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Do Big Beetle Larvae Run Big Thermal Risks?

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DO BIG BEETLE LARVAE RUN BIG THERMAL RISKS?

By

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ABSTRACT

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Faculty Mentors: H. Arthur Woods and Douglas J. Emlen

Extremes of body size captivate biologists. In insects, the lack of extant giants has prompted the question, what is constraining insect size? While multiple physiological and ecological hypotheses have been presented, there is no widely accepted explanation. One unexplored physiological hypothesis is that large insects are unable to shed metabolic heat rapidly enough and are at increased risk of overheating. My project examines this idea using larvae of the Japanese rhinoceros beetle (*Trypoxylus dichotomus*), chosen for their huge size, simple body plan, and underground lifestyle. Using CO_2 respirometry, I measured larval metabolic rates at room temperature. Although these beetle larvae are among the largest insects ever measured, their metabolic rates fell squarely on the expected values extrapolated from other, smaller insects. This permitted me to build a simple mathematical model of heat balance for insects across a wide range of body sizes. Specifically, I converted my measured rates of gas exchange into rates of metabolic heat production, and used the model to predict how much equilibrium body temperature would increase in insects larger than those that naturally occur. I then used CO_2 respirometry during temperature ramping experiments to determine larval critical thermal maxima (CT_{max}). This showed that larvae could survive temperatures of 43.5-47˚C. Together, these show that for every 100-g increase in body size, there is a 0.5˚C increase in equilibrium body temperature, and that body temperatures are predicted to be well below the thermal maximum for animals ten times larger than any extant insect. In addition, larvae placed on runways extending across a thermal gradient were surprisingly active, and clearly capable of behavioral thermoregulation through movement to cooler locations. Collectively, my results suggest that insect size is not limited by metabolic heat production. This study provides a greater understanding of insect size constraints and behaviors associated with thermal regulation.

Do Big Beetle Larvae Run Big Thermal Risks?

Introduction

Compared to other animals, the diversity of insects is staggering. Not only are there more species of insects than all other animal species combined, there is also a greater diversity of morphologies. However, compared to other taxa, extant insects are relatively small. Even the giant prehistoric insect fossils of the late Paleozoic era do not rival the largest extinct and living reptiles, amphibians, fish, birds or mammals. Given that insects are so diverse in other ways, biologists have long wondered about why they remain so small.

While multiple hypotheses have been presented to explain this paradox, nothing has been resolved. Here, we present and evaluate a hypothesis that has been largely overlooked --the metabolic heat hypothesis – which posits that as insects grow larger, they become increasingly unable to shed the metabolic heat they produce, and this puts them at greater risk of overheating. Although this idea has been discussed in the literature (Blanckenhorn 2000) to our knowledge, it has never been directly tested. This hypothesis makes two predictions. First, because insect metabolic rate increases allometrically with size (Chown *et al.* 2007), large insects in general have higher absolute metabolic rates and should, therefore, produce more metabolic heat, pushing them closer to their critical thermal maxima (CT_{max}). Second, insect body temperatures should increase with metabolic rates independent of body size (Chown *et al.* 2007). This is often thought of in terms of the Q10, or the factorial change in metabolic rate over 10˚C. The Q10 is around 2.0 for most insects, indicating a doubling in metabolic rate over 10˚C. Thus, large insects may operate in a feed-forward loop—in which large size causes them to self-heat which, in turn, causes them to produce still more heat.

Of course, heat production doesn't increase indefinitely with body temperature. Instead, metabolism rises to the CT_{max}, after which it starts to fall, due to tissue damage. Typical critical thermal maxima range from 35˚C to 50˚C, depending on species and ecology (Verble-Pearson *et al.* 2015, Moulton *et al.* 1993). Given these relationships, insect metabolic heat may put an upper bound on body size.

To examine this hypothesis, we use larvae of the Japanese rhinoceros beetle (*Trypoxylus dichotomus*). These larvae are ideal for studying heat constraints for several reasons. First,

Japanese rhinoceros beetle adults are among the largest insects by mass. Larvae, which weigh even more than the adults, can reach up to 40 grams. If any insects risk overheating as a result of high metabolic rates and large body size, these are likely candidates. Second, because they are holometabolous, larvae have much simpler body plans than adults. This means that they are unlikely to be able to regulate local tissue temperatures by shunting hemolymph between hotter and cooler compartments, like some flying insects do (Heinrich 1971*a*, Roberts and Harrison 1998, Verdu *et al.* 2004), or by efficient convective cooling across their body surface, since their "grub-like" form offers relatively little area. Third, these larvae live underground in decomposing organic matter, where there is little potential for evaporative or convective cooling via airflow anyway. All of these factors could raise larval temperatures closer to their CT_{max}. The biology of these beetles suggests that larvae may be limited primarily by their own metabolic heat output, a problem likely to be exacerbated by their extreme size.

We test these ideas by measuring larval metabolic rate and critical thermal maxima. In addition, we examined the ability of larvae to behaviorally thermoregulate. Lastly, we create a mathematical model of larval heat balance to predict upper limits to size based on metabolic heat production.

Materials and Methods

Animals

Larvae of the Japanese rhinoceros beetle were purchased from a commercial insect distributor (Yasaka Kabuto Kuwagata World, Hamada City, Japan) and reared to adulthood in the laboratory. These were placed in plastic jars (1 L) containing substrate made from a 1:1 mixture of organic hardwood compost (personal communication, Hiroki Gotoh) and quick-fermented hardwood sawdust (Emlen *et al.* 2012). Additionally, eggs were collected from a local laboratory colony and allowed to grow to the third instar. A total of 110 larvae were collected and placed in the plastic jars (1 L) containing the previously described substrate mixture. To postpone pupation, we kept approximately 60 of the larvae in a temperature-controlled growth chamber at 10˚C, and another 50 at room temperature. Larvae were pulled from the growth chamber as needed and held at room temperature for 24 h before being used in experiments.

CO_2 respirometry

We estimated larval metabolic rates from rates of carbon dioxide emission, using flow-through respirometry. CO_2 levels were measured by an infrared gas analyzer (LI-7000, LI-COR, Lincoln, Nebraska) set up in differential mode. In this mode, CO_2-free air from a cylinder of compressed breathing air (Norco, Boise, ID, USA) was first passed through the instrument's reference side, then past the larvae, then through the instrument's measurement side. The gas analyzer was calibrated using pure N_2 and 2000 p.p.m. CO_2 in N_2 (NorLab, Boise, ID, USA). Flow rates of gas were 200 mL/min (STP) and were regulated by a mass flow controller (Unit UFC-1100, 500 ml/min maximum flow rate, Yorba Linda, CA) controlled by a separate set of electronics (MFC-4, Sable Systems). Analog signals from the LI-7000 were sent to an A/D converter (UI2, Sable Systems) and then recorded using the ExpeData software (Sable Systems). Rates of CO_2 emission were converted to liters of O_2 per minute, using a respiratory exchange ratio of 0.84 (Chown *et al.* 2007). Liters of O_2 per minute were then transformed to µW of heat output using dimensional analysis and a conversion factor of 19,665 joules/liter of oxygen consumed.

Scaling of larval metabolic rates

Carbon dioxide emissions were measured for ten rhinoceros beetle larvae at room temperature, using the flow-through system described above. Each larva was placed into a 110 mL cylindrical glass chamber sealed by a Teflon end cap with two built-in O–rings. Air entered at one end and exited at the other, so that the chamber was flushed approximately twice per minute. Larvae were weighed and placed in the chamber 5-10 minutes prior to measurement to allow them to settle down. Sexes were unknown. Each larva's CO_2 output was measured for 15 minutes, during which time they were free to move. Notes were taken on each larva's movements during the measurements to examine whether movement was creating any significant differences in metabolic rate. CO_2 measurements were reported as the average p.p.m. for the last seven minutes of each experiment.

Critical Thermal Maxima

CT_{max} were determined for six larvae, using thermal ramping. Because these beetle larvae are some of the largest insects ever measured, some in excess of 25 grams, we developed a protocol based on previous work by Terblanche *et al.* (2007). The primary consideration was to ramp slowly enough that larval body temperatures were in thermal equilibrium with the temperature of the air in the flask, and yet quickly enough to reduce exposure effects. To determine a good ramp rate, test ramps were performed in which a larva was placed in the chamber with one thermocouple held in direct contact with the cuticle and another measuring the air temperature of the chamber. We decided to use a ramp rate of 0.08˚C/min, which was fast enough that individual ramps could be done in approximately 6 hours but slow enough that larvae were largely in thermal equilibrium with the flask air. However, even at this slow ramping rate, body temperatures of the larvae will were 1-1.5˚C below air temperature. The temperature ramp began at 20˚C and ended at 50˚C, giving a total ramp time of 6 hours and 25 minutes.

Larvae were measured individually in a 250-ml, water-jacketed, glass Erlenmeyer chamber. The chamber was attached to a programmable Polystat® temperature-controlled recirculating water bath (Cole-Parmer). During each ramp, we measured rates of CO_2 emission (using the system described above, with flow rates set to 350 mL/minute), actual air temperature, and levels of activity by the larva. Temperature was measured by a type T thermocouple inserted into the flask's stopper through a small hole and attached to a thermocouple meter (TC-1000, Sable Systems). Levels of activity were monitored using an infrared activity detector (AD-2, Sable Systems) modified so that the emitter and detector both were mounted on long wires. The wires were glued into the flask stopper so that the emitter and detector both were positioned about 1 cm above the larva. Activity was monitored in four of the six individual ramps. For an objective analysis of our findings, we calculated the absolute difference sums (ADS) of both the CO_2 emission and activity data for each larva (Lighton and Turner 2004).

Behavioral Thermoregulation, Experiment 1

In order to test whether larvae adjusted their body temperature by moving to warmer or cooler locations, we exposed them to composted substrate distributed along a thermal gradient,

which we designed and built ourselves. The gradient was constructed from a 20 kg block of aluminum of dimensions 0.914 m (length) x 0.305 m (width) x 0.0254 m (thickness, 1 inch). Temperatures at the two ends of the bar were fixed by circulating temperature-controlled water through 13 mm diameter threaded holes drilled completely through each end across the width. Water temperature was regulated using a programmable Polystat® temperature controlled recirculating bath (Cole-Parmer) and a digital refrigerated recirculating chiller (VWR Scientific). The temperature on one end was set to 5˚C or 10˚C, while the other end was set to 50˚C. The aluminum block was insulated on the bottom and sides by pieces of Styrofoam. The surface was divided into four lanes using 2.54 cm tall Plexiglas dividers to restrict each larva to lengthwise movement and keep the larvae from interacting. Lanes were filled with a 1:1 mixture of organic hardwood compost and quick-fermented hardwood sawdust substrate that was allowed to equilibrate to the linear gradient temperature.

The temperature of the water flowing through one end was set to 5˚C or 10˚C, while the water on the other end was set to 50˚C. To ensure that we had created a linear thermal gradient in only the lengthwise direction, 16 type T thermocouples were inserted into 3 mm diameter holes drilled into the underside of the aluminum block. The thermocouple holes were distributed along down the middle of the aluminum block every 11.43 cm, as well as across the block (in two places) every 6.1 cm (gradient temperature schematic). To ensure good thermal contact, holes were first filled with thermal paste. The steepness of the gradient was 0.4˚C (range 0.385-0.437) per cm. A total of 35 larvae were used to determine temperature preference along this thermal gradient, while 28 larvae were used in control runs.

All runs were video-recorded from above using a webcam at 30 fps. All movements of the larvae were visible from the camera. Recordings lasted either 1 or 2 hours and still images were recorded every 10 seconds. These images were then analyzed in ImageJ using a manual tracking plugin, allowing me to track the paths of each individual larva. We then converted each position to a temperature, from which we were able to plot the temperature of each larva over time.

Behavioral thermoregulation, Experiment 2

One way larvae might avoid overheating is by emerging above the surface of their substrate, thereby increasing the potential for convective heat dissipation. We tested this by ramping substrate temperatures in a controlled growth chamber. Twelve larvae were placed in one gallon glass jars, halfway filled with substrate. Prior to the experiment, larvae had been kept in the growth chamber at 10˚C, and this was used as the starting temperature for this experiment. Temperature was then increased by 10˚C every 3 hours or until the center of the substrate reached the desired temperature. The highest temperature tested was 40˚C, at which point larvae were returned to their original temperature of 10˚C. At each 10˚C increment, including the initial and final temperatures (i.e., at 10, 20, 30, 40 and 10˚C), jars were checked and all larvae at the surface were recorded.

Analytical model of larval heat balance

A key aspect of our study is to ask about the thermal biology of larvae in the size range we studied (up to approximately 30 g) and of larvae larger than those that we measured directly. The general question is: how does the thermal balance of larvae change as they become very large, up to 5 kg. We approach this problem by extrapolating from our measured values—by scaling them up to hypothetical large larvae. The three specific questions are: (1) Do larvae of *T. dichotomus* produce enough metabolic heat to warm themselves significantly underground? (2) More generally, how big must underground insects evolve to be before heat from their own metabolism raises their temperatures significantly? And (3) at what sizes, if any, would large larvae overheat?

To analyze these questions, we develop and analyze a model of larval heat balance. Any organism's heat content (H) can be described by the following equation (Gates 1980):

$$H = M + Q_a - R + C - G - \lambda E \qquad \text{(Eq. 1)}$$

where H is heat stored by or released from the organism, M is heat produced by metabolism, Q_a is radiation absorbed by the surface, R is infrared radiation emitted by the surface, C is heat gained or lost by convection, G is heat gained or lost by conduction, and λE is heat lost by

evaporation. Changes in temperature can be estimated as changes in H divided by the heat capacity of the organism.

For underground larvae, we can simplify Eq. 1. First, because there is no wind, we assume that $C = 0$. This isn't strictly true because air can move by convection through soil interstices; but this effect will be small. Second, we assume that $Q_a = R$, which will be the case if the larva and the initial thin layer of surrounding soil have the same temperature and emissivity (i.e., they will emit and absorb the same amounts of infrared radiation). The temperatures of soil and larvae are the same because they are in such close contact. We have not measured emissivities of soil or larvae, but both generally are above 0.9 (they are nearly blackbodies) (Hipps 1989; Harrison *et al.* 2012). Finally, we assume that $\lambda E = 0$, which will be the case if there is no evaporation from the larva. Evaporation should be negligible, both because insect cuticle has low permeability to water vapor and because air spaces in soil usually have very high relative humidities (close to saturating).

Given the conditions above, Eq. 1 reduces to

$$H = M - G \qquad \text{(Eq. 2)}$$

which states that larval heat content depends only on the balance between heat produced from metabolism and heat lost to the surrounding soil by conduction (we will use the convention that G is positive when heat moves from the larva to the soil). If the larva is at thermal equilibrium ($H = 0$), then M is balanced exactly by G.

To proceed analytically, we make the simplifying assumption that beetle larvae in soil can be modeled as metabolizing spheres. In other contexts, the problem of fluxes (of heat or chemicals) from spheres are well known, and for many solutions are available. Here we follow Cussler's derivation (Cussler 1997, pp 38, 39). His derivation describes steady-dissolution of a sphere in water, and the problem is to estimate how dissolution rate depends on the sphere's size and the saturating concentration of the chemical right at the surface. Conceptually, this scheme can be transferred to beetle larvae and heat. The larva gives off heat approximately

spherically, and we can arrange the equations so that we predict the equilibrium body temperature of the beetle necessary to give the observed rate of heat production, which we estimate from measured rates of gas exchange.

For an object losing heat spherically, the basic steady-state differential equation is

$$0 = \frac{D}{r^2}\frac{d}{dr^2}r^2\frac{dc}{dr} \qquad \text{(Eq. 3)}$$

where r is the radius, D is the thermal diffusivity, and c is the concentration of heat (in the soil).

The boundary conditions are

$$r = R, \qquad c = c_s$$

$$r = \infty, \qquad c = c_\infty$$

where R is the radius of the beetle larva, c_s is the concentration of heat right at the soil surface adjacent to the beetle, and c_∞ is the concentration of heat in the soil far away from the beetle.

Two integrations give

$$c = b - \frac{a}{r} \qquad \text{(Eq. 4)}$$

Using the two boundary conditions gives the concentration profile of heat in the soil

$$c = c_\infty - \frac{(c_\infty - c_s)R}{r} \qquad \text{(Eq. 5)}$$

The flux of heat can then be found from Fick's law

$$G = -D\frac{dc}{dr} = \frac{-DR}{r^2}(c_\infty - c_s) \quad \text{(Eq. 6)}$$

Solving for c_s, the concentration of heat in the thin surface layer of soil around the spherical larva, gives

$$c_s = \frac{GR}{D} + c_\infty \quad . \quad \text{(Eq. 7)}$$

Because M and G are equivalent when c_s is at equilibrium,

$$c_s = \frac{MR}{D} + c_\infty$$

The heat concentration, c_s (units of joules m^{-3}), can be converted into a temperature by dividing by the volumetric heat capacity of the soil, c_v, so that

$$T = (\frac{MR}{D} + c_\infty)/c_v. \quad \text{(Eq. 8)}$$

Iterating to equilibrium. Although the solution above is analytic, arriving at T by simply plugging in parameters was not possible—because M itself is a function of temperature. Arriving at the equilibrium T therefore required iteration by the following process: (i) assume an initial body temperature of the larva at time 0 that is equivalent to T_∞, the temperature of the soil far away from the larva (assume $T = T_{t=0} = T_\infty$), (ii) compute the larva's metabolic rate at that temperature, (iii) solve Eq. 8 to determine $T_{t=1}$, then repeat steps ii and iii until larval surface temperature stabilized. In practice, this process often led to instabilities, which could be prevented by calculating $T_{t=i+1} = (T_{t=i} + T_{t=i-1})/5$. This portion of the analysis was implemented in R.

Assumptions. Any model contains assumptions about which aspects of a complex situation to include and which to ignore. Here we lay out this model's most important assumptions. (1) The beetle larva is spherical and is in direct contact with the soil surrounding it.

In fact, beetles are cylindrical, although they often curl into more compact masses. Larvae do not excavate large air-filled chambers; usually a significant proportion of the larval cuticle is in contact with the surrounding soil. (2) Beetle body temperature is identical to the soil temperature in a thin layer surrounding the sphere; i.e., there is no gradient in temperature from the cuticle to the first layer of soil. (3) The beetle is isothermic throughout its body. Although this will not be strictly true, the assumption is reasonable because circulation of hemolymph will distribute heat on time scales much shorter than those of heat diffusion in the soil (although actual rates of hemolymph circulation are unknown). (4) The field of soil around the beetle is infinite and has the same physical properties in all directions. This assumption is clearly false but is necessary for deriving the analytic solution above; this assumption will be met approximately by larvae well below the surface of the soil. (5) Levels of O_2 and CO_2 in the soil do not alter or limit rates of metabolism by larvae. For most larvae most of the time, this will be true. Gas levels in soils generally match the composition of ambient local air (aboveground), as long as the soil is relatively dry. In wet soil, high rates of soil respiration together with low rates of gas transport can give low levels of O_2 and high levels of CO_2 (Campbell and Phene 1977; Greenway *et al.* 2006). Moreover, the critical partial pressure of O_2 (below which metabolism is depressed) is usually quite low for resting insects—on the order of 3 – 5 kPa O_2 (Harrison *et al.* 2014).

Parameter values. To run the model, two classes of parameters—for larval metabolism and for heat conduction in the soil—must be specified (all parameters defined in Table 1). The metabolic parameters define how the heat produced by metabolism depends on both larval body mass and temperature. We measured metabolic rates at room temperature (25 °C) for larvae of a range of body masses. Measured metabolic rates fell neatly onto the metabolic scaling lines fitted by Chown *et al.* (2007) to 392 insect species. Those fitted lines (ordinary least-squares and phylogenetically-corrected) had means values of 0.82 and 0.75, respectively, and their joint confidence interval was 0.70 – 0.85. We therefore used 0.75 as the core scaling value. The effects of temperature on metabolic rate were incorporated by fitting a smooth spline to the mean temperature-response curve (between 20 and 45 °C) measured in our temperature-ramping experiments. The second class of parameters (for soil) were based on empirically measured values for composted soil (Ahn *et al.* 2009). These values differ depending on the water content

of the compost. For the core calculations, we used values from the middle of the range. Additional sensitivity analyses were run to explore how strongly estimated body temperatures depended on variation in several soil parameters (the volumetric heat capacity of soil, c_v, and the thermal diffusivity of soil, D) and larval metabolic scaling exponent, b.

Results

Scaling of larval metabolic rates

The 10 larvae used in this experiment weighed an average of 21.062 ± 3.503 g (mean ± S.E.M., range 15.216-26.268 g; figure 1). The linear regression slope was not significantly different from neither zero nor one, using a linear model (confidence interval = -0.127-1.507) and ANOVA test (P= 0.087), and so it is not included. This is most likely because of the variation and small sample size.

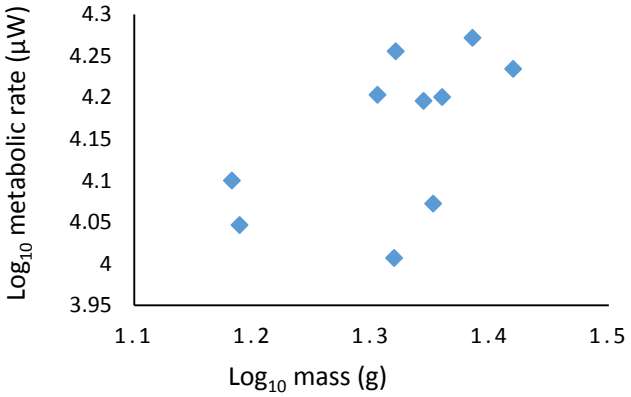

Figure 1. Scaling of larvae metabolic rate with mass.

Average weight and metabolic rate were used to compare our data to previous insect data compiled by Chown *et al.* (2007). While they scaled all the metabolic rates to 25˚C, our data was taken at 22˚C and was not significantly altered by scaling to 25˚C, so we left the data as is. By overlaying our averages on the Chown *et al*. data (figure 2), it is clear both that our beetles are the largest insects measured to date, and that their metabolic rates fall squarely within the expected values. This allows us to project out metabolic rates of insects much larger than actually observed in nature for our mathematical model.

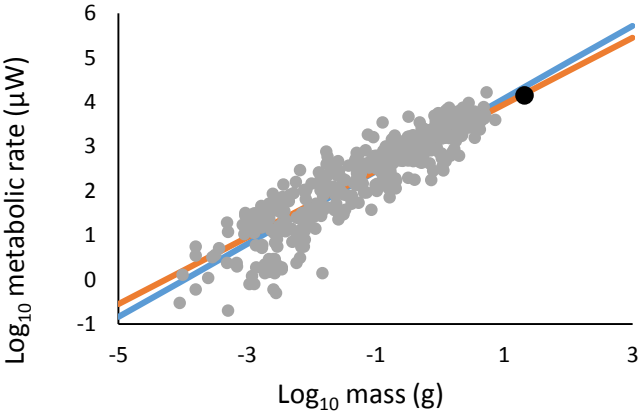

Figure 2. Scaling of *T. dichotomus* metabolic rate compared to other smaller insects metabolic rates gathered by Chown *et al.* (2007).

Critical Thermal Maxima

A representative recording is shown in figure 3. Recordings follow a similar pattern of 1) a gradual increase in CO_2 output 2) followed by one or two peaks and valleys 3) followed by a final peak that ends with a 4) decline at an exponential rate.

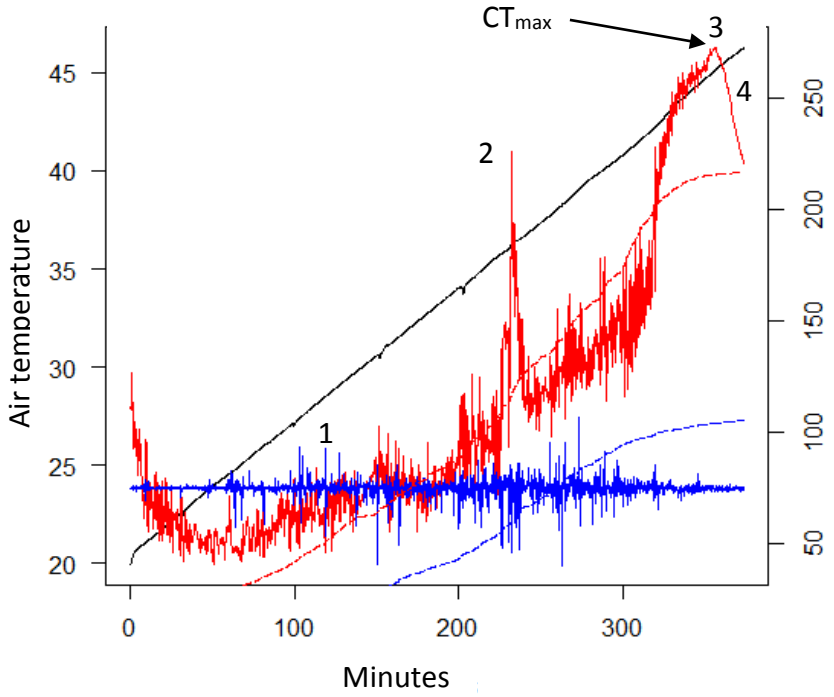

Figure 3. Determination of CT_{max} using absolute difference sums (ADS) of CO_2 and activity. Typical recording showing temperature (black line), larva CO_2 (dashed red line), CO_2 ADS (red line), and activity ADS (dashed blue line). The pattern clearly shows (1; 20-35˚C) a gradual increase in metabolic rate with temperature (2; 35-40˚C) followed by a peak and valley. Finally, there is (3; 45-48˚C) a final peak ending with a (4; >45-48˚C) decline at an exponential rate.

In most recordings, there is an initial decrease in metabolic rate, however, this is likely due to the larva moving and becoming accommodated to the chamber. After this, metabolic rate gradually increases between the temperatures 20 and 35˚C. The first peak, occurs between 35-40˚C. In some cases, a second, smaller peak appears before the final peak. The final peak occurs between 45-48˚C, during which all larva stopped respiration. We determine CT_{max} to fall within these ranges. Therefore, because larva temperature was consistently 1-1.5˚C below air temperature, larva CT_{max} occurs between 43.5-47˚C.

Behavioral Thermoregulation, Experiment 1

Initial runs revealed that larvae may be attracted to light coming from a window in the lab. To control for these effects, we performed experiments with the thermal gradient in both directions. While larvae in the control runs seemed to continue to prefer the side with incoming

light, this did not appear to affect the larvae in experimental runs, as nearly all individuals seemed to show a preference for the heated side. In addition, larvae appeared to have a preference for initial movement in the direction they were originally faced. This was controlled by randomly assigning individuals to face either the hot or cool end.

To test for differences in position between the two groups, we calculated the mean x-coordinate position for each larva, and compared these values for treatment (thermal gradient) and control (no gradient) animals. To test for differences in overall activity we used the standard deviation of x-coordinate position, a reflection of the distances moved, and compared these values for treatment and control animals. Animals exposed to a thermal gradient spent significantly more time at warmer positions (Mean x-cord=426.0982) than control animals (Mean x-cord=287.5359; t=6.5994, P=6.846e-08), despite the fact that the overall activities of treatment and control animals were not different (t=1.6148, P= 0.1115).

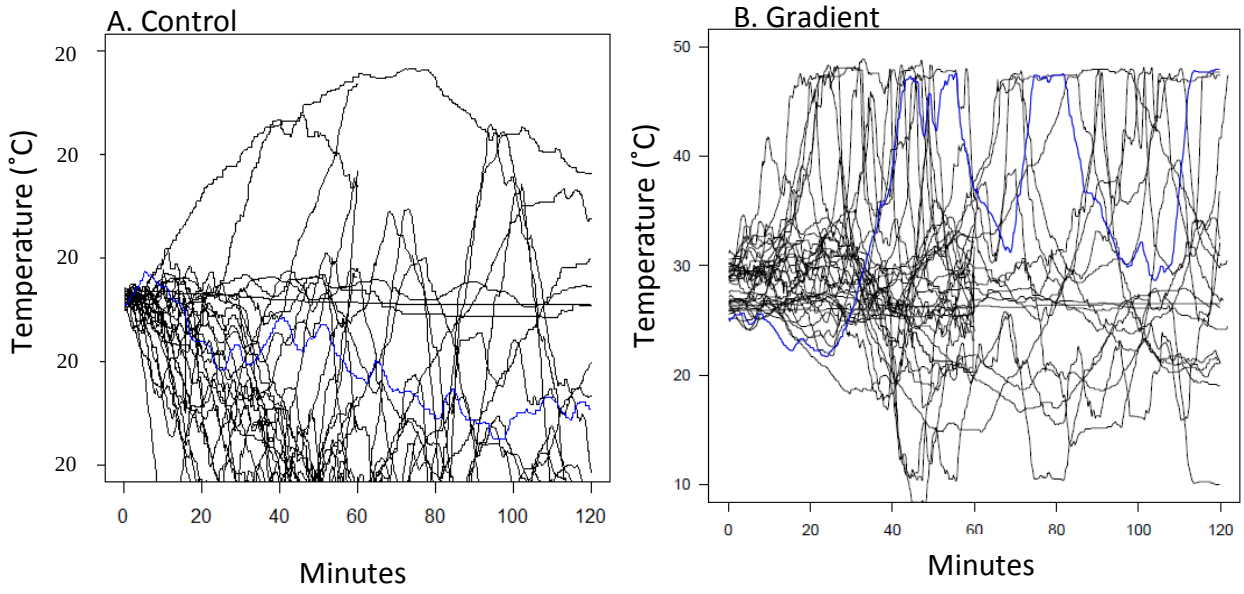

Figure 4: Movements of larvae, based on temperature, over time (control n=25; gradient n=20). Each line represents an individual. A) Control runs showing orientation towards the sunny side. B) Gradient runs showing a preference for the warmer side of the thermal gradient. Blue lines illustrate the general trends.

This experiment demonstrates that larvae move significant distances and, when given the choice of a thermal gradient, preferred an average temperature of 29.29˚C (figure 4). However, it is also clear that temperature is not the only factor prompting larvae to move, since larvae in

the control group were active without the thermal gradient present. Other factors that could contribute to movement include lighting, soil depth, and humidity.

Behavioral thermoregulation, Experiment 2

At the initial temperature of 10˚C, most larvae were buried in substrate, and only three of the twelve larvae were on the surface (figure 5). As temperatures increased to 20˚C and then 30˚C, larvae continued to remain underground, with only one or two animals breaching the surface. However, at 40˚C, seven of the twelve larvae were found at the surface. Upon returning the larvae to 10˚C for 30 minutes, all but one of the larvae returned to the soil.

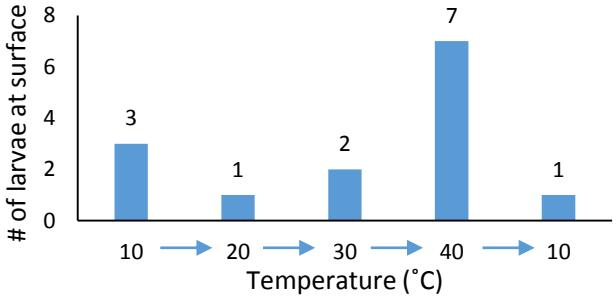

Figure 5. Scoring of larvae position during a temperature ramp.

These results provide further evidence of behavioral thermoregulation by these larvae. 2.33-7 times more larvae were present at the surface when exposed to 40˚C than when larvae were exposed to lower temperatures (10, 20, 30˚C). When the soil temperature grew excessively hot, many individuals came to the surface where they could convectively cool. After being returned to cool temperatures nearly all of them moved back into the substrate, showing that they are sensitive to temperature change and quick to react. We have witnessed behavior similar to this when receiving shipments of larvae. Large numbers of larvae will be at the surface when the container is first opened and the soil is warm to the touch. However, after the soil has had time to equilibriate to room temperature all the larvae will have buried themselves in the substrate (personal communication, Doug Emlen).

Analytical model of larval heat balance

Our model shows that, for larvae within the size range of extant insects (< 100 g), temperature increases due to metabolic rate are not expected to increase larval temperature significantly above soil temperature. This is regardless of the soil temperature larvae are experiencing (figure 6). As size increases, up to 2-3 kg, two effects appear. First, larvae self-heat

(from their metabolism) by 1 – 2 °C. Second, soil temperature begins to significantly affect the amount of self-heating; higher soil temperatures shift the entire larval metabolic curve up, so that relatively more self-heating occurs in hotter soils. Despite this effect, larvae raise their own temperatures by at most 2.5˚C, which, given the CT_{max} we measured, are unlikely to cause significant heat stress. It is only when larvae get up to 4-5 kg that we would expect larvae to self-heat really significantly.

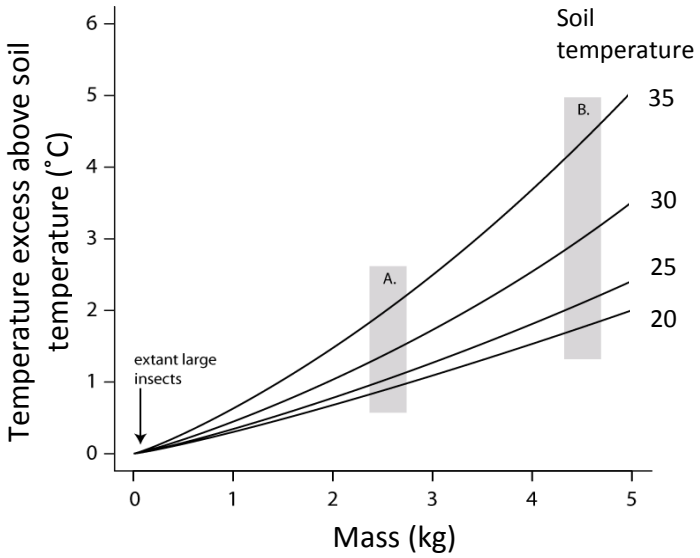

Figure 6. Main result of our mathematical model of larval heat balance. The arrow shows the approximate size of the largest extant insects. Box A) Self-heating of 1-2˚C and where soil temperature begins to have a greater effect. Box B) where significant heating begins to occur.

The predictions of the model depend on multiple parameters, each of which has some uncertainty associated with it. To explore the potential effects of that uncertainty, we did a sensitivity analysis, the results of which are presented in Appendix 1. The conclusion from the sensitivity analysis is that although some combinations of parameters (and especially from low thermal conductivity in very dry soils, and from metabolic scaling coefficients > 0.84) give higher levels of self-heating, the overall magnitude of self-heating still is relatively small; those results change the quantitative but not the qualitative conclusions.

Discussion

Given the huge magnitude of known insect diversity, why are there no really large insects? Even the giant insects of the late Paleozoic Era did not reach sizes comparable to the largest fish, birds, and mammals. Many hypotheses have been proposed to explain this observation, however, tests of those hypotheses are mixed and there is not yet a consensus. Here we present

and test the metabolic heat hypothesis, which states that large insects cannot evolve because they would overheat from their own metabolism.

We tested whether insect size is limited by metabolic heat using the larvae of Japanese rhinoceros beetle which is one of the largest known insects. They are an ideal species to study heat constraints sue to their underground lifestyle, which is likely to promote heating. To do this, we measured larval metabolic rates and critical thermal maxima. We also develop a mathematical model of heat balance which allows us to ask whether extant large insects are on the edge of overheating or around what size this would occur. We also wanted to test for the ability of larvae to behaviorally thermoregulate. This was determined using a thermal gradient to assess temperature preference and temperature ramping experiments to observe behavior during heat stress.

We demonstrate that the metabolic rates of our large insects follow the known metabolic rate scaling of smaller insects (e.g., as compared to previously compiled data by Chown *et al.* 2007). In addition, we found larval CT_{max} to be relatively high. Our model shows that current insect is not constrained by heat production. In fact, based on our model and the CT_{max} of our larvae, we predict that insects living underground could reach up to 4-5 kg before experiencing significant heat stress. In addition, the amount of movement and the response these larvae have to thermal gradients further suggests that if heat became an issue, the insects could move to alleviate the effects.

The mathematical model contains five parameters and, as part of our analysis, we tested for the sensitivity of the model to changes in each one. The parameters with the greatest influence were D, c_v, and M. In particular, decreases in D and c_v, indicating dry soil, led to greater increases in temperature excess above soil temperature. We used intermediate values for D and c_v of, $1x10^{-7}$ m^2/s and $1x10^6$ MJ/m^2, respectively. Higher M values, raised by increases in the metabolic scaling exponent b, also led to greater increases in temperature excess above soil temperature. This is unlikely to affect our conclusions, however, since we have demonstrated the relative consistency of the metabolic rate scaling ratios in insects.

Other insects which may be at risk of overheating due to metabolic heat are the flying adult insects. This is because flight is a highly energetically expensive activity and flight muscles

are known to have high mass specific metabolic rates (Heinrich 1971*b*). For reasons previously discussed in this paper, this leads to incredibly high thoracic temperatures of approximately 38-43˚C (Heinrich 1970). Therefore, it is possible that metabolic heat constraints are more likely to occur in flying adult insects. Although there are examples of adult insects using alternative cooling methods, these insects may also face limitations in terms of upper body size and metabolic heat production. To further examine the effects of metabolic heat production on size, more experiments are needed across taxa and life stages.

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Table 1. Definitions, values, and units of parameters used in the model.

Parameter	Definition	Value (range)	Units
Larval parameters			
b	Scaling exponent of metabolism	0.75	dimensionless
m	Mass of the larva	10 – 5000	g
M	Metabolic rate of the larva	variable	Watts
R	Radius of the spherical larva = $\sqrt[3]{3m/4\pi\rho}$	1.4 – 11	cm
ρ	Density of the larva	0.9	g cm^{-3}
*Soil parameters**			
c_v	Volumetric heat capacity of the soil	1 (0.84 – 1.89)	joules cm^{-3} $°C^{-1}$
c_∞	Concentration of heat infinitely far away = $T_\infty c_v$	variable	joules cm^{-3}
D	Thermal diffusivity	1 x 10^{-3} (dry soil 0.7 – 1.2 x 10^{-3}) (wet soil 1.1 – 5 x 10^{-3})	cm^2 s^{-1}
T	Temperature of the soil adjacent to larva, which is also the larva's temperature	variable	°C
T_∞	Temperature of the soil infinitely far away	variable	°C

* The parameter values of c_v and D are for 'compost soil blend' described in Ahn *et al.* 2009.

Appendix 1: Sensitivity analysis of the model parameters