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A protocol for analysing thermal stress in insects using infrared thermography

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ABSTRACT

The study of insect responses to thermal stress has involved a variety of protocols and methodologies that hamper the ability to compare results between studies. For that reason, the development of a protocol to standardize thermal assays is necessary. In this sense, infrared thermography solves some of the problems allowing us to take continuous temperature measurements without handling the individuals, an important fact in cold-blooded organisms like insects. Here, we present a working protocol based on infrared thermography to estimate both cold and heat thermal stress in insects. We analyse both the change in the body temperature of individuals and their behavioural response. In addition, we used partial least squares regression for the statistical analysis of our data, a technique that solves the problem of having a large number of variables and few individuals, allowing us to work with rare or endemic species. To test our protocol, we chose two species of congeneric, narrowly distributed dung beetles that are endemic to the southeastern part of the Iberian Peninsula. With our protocol we have obtained five variables in the response to cold and twelve in the response to heat. With this methodology we discriminate between the two flightless species of *Jekelius* through their thermal response. In response to cold, *Jekelius hernandezi* showed a higher rate of cooling and reached higher temperatures of stupor and haemolymph freezing than *Jekelius punctatolineatus*. Both species displayed similar thermoregulation ranges before reaching lethal body temperature with heat stress. Overall, we have demonstrated that infrared thermography is a suitable method to assess insect thermal responses with a high degree of sensitivity, allowing for the discrimination between closely related species.

1. Introduction

Changes in the environmental temperature affect most physiological processes and the life traits of insects, causing stress, injury or even death (Cossins and Bowler, 1987; Huey et al., 1990; Frazier et al., 2006; Dell et al., 2011). The influence of temperature on insect physiological responses has generally been assessed by experiments in chambers under controlled temperature conditions; temperatures such as the lower (LLT) or upper lethal temperatures (ULT), which are related to cold or heat injuries, are estimated according to the percentage of surviving individuals (see for example Overgaard et al., 2011 or Andersen et al., 2015). Although the use of these measurements have enabled a breakthrough in the understanding of insect thermal physiology, these estimations are subjected to uncertainties derived from the experimental protocols that are employed (Chown and Nicolson, 2004; Terblanche et al., 2007; Santos et al., 2011), and they require that a large number of individuals are killed or injured to reach a high statistical power. Furthermore, the methods that are limited

Abbreviations: CCR, Chill coma range; CCRR, Chill coma recovery range; CCRT, Chill coma recovery temperature; CCT, Chill coma temperature; CR, Cooling rate; CT_{max}, Critical thermal maximum; HCR, Heat coma thermal range; HR, Heating rate; HRT, Heat regulation temperature; HSR, Heat stress range; iHCR, Differences in the area under the response curves of the model and living individuals for the heat coma range (HCR); iHSR, Difference in the area under the response curves of the model and living individuals for the heat stress range (HSR); iSAR, Differences in the area under the response curves of the model and living individuals for the supra-optimal activity range (SAR); iTR, Differences in the area under the response curves of the model and living individuals for the thermoregulation range (TR); LLT, Lower lethal temperature; OAR, Optimal activity range; PLSR, Partial least squares regression analysis; rHCR, Rate of increase in the body temperature per unit of time during the heat coma range (HCR); rHSR, Rate of increase in the body temperature per unit of time during the heat stress range (HSR); rSAR, Rate of increase in the body temperature per unit of time during the supra-optimal activity range (SAR); rTR, Rate of increase in the body temperature per unit of time during the thermoregulation range (TR); SAR, Supra-optimal activity range; SCP, Temperature at which individuals reached their supercooling point; SST, Start stress temperature; TR, Thermoregulation range; ULT, Upper lethal temperature

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to measuring the temperatures of heat injury may have a low discriminative power between species due to generalized biochemical similarities related to protein perturbation above 50–53 °C (Christian and Morton, 1992; Wu et al., 2002), explaining why heat shock temperatures are narrowly defined as 45–47 °C in flying insects (May, 1976, 1978; Heinrich, 1993; Chown and Nicolson, 2004; Verdú et al., 2006). Therefore, these estimates may not be useful to provide information on the thermotolerance or thermoregulation responses of the analysed individuals to the variations in the real-time experimental temperature. When the body temperature of insect specimens is the parameter of interest, the use of thermocouples has been the classical procedure for estimating the thermoregulatory ability of species (Heinrich, 1993). As an invasive procedure, it has several drawbacks such as the strong influence on the studied individuals, causing injuries or altering their behaviour (Watt, 1997) and preventing the collection of reliable behavioural data associated with body temperature variations.

The need to obtain ecophysiological data that are narrowly distributed or pertain to very rare or endangered species (Bozinovic et al., 2011) urges the design of non-invasive protocols that at the same time are effective and minimize the number of individuals required to estimate physiologically relevant measures. Infrared thermography can help to avoid the problems associated with invasive measurements of body temperature (Stabentheiner and Schmaranzer, 1987; Chown and Nicolson, 2004). Furthermore, it is useful for obtaining a large number of variables that can reflect both the temperature limits and thermal stress curves in situations in which few individuals are available and behavioural changes considered important. Thermal imaging is an alternative method that has important advantages and few limitations for studying the physiology, ecology and natural history of animals (McCafferty, 2007; Cilulko et al., 2013). Several experiments with different groups evaluated their use and commend their capability to be invaluable to research (e.g. Cena and Clark, 1972; Stabentheiner and Schmaranzer, 1987; Farina and Wainselboim, 2001; Kleinhenz et al., 2003; Stabentheiner et al., 2003; Palmer et al., 2004; Kroder et al., 2008; Verdú et al., 2010, 2012; Norris and Kunz, 2012; Stabentheiner et al., 2012; Hartfelder et al., 2013). In this paper, we propose both a working protocol to examine the thermal stress of insects using infrared thermography and a statistical procedure directed to analyse the large number of variables that can be obtained when analysing a small number of specimens with this technique. First, we describe a set of thermal variables to exemplify the capacity of infrared thermography to derive relevant variables capable of describing the thermal responses of insects. These specifically include variables related to the activation temperature of individuals and their responses to both cold and heat conditions. Although these variables can differ according to the taxonomic group under study and the associated methodological circumstances, we illustrate how the use of thermal infrared images can generate a thorough number of variables that have an *a priori* unknown degree of correlation (Andersen et al., 2015). To overcome the problems associated with low sample sizes and the fact that multiple predictor variables are often related, we use a statistical technique that is well suited for examining data with these particular characteristics: partial least regression analyses (hereafter PLSR). Finally, we demonstrate the acquisition and treatment of the data derived from infrared thermography using the thermal stress curves of two congeneric, apterous and narrowly distributed dung beetles that are endemic of the southeastern part of the Iberian Peninsula (*Jekelius hernandezi* (López-Colón, 1988) and *Jekelius punctatolineatus* (François, 1904)).

2. Materials and methods

2.1. Delimitation of thermal variables

All data were obtained using thermographic video sequences, which were recorded with a FLIR ThermaCam P620 thermal infrared camera with a resolution of 640 × 480 pixels and a microbolometer Focal Plane Array detector with a spectral range of 7.5–13 μm and a thermal sensitivity of 0.06 °C at 30 °C. To ensure accuracy, the thermocamera was calibrated using the Standard Calibration service provided by FLIR Systems Inc. We measure the cuticle emissivity at different temperatures (40–70 °C) using fresh cuticles of both *Jekelius* species, obtaining a value of 0.95. For the measurements we take as reference electrical tape (a reference method as described in ISO 18,434-1) and black paint (NEXTEL-Velvet-Coating 811-21; see Kwor and Mattei, 2001). Below, we describe the variables that were extracted in our case using thermographic images under three different experiments as follows: the start of activity, cold and heat response. Although the delimitation of these variables depends on the studied taxonomic group and their associated temperature cut-off points, we aimed to define a thorough set of variables that can be obtained using thermal images in a broad spectrum of insect species.

2.1.1. Start of activity

The temperature at which the activity began (chill coma recovery temperature or CCRT; see Chapman et al., 1926; Krogerus et al., 1932; Hazell and Bale, 2011) corresponds to that measured at the moment in which the focal individual begins to move the legs and head appendices, changing its position with respect to the initial position in which the individual exhibited stupor from cold (i.e., total lack of activity). To induce the stupor state, the individuals were placed into individual bowls that were located within a cooler, which had previously been lowered to a temperature at which the stupor state was attained. This procedure was performed in a portable cooler (Engel Fridge/Freezer MT45F-G3) that was adapted to place the thermocamera on its top for recording the assay development. Once the individuals stopped the activity, the portable cooler was turned off and the sampling began until the beetles became active. During each bioassay, we also calculated the heating rate (HR) of the chill coma recovery range (CCRR) of each individual (in °C min^{-1}) as the temperature change from the loss of activity (the chill coma temperature, see below) to CCRT (see Figs. 1 and 2).

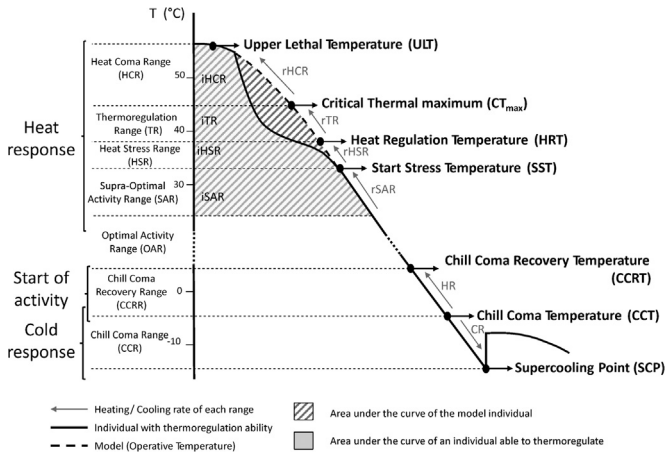

Fig. 1. Thermo-biological scale showing the thermal ranges and variables measured in this study (modified from Vannier, 1994).

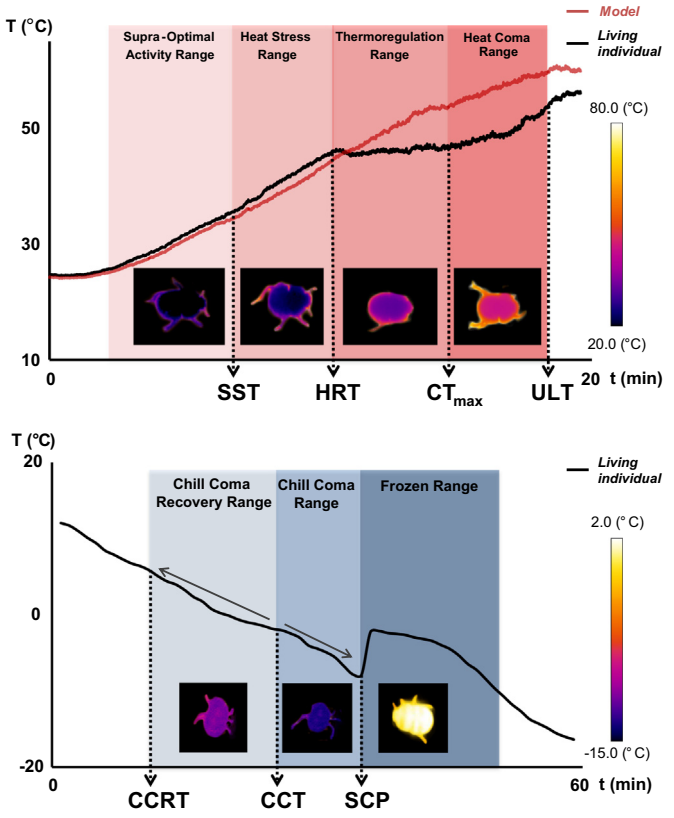

Fig. 2. Graphical representation of the thermal ranges and variables obtained by infrared thermography for heat (above) and cold (below) responses. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.1.2. Cold response

The same individuals that were used in the experiment for start of activity were again gradually cooled (cooling rate of 0.30 °C/min) until they reached their supercooling point (SCP). First, we measured the temperature at which the individuals lost their activity due to cold (the chill coma temperature or CCT; see Hazell and Bale, 2011), as well as the cooling rate (CR), during the chill coma range (CCR), which was defined as the rate of temperature decline from CCT to SCP (see Figs. 1 and 2). The SCP is the moment at which total haemolymph freezing occurs, generating an exothermic reaction that is clearly detected by the thermocamera (Verdú et al., 2010).

2.1.3. Heat response

We examined the heating rate of individuals and their ability to regulate their body temperature with a linear increase in the experimental temperature (heating rate of 1.5 °C min^{-1}). The heat response of living individuals was compared against immediately freshly killed individuals which represent operative temperature measurements (Dzialowski, 2005) and are hereinafter referred to as "model individuals". The resulting response is divided into four different thermal ranges according to both the observed variations in the body temperature of living and model individuals and the behaviour observed in the thermographic video sequences (see Table 1 and Figs. 1 and 2). The proposed thermal ranges are:

- Supra-optimal activity range (SAR), which spans from the selected temperature at which the individuals were active to the start of stress temperature (SST). The SST was delimited at the moment in which we observe a clear acceleration in legs and head movements, reflecting the stress state.
- Heat stress range (HSR), defined as the thermal range from the SST to the heat regulation temperature (HRT), the setpoint at which the temperature of model individuals began to differ from that of living individuals.
- Thermoregulation range (TR), defined as the time that elapsed between the HRT and critical thermal maximum (CT_{max}; see Cowles and Bogert, 1944) at which the individuals began to be unable to regulate their excess of heat (Fig. 1). During this range, individuals may perform abdominal pumping movements while they stretch and shrink their forelegs, decreasing their body temperature. In the case of flying species, the individuals frequently opened their elytra and stretched their wings, making small flight attempts. At the end of this range we observed hind limb paralysis and an evident decrease in the movement of individuals (CT_{max}).
- Heat coma range (HCR), delimited from CT_{max} to the upper lethal temperature (ULT), which is defined as the temperature at which the individual becomes completely paralyzed.

Table 1
Thermoregulation variables derived from the heat response assay. t0, t1, t2, t3 and t4 correspond to the start of the bioassay at 25 °C and the moments in which start of stress (SST), heat regulation (HRT), critical thermal maximum (CT_{max}) and upper lethal (ULT) temperatures were obtained, respectively.

Acronym	Expression
rSAR	$rSAR=\frac{SST-25}{t1-t0}$
rHSR	$rHSR=\frac{HRT-SST}{t2-t1}$
rTR	$rTR=\frac{CT\ max-HRT}{t3-t2}$
rHCR	$rHCR=\frac{ULT-CT\ max}{t4-t3}$
iSAR	$iSAR=\int_{t0}^{t1} model-\int_{t0}^{t1} individual$
iHSR	$iHSR=\int_{t1}^{t2} model-\int_{t1}^{t2} individual$
iTR	$iTR=\int_{t2}^{t3} model-\int_{t2}^{t3} individual$
iHCR	$iHCR=\int_{t3}^{t4} model-\int_{t3}^{t4} individual$

Considering these four ranges, we defined twelve variables to measure the heat response of individuals (Table 1; Figs. 1 and 2). First, we estimated the SST, HRT, CT_{max} and ULT temperatures of each individual. Subsequently, we calculated the slope of the relationship between the body temperature and time (i.e., the rate of increase in the body temperature per unit of time) for each of the four previously delimited ranges (rSAR, rHSR, rTR and rHCR). Subsequently, we calculated the differences in the area under the response curves of the model and living individuals (according to the difference in their integrals) for each of the four thermal ranges (iSAR, iHSR, iTR and iHCR). We use these integrals to quantify the degree of thermoregulation for each thermal range presented by each individual (i.e., the greater the difference between the two response curves, the higher the thermoregulatory efficiency; Table 1).

2.2. Data analyses

The capacity of deriving a large number of thermal variables from the video sequences of thermographic cameras, together with their use for those species with a small number of individuals, hinder the use of classical statistical tests (i.e., many correlated predictors and response variables with few sample units). Under these circumstances partial least regression analysis (PLSR) constitutes an appropriate multivariate technique. PLSR finds latent components that are linear combinations of many multicollinear predictors and can maximize the explained variance in several correlated response variables (see Carrascal et al., 2009

and references therein for the relevance of this statistical tool in ecological studies). Therefore, the thermal response variables are grouped into syndromes that can be explained by a reduced number of new orthogonal components derived from the linear combination of predictors (in this case, the species identity, body weight and ambient temperature). Thus, components or syndromes that are derived from PLSR pursue the maximization of the explained variance according to the response variables. The link between thermal syndromes and response variables allows for the identification of the most relevant parameters that are capable of discriminating between the examined species. This property of PLSR, as well as its ability to work with a small number of sample units, maximizes the likelihood of obtaining significant differences in the thermal responses of species when the available data are scarce. Data were analysed using StatSoft's Statistica 12 (StatSoft Inc, Tulsa, Oklahoma, USA).

2.3. The Jekelius case

2.3.1. Collection, maintenance and acclimation

Individuals of both species were collected in Alicante province during autumn 2012 and 2013 (this is the period of maximum abundance for both species). Specifically, *Jekelius hernandezi* was collected in the Sierra de la Carrasqueta (38.61°N, −0.48°W, 1044 m.a.s.l.) and *J. punctatolineatus* in Xixona (38.49°N, −0.47°W, 254 m.a.s.l.). Both species inhabit semiarid climates in the southeastern part of the Iberian Peninsula, which are associated with rabbit latrines (Fig. 3). Therefore, both species allow for the development of a diagnostic test on the appropriateness of a thermocamera to generate variables that are capable of discriminating between the thermal responses of two species that are closely related from the phylogenetic, biogeographic and ecological points of view.

Individuals were transported to the laboratory in plastic containers (10 × 10 × 10 cm) with soil from the collection site using a portable cooler that was temperature controlled at 15 °C. For the experiments, we selected mature individuals according to external age-grading methods (e.g., tibia and clypeus erosion) that allow for the identification of individuals who are of approximately the same age (see Verdú et al., 2010). Eleven randomly chosen individuals of each species were acclimated at 25 °C for the heat response experiments, which is the minimum temperature required for the proper performance of the neuromuscular system and close to optimal temperature at which individuals are active without reaching heat stress (May 1978, 1985; Heinrich, 1993; Vannier, 1994; Chown and Nicolson, 2004; Verdú et al., 2006). Four individuals of *J. punctatolineatus* and five of *J. hernandezi* were acclimated at 5 °C prior to the trials directed to estimating the responses in the start of activity and cold response experiments. We used a cold temperature at which species can be found in the field, without generating damage; this is the minimum stress temperature before reaching the stage of stupor form cold (Vannier, 1994; Chown and Nicolson, 2004). All individuals were acclimated for 72 h without food supply to eliminate possible mistakes caused by the difference in the feeding states of individuals. Different acclimations were performed using two refrigerated incubators (MIR-153, Sanyo Electric Co. Osaka, Japan). This work conforms to the Spanish legal requirements including those relating to conservation and welfare. Also, beetle collection was made with relevant permissions related to collection and field study.

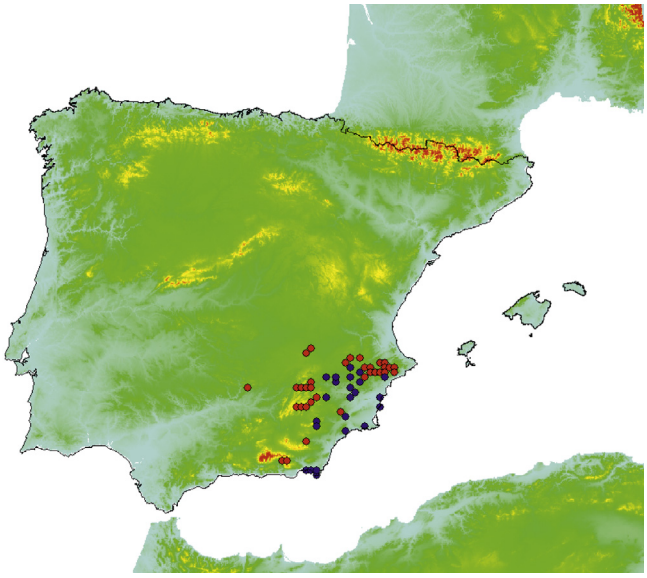

Fig. 3. Geographical distribution of *Jekelius punctatolineatus* (blue circles) and *Jekelius hernandezi* (red circles). The background map represents the elevation from the lowlands (light blue) to mountains (red). Note that in spite of the high degree of regional sympatry both *Jekelius* species live in low altitude and warm localities. The distributional data were compiled thanks to the "Atlas of threatened invertebrates in Spain" (see http://www.magrama.gob.es/). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.3.2. Experimental trials

In each experimental trial, the ambient temperature of the laboratory was recorded using a K/J thermometer Fluke 152 (Fluke Co. California, USA). All individuals were sexed and weighed using a precision balance (AG104 Analytic Balance; Mettler Toledo, Columbus, OH, US). The three experiments were performed with the thermocamera connected to a computer in which the video recording was stored. Subsequently, these video recordings were used to observe the activity of individuals during each experiment and its correspondence with thermal analysis. For thermal analysis, we selected body beetle areas that were approximately 20 mm^2 in the central part of pronotum, where the temperature data were obtained.

In the start of the activity trials, the individuals were placed into individual bowls to induce the stupor state. Individuals were placed within the cooler at a temperature around −8 °C, avoiding reaching the SCP. Immediately after reaching loss of activity (approximately five minutes after starting the assay) of the study specimen, the refrigerator was turned off, and thermal recording began until the start of activity (chill coma recovery temperature, CCRT). The heating rate of the ambient temperature inside the cooler was 0.15 °C/min at a room temperature of 22 °C. After the test of start of activity, the same individuals were cooled at a rate of 0.30 °C min^{-1} until they reached the point of inactivity and SCP. We perform 'the start of activity assay' before the SCP because in this assay the individuals return to normal activity as in the test of supercooling the individuals may be damaged by haemolymph freezing.

For the heat response trials, individuals were placed 2.5 cm above a hotplate (rectangular precision hotplate "Plactronic" stability ± 0.5 °C; J.P. Selecta, Barcelona, Spain), applying a temperature increasing rate of 1.5 °C min^{-1}, from 25 °C (the temperature at which insect species remain unstressed but active; see Vannier, 1994) to the upper lethal temperature (ULT). Beetles were placed 3 cm apart, on a strip of cork, to avoid contact between them, using a pin stuck to the left side of the thorax with a hot melt adhesive. A model individual was used in all heat response experiments. As formerly mentioned, model individuals are freshly killed insects in which a thermocouple is inserted into the abdomen to discriminate the active physiological thermal response of living individuals (Bakken, 1976, 1980; Armbruster and

Berg, 1994; Kovac et al., 2010). Taking into account both the observed variations in the body temperature and behaviour of live and model individuals, we estimated the formerly mentioned twelve thermal variables.

3. Results

The species identity and body mass were tightly related in both *Jekelius* species, and they significantly influenced a thermoregulation PLSR component related to the start of activity ($P=0.0001$). This component was nearly exclusively associated with the heating rate or HR (87.2% of its variance accounted for), but it was not associated with the temperature at which the activity began (CCRT; 0.2% of its variance). The importance of the species identity was higher than the body mass in determining the thermoregulation component, according to the weights of these two predictors (see Table 2; the square of statistical weights was 0.64 for species and 0.36 for body mass). Therefore, CCRT was independent of HR, and the smaller *J. hernandezi* heated more quickly than the larger *J. punctatolineatus*, while the body temperature at the start of activity neither differed between both species nor was it related to body mass (Fig. 4). These results highlighted the importance of body weight in explaining the start of activity of these species in response to the change from cold to hot conditions (a consequence of physical inertia). Moreover, a typical measure such as the CCRT does not allow for discriminating between the thermal responses of the two studied species.

Two thermoregulation PLSR components were obtained in the case of the heat response (Table 3), accounting for 32% of the inter-individual variation in the twelve variables describing the heating response. The first component (including 20% of the variance) defined a thermoregulation syndrome that was negatively associated with the lower heat regulation temperature (HRT) and upper lethal temperature (ULT), and it was positively associated with the heating rate in the thermoregulation range (rTR). It was also positively linked with the difference in the areas under the response curves of model and living beetles during the supra-optimal activity (iSAR) and heat stress (iHSR) ranges (denoting high levels of thermoregulation in these ranges). This thermoregulation syndrome was positively and significantly ($P<0.0001$) related to the ambient temperature during the trials (the most important predictor: square of its weight=0.63) and subtle differences in body mass of individuals (square of weight=0.34). Nevertheless, it was nearly completely independent of the species identity. Therefore, this syndrome does not discriminate between the thermal responses of the two species that have a high physical inertia (Fig. 5A); larger beetles had lower lethal and heat regulation temperatures and more active thermoregulatory responses during the supra-optimal activity and stress ranges as well as heated at faster rates during the thermoregulation range than smaller ones. By contrast, the second PLSR component related to the heat stress response significantly discriminated between the thermal responses of the two species. This component accounted for 13% of the information content of the twelve variables summarizing the heating process, and it was significantly ($P<0.0001$) and mainly related to the identity of the species (square of its weight=0.61; the other two predictors, ambient temperature and body mass, have square weights < 0.23; Table 3). Therefore, *J. punctatolineatus* had higher start stress (SST) and heat regulation (HRT) temperatures as well as faster heating rates in the supra-optimal (rSAR) but slower final heat coma (rHCR) ranges than *J. hernandezi* (Fig. 5B).

Finally, the response to cooling experiments can be

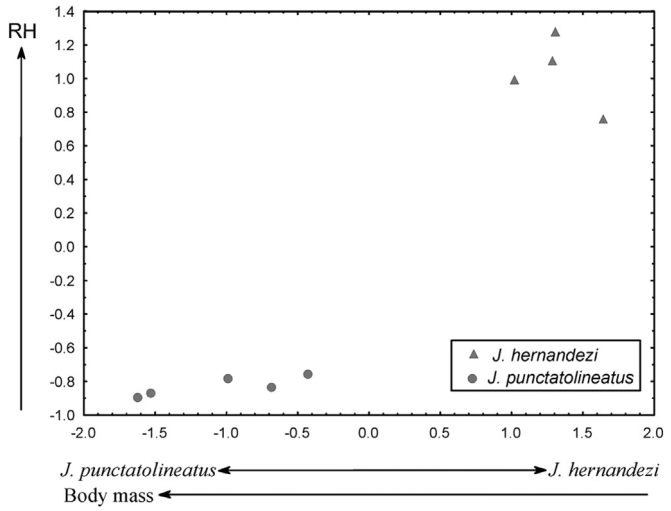

Fig. 4. Relationship between the PLSR components for response and predictor variables obtained for the start of activity assay. Species identity and body mass are the predictors that significantly influence the unique component derived from response variables, which is in turn associated with the heating rate (HR) from the loss of activity to the chill coma recovery temperature. Arrows represent the sign of the relationships (i.e. low values of the predictor component are associated with high body mass values).

Table 2
Results of the PLS regression analysis summarizing the start of activity. The variables whose square weights are larger than 1/number of variables (2 response and 2 predictor variables; i.e., magnitude effect larger than that expected by chance) are indicated in bold type font. The response variables are as defined in the text.

	PLS1	R^2 (%)
Response variables		
HR (°C/min)	**1.00**	87.2
CCRT (°C)	0.05	0.2
R^2 (%)	43.7	
Predictors		
Species	**−0.80**	
Body mass	−0.60	
Eigenvalue	1.72	

Table 3
Results of the PLS regression analysis summarizing the heating response trials. The variables whose square weights are larger than 1/number of variables (12 response and 3 predictor variables; i.e., magnitude effect larger than that expected by chance) are indicated in bold type font. Ambient temperature was included as a control variable due to the impossibility of maintaining air temperature constant throughout the different trials. Response variables as defined in the Table 1.

	PLS1	PLS2	R^2 (%)
Response variables			
SST (°C)	0.21	**0.36**	29.9
HRT (°C)	**−0.38**	**0.33**	51.4
CT_{max} (°C)	−0.17	0.24	15.3
ULT (°c)	**−0.44**	−0.01	46.4
rSAR (°C min^{-1})	−0.11	**0.46**	34.7
rHSR (°C min^{-1})	0.26	0.18	21.5
rTR (°C min^{-1})	**0.38**	−0.17	38.9
rHCR (°C min^{-1})	0.23	**−0.58**	63.0
iSAR	**0.37**	−0.24	41.0
iHSR	**0.30**	−0.15	24.3
iTR	0.13	−0.06	4.7
iHCR	−0.24	0.13	16.1
R^2 (%)	19.69	12.57	
Predictors			
Species	0.17	**0.78**	
Body mass	**0.58**	0.41	
Ambient temperature	**0.79**	−0.47	
Eigenvalues	1.42	0.79	

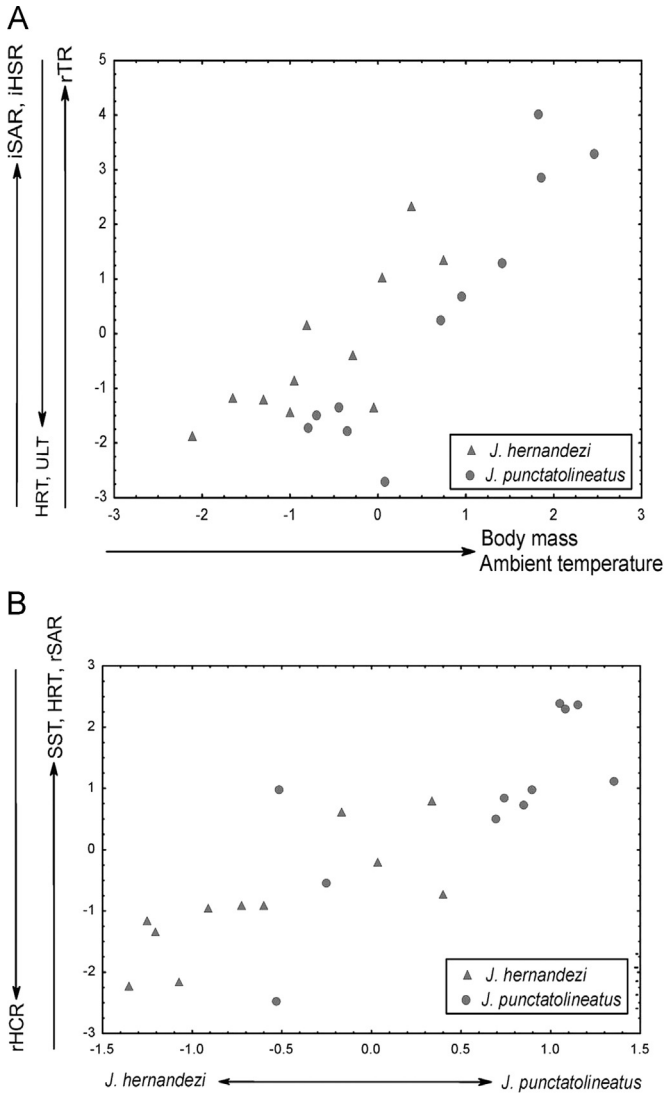

Fig. 5. Relationship between the PLSR components for the response and predictor variables obtained for the heat response assay. The first thermoregulation syndrome (A) is positively associated with the heating rate during the thermoregulation range (rTR) and the differences in response curves of model and living beetles during the supra-optimal activity (iSAR) and heat stress (iHSR) ranges. However, this thermoregulation component is negatively associated with the heat regulation temperature (HRT) and the upper lethal temperature (ULT). Body mass and ambient temperatures are the predictors that significantly influence this component derived from response variables. The second thermoregulation syndrome (B) is positively associated with the start stress (SST) and heat regulation (HRT) temperatures as well as the heating rates in the supra-optimal (rSAR), and negatively with the final heat coma (rHCR) ranges. The only predictor that significantly influence this thermoregulation component is the species identity. Arrows represent the sign of the relationships (i.e. high values of predictor component are associated with high body mass values).

summarized by one significant PLSR component that accounts for 69% of the observed variability in the three considered thermoregulatory variables (Table 4). This component shows that the temperatures at which beetles lost their activity due to cold (CCT), haemolymph freezing (SCP), and the observed cooling rates (CR) were positively associated (Fig. 6). This component was significantly ($P=0.003$) related to a component defined by the two predictors, where the species identity was more important (square of weight = 0.73) and the body mass had a marginally negative influence (0.27). Therefore, *J. hernandezi* cooled slower and reached higher temperatures of stupor and haemolymph freezing than *J. punctatolineatus*. In this case, the association of the three

Table 4
Results of the PLS regression analysis summarizing the cooling response trials. The variables whose square weights are larger than 1/number of variables (3 response and 2 predictor variables; i.e., magnitude effect larger than that expected by chance) are indicated in bold type font. The response variables are as defined in the text.

	PLS1	R^2 (%)
Response variables		
SCP (°C)	0.55	61.0
CCT (°C)	**0.60**	74.4
CR (°C min^{-1})	**0.58**	68.3
R^2 (%)	68.9	
Predictors		
Species	**−0.86**	
Body mass	−0.52	
Eigenvalue	1.66	

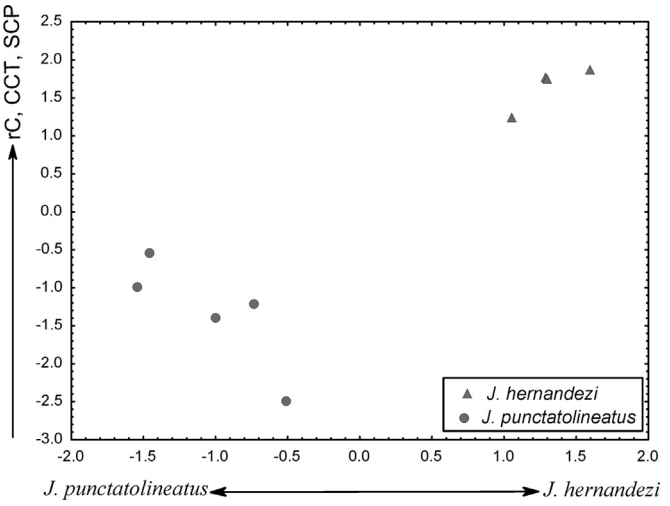

Fig. 6. Relationship between the PLSR components for the response variables and predictors obtained in the cold response assay. Species identity is the only predictor that significantly influence the unique component derived from the response variables, which is in turn positively associated with the temperature at which beetles lost their activity due to cold (CCT), haemolymph freezing (SCP), and the observed cooling rates (CR). Arrows represent the sign of the relationships (i.e. high values of the response component associated with high CCT values).

selected variables and their biological meaning recommends the selection of CCT, which does not require the killing of the insects to obtain that measurement. Tables 5 and 6 show the values of the response and predictor variables for each species.

4. Discussion

The use of infrared thermography provides a greater number of continuous measurements of new variables compared with the classical protocols that are focused on temperature limits. Here, the subset of described variables can be analysed to discriminate between the thermal resistance and stress of species under different scenarios within a dynamic framework. The inevitable correlation among the thus obtained thermal variables can be overcome by analysing the data with partial least regression analyses for defining thermoregulatory syndromes that in turn can maximize the differences among species at low sample sizes. The experimental protocol presented in this paper shows that it is possible to obtain thermal variables for rare, threatened insect species that surpass the frequent ethical and logistic impediments hindering the estimation of the thermal responses that are otherwise necessary to better understand the ecology and biogeography of these species. Our example also shows that we can

Table 5
Mean values of the predictor and response variables for the two *Jekelius* species (± SD in brackets) in the start of activity and cold response experiments. CCRT, CCT and SCP are in °C. CR and HR in °C min^{-1}. Weight is the body mass of fresh individuals in g.

	CCRT	CCT	SCP	CR	HR	Weight
J. hernandezi	5.50	−4.60	−7.64	−0.0037	0.29	0.20
(n=5)	(±0.74)	(±1.01)	(±1.49)	(±0.0003)	(±0.02)	(±0.06)
J. punctatolineatus	5.68	−8.72	−16.96	−0.0220	0.17	0.40
(n=4)	(±0.59)	(±0.90)	(±3.13)	(±0.0060)	(±0.00)	(±0.12)

obtain a set of variables with biological meaning to explain the response of individuals to temperature changes when continuous measurements of the temperature and behavioural data are considered (Cena and Clark, 1972; Stabentheiner and Schmaranzer, 1987; Kroder et al., 2008; Farina and Wainselboim, 2001; Verdú et al., 2010, 2012). Importantly, we show that the proposed protocol provides continuous (dynamic) thermographic measurements that may help when selecting a reduced subset of relevant variables for future studies with closer taxa, minimizing the experimental burden in laboratory conditions. For example, the start of activity experiments in the case of *Jekelius* species indicated that the heating rate (HR), which can only be obtained using continuous thermographic measurements, was the most discriminative variable for describing the thermal stress profile of both species. Variables like the start of the stress temperature (SST) or chill coma temperature (CCT) can also be useful for heat or cold responses, respectively. Therefore, the proposed protocol effectively and objectively guides the detection of the relevant variables.

In the specific *Jekelius* example, this technique could discriminate between the thermal responses of two regionally sympatric and phylogenetically close species (Cunha et al., 2011). Independent of the physical inertia manifested by the differences in body weight (whose effect was controlled by the PLS components), both species mainly differed in their response to cold conditions and the temperature at which both began to be active after cold stupor. Therefore, the species inhabiting lowland areas (*J. punctatolineatus*; see Fig. 3) has remarkably lower heating rates after cold stupor and higher cooling rates for reaching stupor, and its haemolymph freezing points occur at higher temperatures, than the species inhabiting the highland areas (*J. hernandezi*). These results are in agreement with the influence of minimum temperatures in delimiting the species ranges (Sanderson, 1908; Klok and Chown, 2003; Andersen et al., 2015; Sinclair et al., 2015) as well as with cold adaptation (Scholander et al.,1953; Addo-Bediako et al., 2002; Vorhees et al., 2013) and climate variability hypotheses (Addo-Bediako et al., 2000), in which ectothermic species living in colder climates have higher metabolic rates and wider tolerances to compensate for the shorter periods of favourable conditions. A relatively similar pattern has been observed in South African dung beetles, whose critical thermal maximum temperatures showed considerably less variation with altitude than the critical thermal minimum temperatures (Gaston and Chown, 1999). Furthermore, in the neotropical dung beetle, *Canthon humectus hidalgoensis*, the supercooling point is highly correlated with the altitude, and the SCP frequency distributions show notable physiological variability among populations (Verdú, 2011). On the other hand, the heat response of both *Jekelius* species hardly differs, maintaining similarly high body temperatures for a substantial variety of environmental conditions, which is in agreement with the maxithermy hypothesis (Hamilton, 1973; Angilletta et al., 2010) which has been proposed as a mechanism directed to facilitate the metabolic functions of ectothermic species. Moreover, relative similarities in the heat thermal constraints have been observed in a great number of endothermic dung beetles (Heinrich, 1993; Verdú, 2011; Verdú et al., 2012). Endothermic dung beetles begin to regulate the excess of heat at lower body temperatures than ectothermic species when caused by environmental temperatures and/or the internal heat generated by wing muscles during flight. Thus, endothermic dung beetles quickly thermoregulate in order to avoid reaching thermal shock (around 42 °C) and heat damage temperatures (CT_{max}). In the case of wingless ectothermic dung beetle species, such as the two studied *Jekelius* species, with a probable ectothermic pattern, the active thermoregulation range begins at higher temperatures (HRT ≈ 44 °C; see Table 6) and it is directed to regulate the excess of heat due to the ambient temperature since the absence of flight muscles makes it impossible the generation of internal heat (Verdú et al., 2006).

We emphasize that the real-time and continuous analysis of the response to heat allows us to observe the thermoregulation range at which they can maintain their body temperature within limits to avoid reaching temperatures that produce damage and even death. With the proposed protocol, we can analyse, according to behaviour and location, the ability to regulate temperature excess (Kovac and Stabentheiner, 2011; Verdú et al., 2012). The behaviour observed in the thermoregulatory range has been previously studied in flying species (Weis-Fogh, 1967; Heinrich, 1993; Lighton, 1994; Verdú et al., 2012) and now also seems to appear in the studied wingless species. This is very important because it influences their physiology, performance and fitness, allowing them to survive at high temperatures (Cossins and Bowler, 1987; Hochachka and Somero, 2002).

5. Conclusions

The joint use of infrared thermography, combined with an appropriate statistical technique directed to work with low sample sizes and high collinearity among variables, can facilitate the collection of thermal variables capable of discriminating between the thermal responses of closely related species. We hope that the application of this protocol to a larger number of dung beetle species evenly distributed across the phylogenetic tree can enhance our knowledge on the evolutionary strategies of these species, and the role of thermal responses in explaining the current distribution patterns and future probable changes under different climate scenarios.

Conflict of interest

There are no conflicts of interest to declare.

Author contributions

B.G., J.R.V. and J.M.L conceived and designed the research; B.G. and J.R.V. collected the biological samples. B.G., J.R.V. and J.M.L. designed and performed the thermographical and behavioural analyses. L.M.C. and J.M.L. performed data analyses. B.G., J.M.L., J.R.V., and L.M.C. wrote the manuscript.

Table 6
Mean values of the predictor and response variables for the two *Jekelius* species (n = 11 ± SD in brackets) in the heat response experiments. SST, HRT, CT_{max} and ULT are in °C. rSAR, rHSR, rTR and rHCR are in °C min^{-1}. iSAR, iSR, iTR and iHCR are dimensionless because represent the differences in the area under the response curves of the model and living individuals. Weight is the body mass of fresh individuals in g and Ta is the ambient temperature during the trials (in °C).

	SST	HRT	CT_{max}	ULT	rSAR	rHSR	rTR	rHCR	iSAR	iSR	iTR	iHCR	Weight	Ta
J. hernandezi	31.31 (± 3.09)	44.69 (± 1.75)	47.55 (± 1.15)	55.67 (± 2.49)	2.37 (± 0.65)	2.80 (± 0.44)	0.74 (± 0.51)	2.02 (± 0.65)	53.90 (± 85.85)	166.72 (± 589.46)	1552.14 (± 1272.34)	1929.19 (± 1074.38)	0.40 (± 0.06)	21.3 (± 1.2)
J. punctatolineatus	33.63 (± 2.13)	44.70 (± 3.07)	47.19 (± 2.29)	54.18 (± 2.53)	2.57 (± 0.23)	3.31 (± 0.60)	1.30 (± 1.64)	1.45 (± 1.02)	54.57 (± 200.76)	79.10 (± 767.61)	1160.33 (± 1166.44)	1855.44 (± 994.04)	0.46 (± 0.09)	22.2 (± 1.39)

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