4) until spiracular activity ceased (critical thermal maximum, CT_{max}) during the mortal fall, (5) followed by a pre-mortal valley and (6) a post-mortal burst of CO_2 release, and (7) exponential decay. The black arrow indicates CT_{max} measured at the cessation of spiracular activity.

MATERIALS AND METHODS

Study animals

Larval, pupal and adult *T. molitor* L. beetles from a colony maintained in our laboratory at the University of California, Irvine, were used for these experiments. All life stages were kept together in a glass terrarium containing old-fashioned rolled oats (Quaker, Chicago, IL, USA) to a depth of 3 cm, Brewer's yeast flakes (Lewis Laboratories International, Southport, CT, USA) to supplement nutrition, and a beaker filled with water to increase relative humidity. Beetles were kept at room temperature (25°C) on a 12 h:12 h L:D cycle. Prior to each experimental trial, the beetle subject was weighed on an analytical balance (Mettler Toledo AB104, Riverside, CA, USA) to the nearest 0.1 mg. Live mass (mean ± s.e.m.) was 180.1±8.8 mg for larvae, 167.8±7.1 mg for pupae and 132.3±6.8 mg for adults.

Respirometry and manipulating temperature

Thermolimit respirometry (Lighton and Turner, 2004) was employed using a flow-through respirometry system to measure CO_2 release from each beetle while the air temperature was steadily increased. Respirometry chambers (2 ml volume) were constructed from plastic syringes and sealed with a single-hole rubber stopper (no. 1). Each chamber was lined with fine mesh to prevent the beetles from escaping through the entrance and exit ports, and to promote turbulent flow of air within the chamber.

Air was pushed across two columns of silica gel and one column of Ascarite to remove water and CO_2, respectively, and through a mass flow controller (0–200 ml model, Side-trak, Sierra Instruments, Monterey, CA, USA) to maintain the air flow at 100 ml min^{-1}. The air stream then entered a temperature-controlled cabinet (Sable Systems International, Las Vegas, NV, USA), where it passed through a coil of copper tubing (160 cm length) to ensure thermal equilibration of the air before entering the respirometry chamber where the insect was held. The air leaving the chamber then exited the temperature-controlled cabinet and entered an infrared CO_2 analyzer (LiCor, Model LI-6251, Lincoln, NE, USA).

The air temperature was manipulated using a temperature-controlled cabinet wired to a PELT-5 temperature controller (Sable Systems International). The temperature of the airstream flowing across the beetle was measured using a thermocouple wire inserted

into the tubing at the exit of the respirometry chamber, and monitored using a TC-2000 thermocouple meter (Sable Systems International). The outputs from the thermocouple meter and CO_2 analyzer were connected to a laptop computer *via* a data acquisition interface (UI2 Interface, Sable Systems International).

Recording was initiated as air was pushed through an empty chamber to establish baseline CO_2 values for 1–2 min. Recording was paused as the beetle was placed into the respirometry chamber, then resumed after 2 min. At this point the temperature profile programmed into the PELT-5 was initiated. The profile began with a 20 min equilibration period at 25°C, followed by a temperature ramp at 0.5°C min^{-1} for 50 min, a 10 min period at 50°C, and finally a decrease in temperature at –10°C min^{-1} until the chamber returned to 25°C. Although 0.5°C min^{-1} is likely much greater than rates of temperature increase experienced by these insects under field conditions (see Terblanche et al., 2011), these experiments were designed to detect physiological differences in CT_{max} among life stages rather than to mimic ecological conditions. Therefore, the fastest rate of change that did not produce a lag effect [see Lighton and Turner for discussion of lag effects (Lighton and Turner, 2004)] was selected. Temperature (°C) and CO_2 concentration (p.p.m.) were recorded once per second for the duration of each trial using Expedata Software (Expedata PRO, version 1.3.4, Sable Systems International). This procedure was repeated in beetles at each life stage.

$\dot{V}_{CO_2}$ data were used exclusively to determine CT_{max} values in this study. Infrared activity detection was not used as we made comparisons between mobile and immobile life stages. We did not wish to use a form of analysis for two of the life stages (larvae and adults) that could not be used for the third (pupae). Therefore, we elected to use the $\dot{V}_{CO_2}$ patterns to determine CT_{max} in all three life stages. Although prior studies using thermolimit respirometry have been conducted exclusively in adult insects, these studies have shown that $\dot{V}_{CO_2}$ and activity measurements produce equivalent estimates of CT_{max} in tracheated insects tested under normoxia (Lighton and Turner, 2004; Folk et al., 2007; Klok et al., 2004; Stevens et al., 2010).

Mortality assays

All three life stages of *T. molitor* were used to determine the point of death during heating in order to map lethal temperatures onto the patterns of CO_2 release. To do this, we assessed recovery in individual beetles from each life stage after exposure to temperature profiles similar to those used for CT_{max} trials (25–50°C at 0.5°C min^{-1}). The same respirometry protocol used during CT_{max} experiments was employed, with the exception that the temperature increase was stopped at a specific, predetermined temperature between 46 and 50°C. Each beetle was then kept inside the respirometry chamber at room temperature (25°C) for 30 min for recovery. Beetles were scored as alive if a pre-ramping $\dot{V}_{CO_2}$ release pattern resumed within 30 min of returning to 25°C, or as dead if a pattern of controlled $\dot{V}_{CO_2}$ release did not resume. This procedure was repeated for multiple individual beetles of each life stage at each test temperature. To determine the relationship between the lethal temperatures and CT_{max}, we determined the percentage of individuals for which CT_{max} fell within the range of lethal temperatures for each life stage.

Determination of CT_{max}

Data analysis was performed using Expedata analysis software (version 1.3.4, Sable Systems International). $\dot{V}_{CO_2}$ data were first copied into a new channel for analysis, baseline corrected, and converted from p.p.m. to µl CO_2 h^{-1}. Corrected data were analyzed

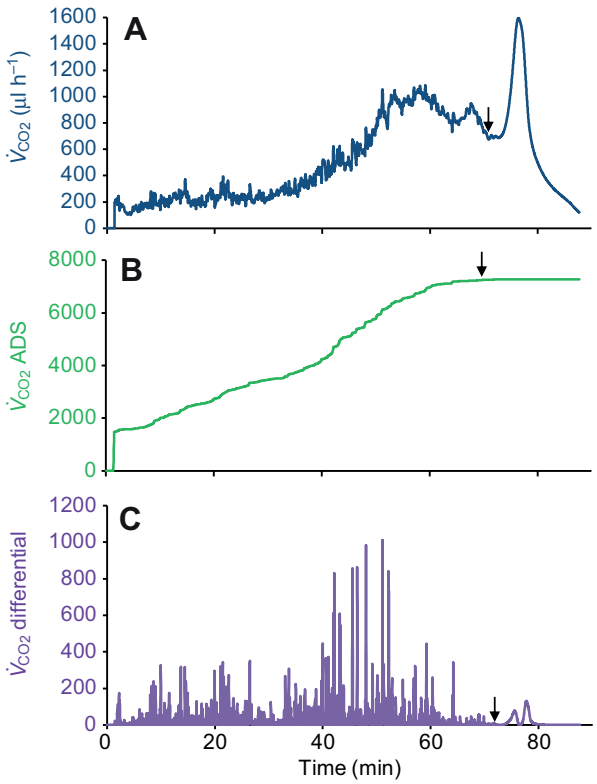

Fig. 2. The three analytical methods used to determine CT_{max} using thermolimit data. (A) CO_2 release (blue) from an adult beetle as temperature (not shown) increased over time during a CT_{max} test. This blue trace was used to determine CT_{max} as the lowest 10 consecutive $\dot{V}_{CO_2}$ data points in the pre-mortal valley (PMV). (B) The $\dot{V}_{CO_2}$ data were transformed into the absolute difference sum (ADS) (green), then fitted to a linear regression to identify CT_{max} as the 10 highest consecutive ADS residuals. (C) The differential of the $\dot{V}_{CO_2}$ data was calculated and squared to show the cessation of spiracular activity (CSA) (purple), and used to determine CT_{max} as the 10 data points immediately prior to where the differential flattens. The black arrows in each panel indicate the CT_{max} determined for this animal using each analytical method.

to determine CT_{max} using methods modified from Lighton and Turner (Lighton and Turner, 2004). Lighton and Turner propose identifying the inflection point in the absolute difference sum (ADS), which is the cumulative sum of the absolute differences between adjacent $\dot{V}_{CO_2}$ data points. This inflection point is suggested to be an objective method for identifying the point at which short-term variability in the $\dot{V}_{CO_2}$ data declines abruptly, indicating the cessation of spiracular activity by the insect. This technique has the advantage of being highly repeatable, and as objective as may be possible with these types of data. However, in employing these analyses we discovered that this method does not always identify the 'last breath' produced by the insect, especially in the pre-adult life stages. Therefore, we compared CT_{max} values identified using three distinct methods (see Fig. 2). We first identified CT_{max} as the ADS inflection point described above. In brief, the ADS inflection was determined by selecting the 10 min interval surrounding the expected CT_{max} point, and fitting a linear regression to these ADS data points. We identified the 10 highest consecutive residuals of this regression, and the mean temperature of these residuals was recorded as the ADS CT_{max} (see Fig. 2B). Second, to determine the temperature at which voluntary control of the spiracular muscles failed, we

identified CT_{max} as the mean temperature of the final 10 s before the cessation of spiracular activity (CSA). This point was located using the 'differentiate' and 'square' functions in Expedata to magnify differences between spiracular activity and electrical noise. The point at which this differentiated trace leveled to zero was then marked as the point of spiracular failure, and the final 10 data points prior to this point were selected. The mean temperature of these data points was recorded as the CSA CT_{max} (see Fig. 2C). Both of these CT_{max} measurements were completed using an Expedata macro (supplementary material Table S1). Lastly, we identified the temperature at the pre-mortal valley (PMV), a metric that has been favored by previous authors estimating CT_{max} with thermolimit respirometry in *T. molitor* (Stevens et al., 2010). The PMV was measured in Expedata using a nadir search function to find the lowest 10 consecutive points between the ramping phase and the post-mortal peak. The mean temperature of these points was recorded as the PMV CT_{max} (Fig. 2A).

Data analysis

Analysis of variance (ANOVA) was used to compare each of the three metrics (ADS, CSA, PMV) across life stages, and Tukey's HSD *post hoc* tests were used to separate statistically different groups. Prior to analyses, data were assessed for normality and equality of variance using Shapiro–Wilk and Barlett's tests, respectively. These analyses were completed in SAS (v. 9.2, SAS Institute, Cary, NC, USA). Data are presented as means ± s.e.m., and significance was set at P=0.05. For mortality assays, percentage survival at each test temperature was calculated and plotted separately for larvae, pupae and adults. The temperature at which death occurred during heating for each life stage was identified as the range of temperatures at which survival was less than 100% but greater than 0%. This range of lethal temperatures was mapped onto the pattern of CO_2 release for each life stage to compare the temperature at which death occurred with CT_{max}. A two-proportion z-test (Sheskin, 2004) was used to determine whether the proportion of surviving animals at each test temperature was significantly different between the life stages.

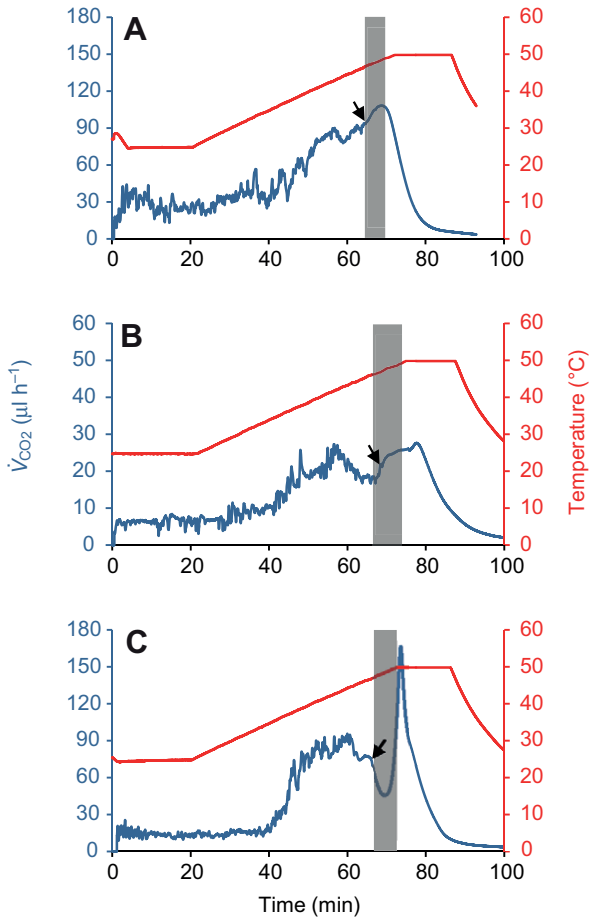

Fig. 3. Representative thermolimit respirometry trials for individual *T. molitor* beetles at each life stage. $\dot{V}_{CO_2}$ (blue) and temperature (red) are shown over time for an individual larva (A), pupa (B) and adult (C). Black arrows indicate CT_{max}. Shaded regions illustrate the range of temperatures during which mortality occurs for each life stage (see Fig. 4).

RESULTS

Patterns of CO_2 release

We compared the patterns of CO_2 release in larval, pupal and adult life stages of *T. molitor* using thermolimit respirometry. Each animal was acclimated for 20 min at 25°C before temperature was increased from 25 to 50°C at 0.5°C min^{-1}. The actual measured rate of temperature change was compared across life stages using analysis of variance (ANOVA), which confirmed that the ramp rate was identical for all three groups at 0.48±0.01°C min^{-1} (P=0.960). At all three life stages, *T. molitor* showed an increase in $\dot{V}_{CO_2}$ with temperature until a maximum $\dot{V}_{CO_2}$ was reached (Figs 1, 3). Maximal $\dot{V}_{CO_2}$ was typically followed by a drop in $\dot{V}_{CO_2}$, i.e. the CO_2 valley. In adults, this drop was deep and quite evident (Fig. 3C), while in larvae and pupae this feature was less evident and in fact often absent (Fig. 3A,B). All three life stages showed a subsequent burst of CO_2 as temperature continued to increase, followed by an exponential decline in $\dot{V}_{CO_2}$ to near-zero values (Fig. 3).

Adult *T. molitor* produced the expected pattern of CO_2 release (Fig. 1, Fig. 3C) described by several studies using thermolimit respirometry in insects. This pattern is characterized by seven phases: equilibration, ramping, plateau, mortal fall (CT_{max}), post-mortal valley, post-mortal peak and exponential decay (Lighton and Turner, 2004) (Fig. 1). Prior work (Stevens et al., 2010) that used thermolimit respirometry to assess CT_{max} in adult *T. molitor* reported the same pattern of CO_2 release from these animals.

By contrast, the patterns of CO_2 release produced by *T. molitor* larvae and pupae differed from those of the adults in two ways. First, CT_{max} estimates (determined using the ADS and CSA methods) occurred during the post-mortal peak phase in larvae and pupae, rather than during the pre-mortal valley as in adults (Fig. 3A,B). Additionally, the smooth and deep decline in $\dot{V}_{CO_2}$ (i.e. the pre-mortal valley) prior to the post-mortal peak seen in adults (Fig. 3C) did not appear in larvae and pupae (Fig. 3A,B). While a decrease in $\dot{V}_{CO_2}$ was evident in some pre-adult animals, spiracular control was maintained throughout this phase, and instead CT_{max} occurred during the first half of the post-mortal peak. In other words, spiracular failure consistently occurred during different phases of the $\dot{V}_{CO_2}$ pattern in pre-adult and adult life stages of this species. Nonetheless, the cessation of spiracular control (CT_{max}) was clearly evident in the CO_2 traces of all three life stages.

Second, the pattern of CO_2 release for larvae and pupae differed from that of the adults in terms of the size and shape of the post-mortal peak. In adults, this sharp peak was invariably greater in amplitude than maximal $\dot{V}_{CO_2}$, and occurred after CT_{max} and the valley phase (Fig. 3). By contrast, the post-mortal peak in larvae and pupae appeared as a gradual rise in $\dot{V}_{CO_2}$ that was typically of equal or only slightly greater amplitude than maximal $\dot{V}_{CO_2}$

Table 1. Mean (±s.e.m.) CT_{max} determined by three distinct analytical methods in *Tenebrio molitor* at each life stage

Analytical method	Larvae	Pupae	Adults	SS	*F*-value	d.f.	*P*-value
$\dot{V}_{CO_2}$ ADS	45.9±0.2[a] (*N*=7)	46.5±0.2[a] (*N*=8)	48.0±0.3* (*N*=6)	15.756	17.58	2	<0.001
CSA	46.4±0.1[a,b] (*N*=7)	47.0±0.2[a] (*N*=8)	48.2±0.3* (*N*=6)	10.940	21.55	2	<0.001
PMV	46.5±0.2[b] (*N*=7)	45.5±0.3[b] (*N*=8)	48.0±0.6* (*N*=6)	30.131	19.63	2	<0.001
P-value	0.049	0.002	0.626	–	–	–	–

CT_{max}, critical thermal maximum; ADS, absolute difference sum; CSA, cessation of spiracular activity; PMV, pre-mortal valley.
Single-factor ANOVA was used to compare CT_{max} values across life stages and across analytical methods (α=0.05), followed by Tukey HSD *post hoc* tests.
*Significantly different from the other life stages.
Different lowercase letters indicate a statistically significant difference among methods for determining CT_{max}.
CT_{max} in adults was different from that in both pre-adult life stages using all three analytical methods (*P*-values shown in right-hand column).
Although CT_{max} estimates differed across analytical methods in pre-adult life stages, the three analytical methods produced statistically identical estimates of CT_{max} in adult *T. molitor* (*P*-values shown in bottom row).

(Fig. 3A,B). While much less pronounced in larvae and pupae, the post-mortal peak was present in all the animals we tested.

Identifying CT_{max}

CT_{max} has been defined (Lighton and Turner, 2004) as the temperature at which spiracular control ceases. We used Expedata to determine CT_{max} using this criterion as the average temperature of the point during which spiracular failure occurred for each animal. We compared three methods for estimating this point: (1) the inflection point in the ADS residuals, (2) the 10 s interval prior to the CSA and (3) the low point in the PMV. ANOVA was used to compare these metrics among the life stages, followed by Tukey *post hoc* tests to distinguish between statistically different groups. We found that CT_{max} determined using all three methods was significantly higher in adult *T. molitor* than in either larvae or pupae (P<0.001), but statistically identical between larvae and pupae (Table 1). When comparing the three methods for estimating CT_{max} with an ANOVA, we found that CT_{max} values varied significantly with analytical method in pupae (P=0.002), while values were marginally different across methods in larvae (P=0.049), and identical across methods for adults (P=0.626). According to *post hoc* tests, in both larvae and pupae the ADS estimate was identical to the CSA estimate. By contrast, the PMV estimate was significantly lower than the ADS in both larvae and pupae, and was significantly lower than the CSA estimates in pupae. This last result is primarily due to the fact that the pupae (and a small percentage of larvae) did not display a clear pre-mortal valley like the adults. Instead, spiracular activity continued steadily in these animals until the post-mortal peak (Fig. 3B). As a result, PMV estimates occurred well in advance of ADS and CSA measurements in pupae, and may not actually represent a corresponding point to the PMV values measured in adults. By contrast, CT_{max} values did not differ across analytical methods in adults, as spiracular failure occurred simultaneously with the pre-mortal valley in this life stage.

During data analyses, it was observed that the ADS method, unlike the CSA method, did not consistently identify the point of spiracular failure in this species, particularly in the pre-adult life stages. We quantified this by determining the percentage of animals for which ADS and CSA estimates of CT_{max} differed by more than 0.5°C. We found that differences greater than 0.5°C occurred in 48% of the animals we measured, the majority of which were larvae and pupae. In all cases for which the ADS and CSA differed by more than 0.5°C, the ADS value was lower than the CSA value.

Mortality

In a separate set of measurements, we compared mortality during heating among life stages by removing the insects from the temperature ramp at a precise, predetermined temperature. Each insect was scored for survival based on whether pre-CT_{max} spiracular activity resumed within 30 min of the return to 25°C. From these data, we calculated percentage survival for each life stage at each test temperature, and identified the range of temperatures at which recovery was not possible for each life stage (temperatures between 0 and 100% survival) (Fig. 4). Survival was 100% for all life stages at 46.5°C and below. The range of lethal temperatures for adults

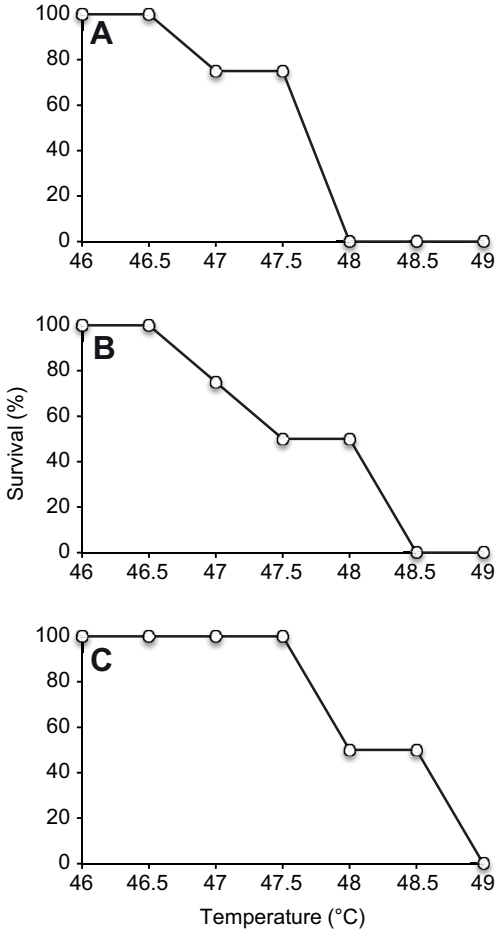

Fig. 4. Mortality for larvae (A), pupae (B) and adults (C) following removal from a thermolimit respirometry trial at specific temperatures. Survival is shown for each temperature at which beetles were removed. The range of lethal temperatures (between 0 and 100% survival) occurs shortly following CT_{max} (see Fig. 3).

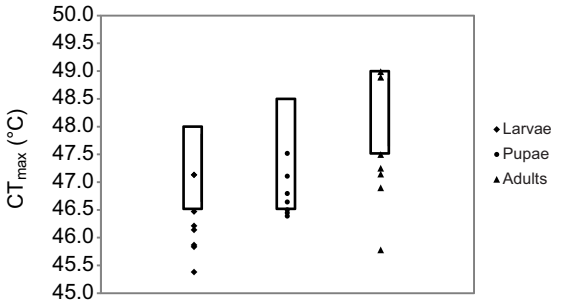

Fig. 5. The ranges of lethal temperatures (open boxes) and CT_{max} measurements (symbols) for individual beetles are shown for each life stage. More than 90% of the CT_{max} values we measured fell within 1°C of the range of lethal temperatures for that life stage, while the remainder occurred just below this range. These results suggest that CT_{max} measured with thermolimit respirometry is closely related to the time of death during heating.

spanned a higher range (47.5–49°C) than that for larvae (46.5–48°C) or pupae (46.5–48.5°C). To assess whether lethal temperatures differed with life stage, we compared the proportion of surviving animals from each life stage at each test temperature using a two-proportion *z*-test, which showed that survival was significantly greater in adults at 48.5°C than in either larvae or pupae at that temperature (P<0.050; N=4 for larvae, N=4 for pupae, N=10 for adults) (Fig. 5).

With the CT_{max} and mortality data combined, we were able to discern a clear association between the range of lethal temperatures and CT_{max} for each life stage. More than 90% of the beetles tested displayed a CT_{max} that occurred within 1°C of the range of lethal temperatures for its respective life stage, with 50% occurring just below the lethal range and 50% occurring within this range (Fig. 5). We also found that lethal temperatures spanned the period between CT_{max} and the zenith of the post-mortal peak in more than 77% of the individuals tested (Fig. 3).

DISCUSSION

Patterns of CO_2 release

This paper is the first to report distinct patterns of CO_2 release in different life stages of a single insect species during thermolimit respirometry. Adult *T. molitor* displayed a pattern of CO_2 release during thermolimit respirometry that has been well described in the literature (Lighton and Turner, 2004; Klok et al., 2004; Folk et al., 2007; Stevens et al., 2010). However, we found that the CO_2 release pattern produced by larvae and pupae differed from that of adults in terms of both when spiracular failure occurred and the characteristics of the post-mortal peak. Most notable was that the point of spiracular failure in pre-adult *T. molitor* occurred during the post-mortal peak phase rather than during the CO_2 valley as it did in adults.

This study is the first to use thermolimit respirometry in pre-adult insects. Prior studies have used this technique in adults and found remarkably little variation in the pattern of CO_2 release across the species that have been examined. The $\dot{V}_{CO_2}$ pattern reported for harvester ants [*Pogonomyrmex rugosus* and *P. californicus* (Lighton and Turner, 2004)], drosophilid flies [*Drosophila melanogaster* (Folk et al., 2007; Lighton, 2007)] and tenebrionid beetles [*Gonocephalum simplex* (Klok et al., 2004); *T. molitor* (Stevens et al., 2010)] all resemble each other closely and match the result found for adult *T. molitor* in this study. Patterns of CO_2 release have also been reported during thermolimit respirometry for two non-insects, the isopod species *Armadillidium vulgare* (Klok et al., 2004) and *Porcellio scaber* (Stevens et al., 2010). In isopods, gases are exchanged through pleopodal exopodites, and circulated to the tissues by respiratory pigments in the hemolymph (Wright and Ting, 2006). This differs markedly from insects, in which gases are delivered directly to the tissues *via* the air-filled tracheal system. During rising temperature, isopods showed a smooth rise in CO_2 release until CO_2 plummeted at CT_{max}, followed by a broad post-mortal peak (Klok et al., 2004; Stevens et al., 2010). This variation in pattern between insects and isopods has been suggested to result from differences in respiratory morphology, and to date has been the only example of variation in $\dot{V}_{CO_2}$ patterns during heating.

We have demonstrated here that the pattern of CO_2 release during heating described in prior studies of adult insects can vary in other life stages within the same species. The source of these differences in CO_2 release across development remains unclear, however. Variation may be related to developmental differences in either the structure of the tracheal system or control of the spiracles. Mölich and Lighton have found that the post-mortal peak is abruptly truncated in adult *Drosophila* if, during thermolimit respirometry, the incurrent gas is changed from air to pure nitrogen shortly after the post-mortal rise in CO_2 output begins (Mölich and Lighton, 2007). They interpreted this to mean that the formulation of CO_2 in the post-mortal peak requires oxygen and thus is a product of aerobic respiration. We have obtained the same result using adult mosquitoes (A.S.V., E. M. Gray and T.J.B., unpublished). It is possible that the post-mortal peak results from run-away aerobic respiration in the mitochondria at the point of, or just after, death of the organism. In their recent paper, Mölich and colleagues discuss the origin of this phenomenon, which they refer to as mitochondrial ‘hyperthermic overdrive’ (Mölich et al., 2012). They explain that hyperthermic overdrive may originate from the breakdown of the mitochondrial membrane under high temperatures, thereby increasing the permeability of the inner mitochondrial membrane to protons and causing the uncoupling of oxidative phosphorylation (Mölich et al., 2012). This process could result in the appearance of a post-mortal burst of CO_2 as the gas diffuses out of the open tracheal system, particularly in insects with well-developed tracheal systems. For example, adult insects such as flying mosquitoes, tenebrionid beetles and foraging ants can be highly active and often have well-developed tracheal systems. Therefore, the CO_2 produced in these insects can readily diffuse out of the tracheal system if the spiracles are open. Pupal life stages are similarly developing the adult tracheal system and have a well-developed capacity for gas exchange. By contrast, larvae tend to be less active and do not experience the extremes of aerobic activity found in the adults. It may be, therefore, that the larvae have fewer mitochondria and/or a less extensive tracheal system, and that the CO_2 formed when the mitochondria become more permeable at high temperature does not readily escape as a ‘post-mortal peak’.

Identifying CT_{max}

We found that CT_{max} differed across life stages in *T. molitor*, with adult beetles showing significantly higher CT_{max} compared with larval and pupal beetles (P<0.001). A variety of studies have reported differences in thermal tolerance among insect life stages, although the majority of this work examined cold tolerance rather than heat tolerance (reviewed in Bowler and Terblanche, 2008). Studies that have investigated the effects of ontogeny on heat tolerance have

typically shown that tolerance declines in later stages. Authors have attributed this trend to increasing mobility across ontogeny, allowing adults to compensate behaviorally for changes in temperature and thus avoid dangerous extremes (Bowler and Terblanche, 2008; Marais et al., 2009). However, there are exceptions to this generality, such as a study in *Drosophila buzzatii* which demonstrated that variation in temperature tolerance across life stages does not always proceed chronologically, with heat resistance being highest in pupae, followed by eggs, first instar larvae and finally third instar larvae (Krebs and Loeschcke, 1995). In their recent review, Bowler and Terblanche cautioned against generalizations regarding the effects of ontogeny on heat resistance, as these relationships vary significantly across studies (Bowler and Terblanche, 2008). Our finding that CT_{max} is highest in adult *T. molitor* could reflect the interaction of multiple factors, such as the microclimate conditions experienced by each life stage and the degree to which behavioral compensation can be used to avoid extreme temperatures (Bowler and Terblanche, 2008).

The CT_{max} values we obtained for *T. molitor* differ from those reported previously for this species. Stevens and colleagues used both the ADS method and the PMV method to estimate CT_{max} in *T. molitor*, and reported values of 44.0 and 44.9°C, respectively (Stevens et al., 2010). These values are approximately 4°C lower than those found using the ADS and PMV methods in our study. However, these differences are to be expected given the differences in our experimental design, notably the rate of temperature change. While Stevens and colleagues elected to use a ramp rate of $0.25°C\,min^{-1}$ (Stevens et al., 2010), here we used a rate of $0.50°C\,min^{-1}$. Terblanche and colleagues have shown that higher ramp rates produce higher estimates of CT_{max} (Terblanche et al., 2007). Differences in ramp rate of about $0.2°C\,min^{-1}$ resulted in CT_{max} values that differed by as much as 3.5°C, depending on the start temperature. Therefore, the observed differences in CT_{max} values reported for *T. molitor* by Stevens and colleagues (Stevens et al., 2010) and in the present study are to be expected. Nonetheless, it is important to acknowledge that we cannot rule out the possibility that population differences may be a source of variation in the CT_{max} values reported in the two studies.

We compared three distinct methods for estimating CT_{max} based on thermolimit data, two of which have been used in prior studies. The ADS method, which is based on identification of the inflection point in the residuals of the absolute difference sum, has previously been favored by authors because of its perceived objectivity. In their original paper describing thermolimit respirometry, Lighton and Turner explain that the inflection point for each ADS corresponds to the point at which spiracular control ceases (Lighton and Turner, 2004). Although this is often the case, we discovered that estimations of CT_{max} using the ADS method did not consistently identify spiracular failure in this species, particularly in the pre-adult life stages. Therefore, we sought to identify the CSA more directly by measuring CT_{max} as the point where the $\dot{V}_{CO_2}$ differential becomes flat. Our results showed that although the ADS method produced slightly lower estimates of CT_{max} than the CSA method in larvae and pupae, the two methods produced similar, low-variability estimates of CT_{max}. Importantly, the difference we found in CT_{max} between adult and pre-adult life stages was evident using either method. Therefore, we conclude that the ADS and CSA methods are both appropriate for analysis of thermolimit data in any life stage.

By contrast, we found that the PMV was not a reliable measure of CT_{max} in pre-adult life stages of *T. molitor*. Both larvae and pupae lack a true pre-mortal valley phase, in which CO_2 declines to a smooth valley preceding the post-mortal peak. Although a small decline in $\dot{V}_{CO_2}$ can be observed in these stages, the lack of a clear nadir at this point results from the fact that spiracular activity continues throughout this phase until the post-mortal peak is reached. Therefore, we found that estimates of CT_{max} using this technique in the pre-adult phases often preceded the point of spiracular failure by 1–2°C, and hence did not represent a corresponding value to the PMV recorded in adults. We conclude that although the PMV method is useful for analysis in *T. molitor* adults, it may not be appropriate for comparisons of CT_{max} across developmental stages.

Mortality

We examined the point at which death occurred during heating in *T. molitor* to determine how mortality related to the metrics we used to estimate thermal limitation. We determined percentage survival at each test temperature to identify the range of lethal temperatures, i.e. temperatures at which survival fell between 0 and 100%, in each life stage. Our results showed that the lethal temperature range was closely related to CT_{max} within each life stage. The range of lethal temperatures was higher in adults than in larvae and pupae, which was consistent with our finding that CT_{max} was statistically higher in adults than in the earlier life stages. We also found that the vast majority of CT_{max} values occurred within 1°C of the lethal temperatures measured for each life stage, demonstrating that heat death strongly correlates with CT_{max} in this species. Additionally, despite differences in $\dot{V}_{CO_2}$ patterns and CT_{max} across life stages, we found that the range of lethal temperatures spanned the same phases of the $\dot{V}_{CO_2}$ pattern in all three life stages, between CT_{max} and the zenith of the post-mortal peak in most animals. We interpret this to mean that heat death occurs between CT_{max} and the post-mortal peak during heating in this species, regardless of life stage.

This is the first time that CT_{max} and mortality have been directly compared in one species under identical experimental conditions. Our results show that under sustained heating regimes that surpass lethal limits, death shortly follows CT_{max} in these animals. The close association between these two endpoints demonstrates that the cessation of spiracular activity successfully identifies the point of physiological limitation during heating in this insect, rather than a behavior associated with thermal stress.

CONCLUSIONS

We conclude that thermolimit respirometry can be used to measure CT_{max} in different developmental stages of insects exhibiting variable patterns of CO_2 release. CT_{max}, when measured as the loss of spiracular control, does correlate with lethal temperatures in this species under sustained thermal ramping, and thus represents physiological failure due to heat stress. We caution that the CO_2 release patterns obtained using this method can vary even within a species, particularly across developmental stages, and thus care needs to be taken when interpreting results obtained using thermolimit respirometry. Finally, we confirm that measuring thermal limitation as the cessation of spiracular activity provides an estimate of CT_{max} that closely approximates the actual point of death.

ACKNOWLEDGEMENTS

We thank John Lighton for his guidance with our thermolimit respirometry experiments and analysis of the $\dot{V}_{CO_2}$ data. We also thank Erica Heinrich and anonymous reviewers for helpful comments on earlier versions of this manuscript.

FUNDING

This work was supported by the National Science Foundation [grant number IOS0903757 to T.J.B.].

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