Thermal tolerance and recovery behaviour of *Thorectes lusitanicus* (Coleoptera, Geotrupidae)

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Abstract. 1. Population differences in physiological responses are examined in *Thorectes lusitanicus*, an endemic Iberian dung beetle species, by submitting individuals of different populations to the same experimental and acclimation conditions.

2. An infrared thermography protocol was used, consisting of three assays: start of activity, cold response, and heat response. Individuals of 12 populations were studied and the comparative explanatory capacities of several environmental factors in relation to the observed inter-population differences were examined.

3. The heating rate from chill coma to the beginning of activity was the variable with the highest discrimination power among the studied populations, accounting for 94% of the observed variance. Regarding the heat response, only six of the 16 thermal variables reached significance (inter-population differences accounted for 52–74% in these six thermal parameters).

4. From the three considered environmental factors (Mediterranean climate, land cover, and trophic characteristics) only land cover characteristics remain statistically significant, affecting the cold response of individuals.

5. *Thorectes lusitanicus* is a species characterised by a high diversity of thermotolerance and recovery traits across populations with a low degree of association with broad environmental factors. Finally, it is suggested that the apterous character of this species could be a determinant factor explaining the high diversity of ecophysiological traits related to thermal stress tolerance and the recovery time.

Key words. Infrared thermography, insect physiology, physiological diversity, thermal stress, thermal tolerance, thermoregulation.

Introduction

Physiological diversity is the variability in physiological traits among animals, plants, and other organisms (Spicer & Gaston, 2009). The existence of this diversity is the foundation of evolutionary physiology, which aims to understand the origin and maintenance of the variety of physiological responses among populations or species inhabiting different environmental conditions (Garland & Adolph, 1991; Schmidt-Nielsen, 1997; Chown, 2001), due to biotic (Nosil *et al.*, 2005) and abiotic factors (Gandon *et al.*, 1998; Rieseberg *et al.*, 1999; Hall, 2005). Among these abiotic factors, environmental temperature has a widespread effect, as it affects almost all biological processes, from molecular kinetics to organismal fitness and

global distribution patterns (Angilletta, 2009; Kingsolver, 2009; Keller & Seehausen, 2012; Ma *et al.*, 2015; Buckley & Huey, 2016).

The response to environmental temperature of different populations may vary as a consequence of phenotypic plasticity (i.e. the same genotype with different phenotypic responses; see West-Eberhard, 1989; Chown & Terblanche, 2006) or because of the existence of ecotypes (i.e. genetically distinct sub-populations restricted to specific areas and habitats manifesting specific tolerance limits to local factors; Turrill, 1946; Lowry, 2012). These responses can also be different under natural conditions (Spicer & Gaston, 2009), but similar after the individuals are subjected to common laboratory conditions (acclimation). This can happen as a consequence of reversible local physiological acclimatisation processes, in which case phenotypic plasticity can play a significant role. For example, the dung beetle *Canthon humectus hidalgoensis* Bates, 1887 exhibits inter- and intra-population variability in its thermal

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tolerance, suggesting phenotypic plasticity in its response to altitude involving a compensatory modification of its low thermal tolerance (Verdú, 2011). By contrast, populations can show persistent physiological differences (ecotypes) that are not easily reversible under acclimation.

Using a recently published thermographic protocol (Gallego *et al.*, 2016), the population variability in the physiological responses of *Thorectes lusitanicus* (Jekel, 1866), an endemic Iberian dung beetle species, was studied. Within the general cold-temperate Geotrupidae family (Zunino, 1984), *Thorectes* species (*sensu lato*) can be considered as paleoendemisms (Cunha *et al.*, 2011) well adapted to Mediterranean conditions (Martín-Piera & López-Colón, 2000; Lobo *et al.*, 2006; Lobo *et al.*, 2015), showing adaptations to arid environments such as elytra fusion, wing loss, or polyphagous habits. Regarding *T. lusitanicus*, these adaptations have recently gained increased prominence, because this coprophagous species is able to consume and bury oak acorns. This surprising dietary shift promotes the dispersal of Mediterranean *Quercus* forests (Pérez-Ramos *et al.*, 2007), also extending the thermal niche of the beetle as well as increasing its reproductive rate (Verdú *et al.*, 2010). Furthermore, this diet shift improves the health condition of beetle individuals through the increase of prophenoloxidase levels, an inactive proenzyme important for the immunity of insects (Verdú *et al.*, 2013).

Considering the distribution range of *T. lusitanicus* in the south of the Iberian Peninsula, where the species can be found in different habitats, the main purpose of this study was to analyse variations in the thermal tolerance and recovery time response of different populations found at 12 broadly separated localities under a common experimental arena. Subsequently, a preliminary assessment was made of the comparative explanatory capacity of several simple macroenvironmental factors with regard to the observed inter-population differences in thermal responses.

Materials and methods

Capture and maintenance

We collected individuals of *Thorectes lusitanicus* in 12 different locations distributed throughout its geographical range in the south of the Iberian Peninsula during 2012, 2013, and 2014 (Table 1; Fig. 1 and Table S1). These individuals were maintained in $10 \times 10 \times 10$ cm plastic containers with soil from the collection site and stored at 15 °C inside a portable cooler (Thermo Engel; 18 litres, Perth, Australia), during transport to the laboratory. We estimated both the cuticular deterioration of the anterior tibia and the hardness of the pronotum and elytra, to select individuals of approximately the same medium age (Tyndale-Biscoe, 1984) avoiding the collection of both young and older individuals. Furthermore, in order to homogenise the effect of diet and gut content on the physiological measures obtained during experimental trials, all the individuals were starved for 5 days at the acclimation temperature prior to the thermal assays (see Verdú *et al.*, 2010 and Gallego *et al.*, 2016 for a complete description of the protocol).

Acclimation temperatures

Individuals from each locality were separated into two groups according to acclimation temperatures. One group of individuals was acclimated at 25 °C for 5 days before the beginning of the heat response assay. This temperature is the minimum required for the proper function of the neuromuscular system, being the temperature at which insect individuals are active without heat stress (May, 1979, 1985; Vannier, 1994; Chown & Nicolson, 2004; Verdú *et al.*, 2006; Heinrich, 2013; Gallego *et al.*, 2016). The other group was acclimated at 5 °C before the beginning of the cold response trials, as this temperature is the recommended minimum temperature before reaching the torpor state as a result of cold (Vannier, 1994; Chown & Nicolson, 2004; Gallego *et al.*, 2016). These two acclimation temperatures were achieved using two refrigerated chambers (MIR-153; Sanyo Electric Co., Osaka, Japan). These experimental procedures conform to Spanish legal requirements, including those relating to conservation and welfare. Beetle capture was carried out with the relevant permits related to animal collection and laboratory study. Both collection and maintenance procedures prevented beetle death, being designed to minimise the mortality and stress of the individuals.

Thermal analyses

The collected individuals were submitted to a standardised protocol of infrared thermography capable of providing continuous thermal information on rare, threatened insect species without necessarily causing death or injury (Gallego *et al.*, 2016). Three different thermal assays were designed following this protocol to estimate the thermal stress responses of dung beetles: start of activity, cold response, and heat response (see Fig. 2 and Gallego *et al.*, 2016). The start of activity assay measures the temperature at which activity begins in individuals coming out of a paralysed state due to cold (chill coma recovery temperature, or CCRT), and corresponds to the moment at which the focal individual begins to move the legs and head appendices, changing its position with respect to the initial position (obtaining the heating rate, or HR, to that moment). The cold response assay starts when individuals are paralysed due to cold (chill coma temperature, CCT) and the temperature continues decreasing at a constant rate (0.30 °C min^{-1}) until beetles reach the supercooling point (SCP, when total freezing of the haemolymph occurs); the cooling rate (CR) between these two points is calculated. The thermal variables arising from the cold response and the start of activity assays were analysed jointly (hereafter, the cold response) and these tests were performed in a portable cooler with the same individuals. First, the activity assay was started, with the chamber being cooled (-8 °C) until the individuals reached a state of torpor. Subsequently, the temperature was increased (at a rate of 0.15 °C min^{-1}) until recovery. Finally, we examined the ability of individuals to regulate their body temperature from 25 °C in the heat response assays. By linearly increasing the temperature (at a heating rate of 1.5 °C min^{-1}) from 25 °C, four threshold temperatures were estimated for each animal: start of stress temperature (SST), heat regulation temperature (HRT), critical thermal maximum (CT$_{max}$), and upper

Table 1. Locality name (letter corresponding to its geographic location in Fig. 1), number of individuals physiologically analysed in each locality (*N*), Spanish province in which each locality is located, longitude and latitude of these localities, and main environmental characteristics according to land cover, trophic quality and Mediterranean climate subtype.

| Location	N	Province	Longitude	Latitude	Land cover	Trophic quality	Mediterranean climate subtypes
Charco Redondo (A)	7	Cádiz	−5.5854	36.2630	Forest	Cow/Acorns	Thermo-mediterranean
Casares (B)	13	Málaga	−5.2790	36.4114	Grasslands	Goat/Sheep	Thermo-mediterranean
Los Alcornocales (C)	9	Málaga	−5.5934	36.5151	Forest	Cow/Acorns	Meso-mediterranean
Alhaurín de la torre (D)	6	Málaga	−4.6317	36.6637	Grasslands	Rabbit	Thermo-mediterranean
Carratraca (E)	7	Málaga	−4.7869	36.8532	Scrub	Goat/Sheep	Meso-mediterranean
La Contadora (F)	10	Málaga	−4.3762	36.8177	Forest	Goat/Sheep	Meso-mediterranean
Las Lagunas (G)	9	Málaga	−4.3135	37.0957	Scrub	Goat/Sheep	Meso-mediterranean
Iznalloz (H)	12	Granada	−3.4810	37.3933	Scrub	Rabbit	Meso-mediterranean
Las Mimbres (I)	15	Granada	−3.4578	37.3157	Forest	Cow/Acorns	Meso-mediterranean
Alcolea (J)	6	Almería	−2.9326	36.9108	Grasslands	Rabbit	Thermo-mediterranean
Baza (K)	12	Granada	−2.8713	37.4601	Scrub	Goat/Sheep	Meso-mediterranean
Sierra Mágina (L)	7	Jaén	−3.6050	37.7630	Forest	Rabbit	Meso-mediterranean
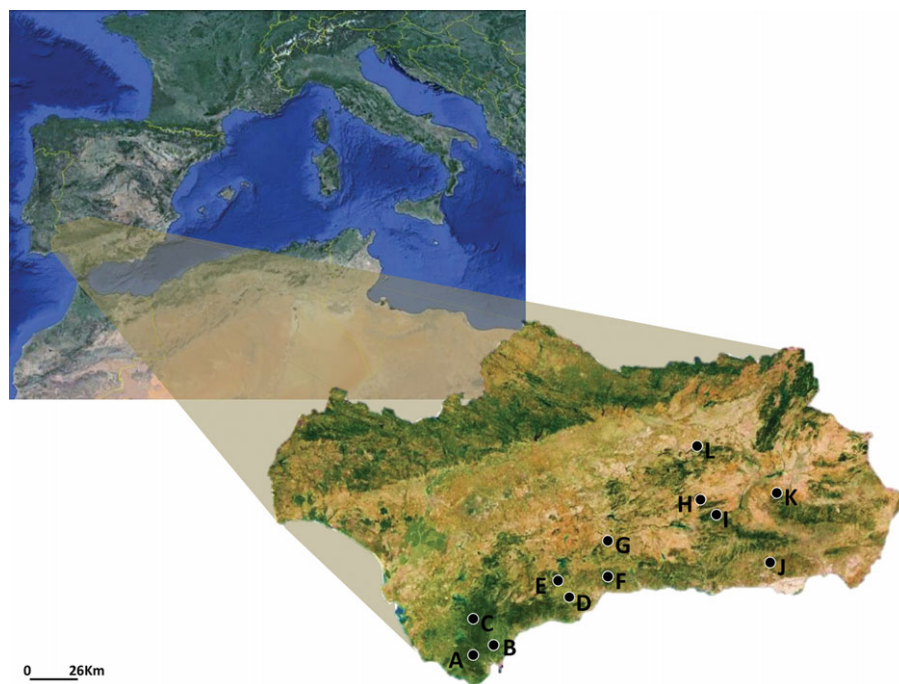
Fig. 1. Map showing localities of study populations of *Thorectes lusitanicus* on the southern Iberian Peninsula (Andalucia; see the Materials and methods section for more details). For localities corresponding to capital letters, see Table 1. [Colour figure can be viewed at wileyonlinelibrary.com].

lethal temperature (ULT). The SST is the moment at which we observed a clear acceleration in leg and head movements, reflecting the stress state; the HRT is the temperature at which the individuals decreased body temperature with abdominal pumping movements and stretching and shrinking their forelegs; CT_{max} is the temperature at which the individuals began to be unable to regulate their excess of heat; and ULT is the temperature at which individuals became completely paralysed. In the heat response assays, the temperatures of living individuals were compared against those of freshly killed (by freezing) individuals of the same species acclimated under the same conditions, which represent operative temperature measurements (i.e. body temperatures theoretically equilibrated with the environment;

Hertz *et al.*, 1993; Dzialowski, 2005). These freshly killed individuals are hereafter referred to as 'model individuals' without thermoregulation ability, used to obtain information on the active thermoregulation of live beetles. Model individuals were also considered when examining the cold response, but their thermal patterns were the same as those of living individuals, and thus they were not used in data analyses (Fig. 2). Based on these four temperatures, we identified four phases or ranges in the heat response (see Fig. 2 and Gallego *et al.*, 2016): supra-optimal activity range (SAR, which spans from the selected temperature at which the individuals were active to the SST); heat stress range (HSR, from the SST to the HRT); thermoregulation range (TR, the time elapsed from HRT to CT_{max}); and heat coma range

(HCR, delimited from CT_{max} to the ULT). Rates of temperature increase of living individuals within these four phases (rSAR, rHSR, rTR and rHCR) were also used as thermal parameters. Additionally, we calculated the differences between model and living individuals in the area under the response curves for each of the four thermal phases by means of integrals (iSAR, iHSR, iTR and iHCR). The differences in the increment of temperature between model and living individuals for each phase were also calculated (ΔSAR, ΔHSR, ΔTR and ΔHCR), as a measure of the thermoregulation ability of each individual (see Fig. 2, Table S2; and Gallego *et al.*, 2016). In total, 16 continuous thermal variables were considered in the heat response experiments and five in the cold response experiments.

All data were obtained using infrared thermography with a FLIR ThermaCam P620 (Melrose, Massachusetts) thermal infrared camera (resolution of 640×480 pixels) and a microbolometer Focal Plane Array detector (with a spectral range of $7.5–13\,\mu m$ and a thermal sensitivity of $0.06\,°C$ at $30\,°C$). The thermocamera was calibrated with the standard calibration service provided by FLIR Systems Inc. We measured the cuticle emissivity at different temperatures ($40–70\,°C$) using fresh cuticles of different locations of *T. lusitanicus*, obtaining an average value of 0.96. For the measurements we took as reference electrical tape (a reference method as described in ISO 18434-1) and black paint (NEXTEL-Velvet-Coating 811-21, Hamburg, Germany). In each experimental trial, the ambient temperature of the laboratory was recorded using a K/J thermocouple Fluke 152 (Fluke Co., Everett, Washington). All individuals were weighed with an AG104 analytic balance (precision error of 0.1 mg; Mettler Toledo, Columbus, Ohio). The colour variability within the individuals of *T. lusitanicus* was not considered because all collected individuals were black and the colour variability in dung beetles is structural and not pigmentary (see Kinoshita, 2008; Akamine *et al.*, 2011). Moreover, the possible structural differences in the beetle exoskeleton of different Geotrupidae species do not seem to significantly influence inner body temperatures (Carrascal *et al.*, 2017). The three experiments were carried out with the thermocamera connected to a computer in which the video recording was stored. Subsequently, we selected areas in the central part of the pronotum ($\sim 20\,mm^2$) where temperature data were obtained using ThermaCAM Researcher Pro 2.9 (FLIR software).

Environmental factors

The 12 studied localities were assigned to one of the levels of three different factors related to the identity of trophic resources, land cover characteristics, and the Mediterranean climate subtype to which each locality belongs (Table 1; Fig. 1). The trophic resource factor contains three levels according to the main food resources available in each locality: rabbit pellets, goat and sheep dung, and cow dung with acorns. Rabbit pellets have special characteristics such as small size, low content of nutrients (mean $\pm$ SD; $1.2\% \pm 0.2\%$ nitrogen), and very low hydric content ($15.0\% \pm 0.5\%$ water content) (Verdú & Galante, 2004), and the availability and abundance of these small dry pellets seem to have been fundamental for the maintenance and evolutionary success of *Thorectes*
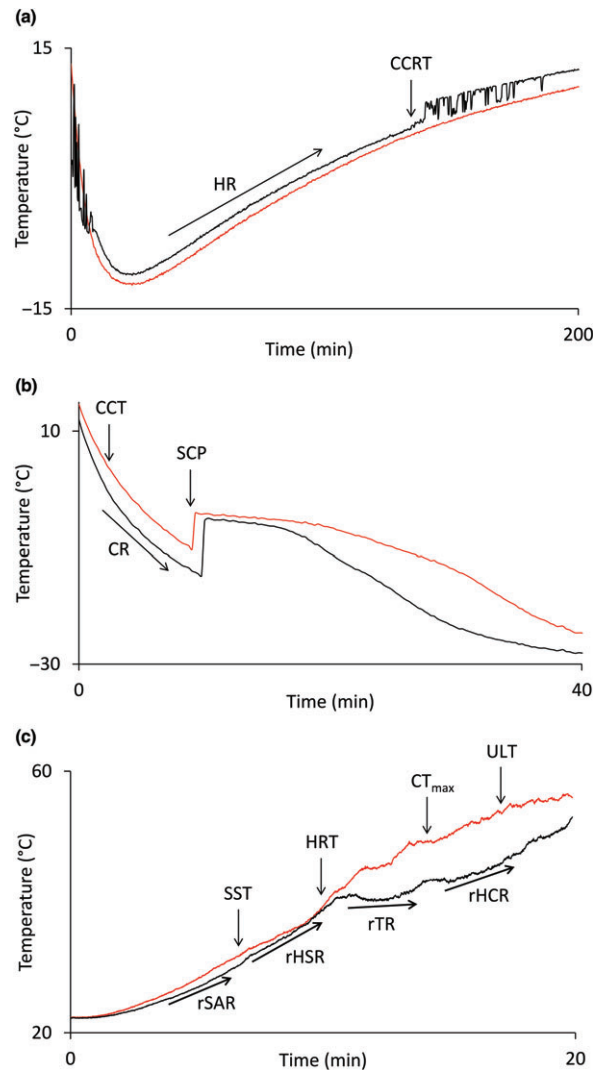

Fig. 2. Thermal response obtained at each assay with main variables measured: (a) start of activity assay; (b) cold response; and (c) heat response. CCRT, chill coma recovery temperature; HR, heating rate; CCT, chill coma temperature; CR, cooling rate; SCP, supercooling temperature; SST, start stress temperature; HRT, heat regulation temperature; CT_{max}, critical thermal maximum; ULT, upper lethal temperature; rSAR, heating rate on supra-optimal activity range; rHSR, heating rate on heat stress range; rTR, heating rate on thermoregulation range; rHCR, heating rate on heat coma range. Continuous line, living individuals; dashed red line, model individuals. [Colour figure can be viewed at wileyonlinelibrary.com].

dung beetles in Mediterranean environments (Verdú & Galante, 2004); sheep and goats produce medium-sized dung pellets with a relatively high nutrient content ($2.4\% \pm 0.2\%$ nitrogen) and medium hydric content ($53.5\% \pm 0.9\%$ water content) (Verdú & Galante, 2004); cow dung is characterised by a moderate nutrient content ($1.9\% \pm 0.1\%$ nitrogen) and a high hydric proportion ($70.1\% \pm 1.0\%$ water content) (Verdú & Galante, 2004). The cow dung trophic level also includes the presence of acorns, as oak forests are linked to the humid conditions needed for cattle farming in the south of the Iberian Peninsula.

Moreover, consumption of acorns by *T. lusitanicus* has proved to be decisive in the increase in the thermotolerance and health status of this species (Verdú *et al.*, 2010). The three levels of the land cover factor represent landscapes within an increasing gradient of vegetation cover (Moreira, 2007): scattered scrubs and grasslands (first level, mainly dominated by *Ulex baeticus* Boiss, *Rosmarinus officinalis* L., *Cistus albidus* L., and *Phlomis purpurea* L.), scattered scrubs and trees (second level; *Quercus ilex* L., *Pinus pinaster* Aiton, *Thymus zygis* L., *Quercus coccifera* L., and *Retama sphaerocarpa* (L.) Boiss), and forests (third level; *Quercus suber* L., *Quercus ilex*, and *Pinus halepensis* Mill.). Levels of trophic and land cover factors were assigned by examining the general characteristics of the study locations in an area 500 m around collecting places. Finally, the Mediterranean climate subtypes were based on the thermicity index, It = $[T + m + M] \times 10$, where T is the annual average temperature, m is the average minimum temperature of the coldest month of the year, and M is the average maximum temperature of the coldest month of the year. The following two levels were defined considering It: thermo-mediterranean (It from 350 to 450) and meso-mediterranean (It from 220 to 350). This factor is usually related to the physiological effects of climate on individuals (Verdú & Galante, 2002; Chown & Nicolson, 2004; Rivas-Martínez, 2004).

Statistical analyses

We first used general linear mixed models (GLMMs) to select those thermal variables capable of discriminating the differences in cold and heat responses of the individuals belonging to the 12 sampled localities. The measures of each variable were tested against a random factor defining each of the collecting localities. Body mass of beetles and subtle, random variations in the ambient temperature during assays were included as covariates to control for their effects on inter-population differences ($n = 53$ individual beetles for the heat response and $n = 60$ for the cold response). Ambient temperature was not included in the GLMMs aimed at examining cold responses, because essays were conducted inside refrigerated chambers with controlled conditions. Location was considered a random factor as the 12 selected localities aimed to represent a sample of the distributional range of the species. Post hoc unequal N honest significant difference (HSD) tests were subsequently used to determine the amount or quantity of significant differences among the means of all possible pairs of localities. In all, 21 related significance tests were performed to estimate inter-population differences using 21 thermal variables measured on the same beetle populations. To control for type I errors, increasing the power of individual tests while maintaining fixed experiment-wise error rates, the sequential Bonferroni adjustment (Rice, 1989; Chandler, 1995) with an experiment-wise error rate of 0.05 was used.

Two-way mixed mancovas were used to test whether the thermal parameters for which significant inter-population differences were found differed between the levels of each of the three fixed factors (trophic resource, land cover, and Mediterranean climate subtypes). Again, location was considered a random factor (in this case, nested within the levels

of each environmental factor), while the covariates included in the models were body mass of individual beetles and, in the case of heat response assays, ambient temperature. These mancova models use all the observed variability among individual beetles, partition the original variance in the components attributable to environmental factors while considering the among-individual and among-population components, and avoid pseudoreplication by establishing the d.f. for the error terms according to the 12 studied populations. Three models were carried out with four cold variables (one per environmental factor), and the other three models with six heat variables. These variables were statistically significant in the GLMM models. Due to the large number of significance estimations derived from these six models (three environmental factors × four plus six thermal variables = 30), the multivariate Wilks' lambda test was carried out as an omnibus test of global significance of each environmental factor. Only if Wilks' lambda test was significant did we proceed with univariate F tests for each particular thermal variable, thus providing protection against the inflation of type I error in each analysis (with d.f. = 2,9 in F tests for land cover and trophic environmental factor, and d.f. = 1,10 for Mediterranean climate subtypes). The amount of variance for each environmental factor was estimated by dividing its sum of squares (SS) by the total SS of each thermal variable.

All the analyses were carried out using StatSoft's statistica v12.0 (StatSoft Inc, Tulsa, Oklahoma).

Results

Body mass did not exert a significant role in any of the 16 considered heat response variables, while subtle random variations in ambient temperature in the experimentation room reached statistical significance in only CT_{max}, SAR and ΔSAR.

Except for the temperature at the supercooling point (SCP), all physiological variables for the cold response significantly discriminated among the 12 populations of *T. lusitanicus*. Heating rate from chill coma to the beginning of activity (HR) was the variable with the highest discrimination power among the studied populations, accounting for 94% of the observed variance among individual beetles (50 out of 66 differences between population pairs reached significance in the *post hoc* HSD tests; Table 2; Fig. 3). Regarding the heat response, only six of the 16 thermal variables reached significance after controlling for the multiple testing. Inter-population differences accounted for 52–74% of the variance in rHSR, SST, ΔHSR, rSAR, iSAR, and CT_{max}, and significantly discriminated approximately one-sixth of differences between population pairs (Table 2).

The influence of the three coarse-grained environmental factors on the above selected 10 variables characterising the different thermal responses among the 12 studied beetle populations is shown in Table 3. Globally, the unique, significant and measurable environmental effect was registered for the response to cold considering the three landscape categories (grasslands, scrub, and forests). Nevertheless, this environmental factor explained only an average of 23% of the observed variance in the studied thermal variables. Land cover differences significantly influenced the heating rate from chill coma to the beginning of activity (HR), as shown in Fig. 4, being responsible for 52% of the

Table 2. Intraspecific differences of cold and heat response variables (five and 16 thermal parameters, respectively) among populations of *Thorectes lusitanicus*, after controlling for body mass of individual beetles and subtle random variations in ambient temperature in the experimentation room during assays (included as covariates in the general linear mixed ANCOVA models, using population as a random factor).

Cold variables	$F_{(11,47)}$	P	R^2	No. of pairs
HR ($^{\circ}$C min^{-1})	71.86	<0.0001*	94.3	50
CCRT ($^{\circ}$C)	5.99	<0.0001*	58.4	8
CCT ($^{\circ}$C)	4.77	<0.0001*	47.3	9
CR ($^{\circ}$C min^{-1})	3.93	0.0005*	47.3	7
SCP ($^{\circ}$C)	1.29	0.261	23.0	0

Heat variables	$F_{(11,39)}$	P	R^2	No. of pairs
rHSR ($^{\circ}$C min^{-1})	6.96	<0.0001*	62.7	12
SST ($^{\circ}$C)	5.92	<0.0001*	58.6	10
ΔHSR ($^{\circ}$C)	5.38	<0.0001*	54.7	8
rSAR ($^{\circ}$C min^{-1})[†]	9.23	<0.0001*	51.3	13
iSAR[†]	5.16	<0.0001*	50.6	8
CT$_{max}$ ($^{\circ}$C)[†]	5.21	<0.0001*	48.8	9
HRT ($^{\circ}$C)	2.62	0.013	42.2	0
ΔHCR ($^{\circ}$C)	2.35	0.024	37.9	0
iHSR	2.06	0.048	34.9	1
ΔTR ($^{\circ}$C)	1.66	0.120	30.8	0
iHCR	1.35	0.237	26.6	0
ULT ($^{\circ}$C)	1.71	0.108	26.4	0
rHCR ($^{\circ}$C min^{-1})	1.25	0.288	24.9	0
ΔSAR ($^{\circ}$C)	1.40	0.213	23.9	0
iTR	0.78	0.655	17.2	0
rTR ($^{\circ}$C min^{-1})	0.71	0.724	15.4	0

∗Variables that remain significant after the sequential Bonferroni adjustment, to control for the increase in type I error while performing multiple related, non-independent, tests.

†Variables for which the ambient temperature during assays had a significant and positive effect ($P < 0.05$, accounting for <14% of total variance; for iSAR the effect remained significant after the sequential Bonferroni adjustment).

R^2, variance accounted for by the random factor population (in %). The right column shows the number of population-pairs with significant differences in the *post hoc* honest significant difference tests (the total number of pairwise comparisons are 66).

HR, heating rate; CCRT, chill coma recovery temperature; CCT, chill coma temperature; CR, cooling rate; SCP, supercooling point; rHSR, heat stress range rate; SST, start stress temperature; rSAR, supra-optimal activity range rate; iSAR, supra-optimal activity integral; CT$_{max}$, critical thermal maximum; HRT, heat regulation temperature; ΔHCR, heat coma range increment; iHSR, heat stress range integral; ΔTR, thermoregulation range increment; iHCR, heat coma range integral; ULT, upper lethal temperature; rHCR, heat coma range rate; ΔSAR, supra-optimal activity range increment; iTR, thermoregulation range integral; rTR, thermoregulation range rate.

observed variation under controlled laboratory conditions: populations of beetles inhabiting grassland areas heated at lower rates than those living in woodlands or scrub with scattered trees. For the remaining non-significant combinations of environmental factors and cold/heat thermal response variables, the amount of variation attributable to environmental factors decreased to 6–16%.

Discussion

Taking into account the intraspecific and interindividual variability of the data used, our results suggest that *T. lusitanicus* is a species characterised by a high diversity of thermotolerance mechanisms across populations, even after being acclimated and tested under homogeneous laboratory conditions. These different intraspecific thermotolerances can be viewed as persistent physiological differences, taking into account the fact that all individuals were submitted to the same acclimation temperatures for 5 days prior to the physiological analyses. Thus, it is

reasonable to assume that this species has different thermotypes (Matthews, 1986; Kawecki & Ebert, 2004; Spicer & Gaston, 2009), which may or may not have a genetic basis. It is therefore necessary to carry out additional research to examine whether the detected thermal variations change within the lifetime of an individual or along successive generations when exposed to different conditions from those prevailing in the original habitat (Fischer *et al.*, 2003, 2006; Rako & Hoffmann, 2006). In that case we should expect local adaptations of plastic phenotypes that are not easily reversible, but additional genetic and physiological studies are needed to corroborate this possibility (Tufto, 2000; Chown & Terblanche, 2006).

The thermal variable with a greater ability to discriminate among the 12 populations was the heating rate from chill coma to the beginning of activity. Although nine other thermal variables were also able to discriminate between some of the populations considered, it is worth noting the comparatively weak role played by the classical physiological variables related to lethal temperature limits, such as the chill coma and critical thermal
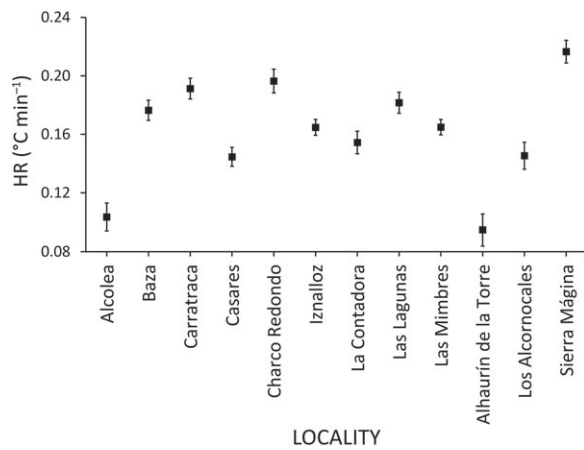
Fig. 3. Adjusted means (±95% CI) of the HR variable (heating rate from the state of paralysis due to cold to the state of activity recovery) in the 12 studied populations. The adjusted means derive from general linear mixed ANCOVA models, controlling for the effects of body mass of individual beetles.

maxima temperatures, which cause damage in the individuals. The study of population differences in insect thermal responses has traditionally been focused on examining sublethal or lethal temperatures (Neargarder *et al.*, 2003; Bahrndorff *et al.*, 2006; Hidalgo-Galiana *et al.*, 2014; García-Robledo *et al.*, 2016), despite the methodological problems in the estimation of these thermal limits (Santos *et al.*, 2011). However, the use of infrared thermography in insects allows for the detection of a broad spectrum of relevant thermal variables, widening the knowledge

of the interaction between temperature and behavioural data. Furthermore, the ease with which this methodology enables us to take measurements allows for research on endemic and endangered species or restricted populations (Gallego *et al.*, 2016; Tattersall, 2016). Our results suggest that the detection of inter-population differences in thermal biology may have a much better chance of succeeding if we are able to include variables able to reflect the thermal responses beyond thermal limits.

According to our results, the main trophic resources available and the Mediterranean climate subtypes factors were not associated with the thermal response variables analysed, while the factor related to land cover characteristics was slightly related to inter-population differences in the thermal response to cold of *T. lusitanicus*. The variations in thermotolerance and recovery time to cold (as HR) under controlled laboratory conditions do not seem to follow clearly defined gradients or clines tracking broad geographical environmental variations throughout the distribution area of this species. Thus, we suggest that these physiological characteristics should be explored from a microclimatic and local point of view. The apterous character of this species could be determinant to explain the high diversity of ecophysiological traits related to thermal stress tolerance and the recovery time from a chill coma temperature to a temperature of optimal activity or performance. In dung beetles, as in other Coleoptera, the apterism related to the fusion of elytra is considered an adaptation to dry and arid conditions, because it reduces the loss of hydric content, thus allowing the use of water in thermoregulation processes by means of evaporative cooling mechanisms (Cloudsley-Thompson, 1975; Draney, 1993; Verdú & Galante, 2002). In the case of Geotrupidae, including *T. lusitanicus*, the presence of this subelytral cavity is crucial to prevent

Table 3. Multivariate mixed MANCOVA models testing for the influence of three environmental factors on the thermal variables measuring the cold and heat responses of *Thorectes lusitanicus* in 12 different populations (controlling for body mass of individual beetles and subtle random variations in ambient temperature in the experimentation room during assays – covariates – and using population as a random factor).

	Land cover	Trophic quality	Mediterranean climate subtypes
Cold variables			
Wilks' multivariate test	$F = 2.50, P = 0.050$	$F = 1.94, P = 0.115$	Cannot be estimated
HR (°C min^{-1})	52.2*	10.3	28.0
CCRT (°C)	9.3	12.9	1.7
CCT (°C)	12.9	20.0	4.1
CR (°C min^{-1})	18.3	20.5	8.5
Heat variables			
Wilks' multivariate test	$F = 1.85, P = 0.136$	$F = 2.16, P = 0.085$	Cannot be estimated
rHSR (°C min^{-1})	19.8	35.6	13.0
SST (°C)	5.2	0.8	0.6
ΔHSR (°C)	3.8	4.5	0.8
rSAR (°C min^{-1})	3.6	11.5	15.0
iSAR	18.4	11.2	5.0
CT$_{max}$ (°C)	17.4	3.1	3.2

*Thermal variable with a statistically significant influence for the considered environmental factor.
The figures for each thermal variable are the amounts of variance accounted for by each environmental factor (expressed as a percentage). The thermal response variables considered are those with significant inter-population differences in Table 2. Degrees of freedom for the Wilks' multivariate tests are eight, 18 for cold variables, and 12, 14 for heat variables. Wilks' multivariate test cannot be estimated for Mediterranean climate subtypes because this factor has only two levels; nevertheless no variable reached the significance level.
HR, heating rate; CCRT, chill coma recovery temperature; CCT, chill coma temperature; CR, cooling rate; rHSR, heat stress range rate; SST, start stress temperature; ΔHSR, heat stress range increment; rSAR, supra-optimal activity range rate; iSAR, supra-optimal activity range integral; CT$_{max}$, critical thermal maximum.
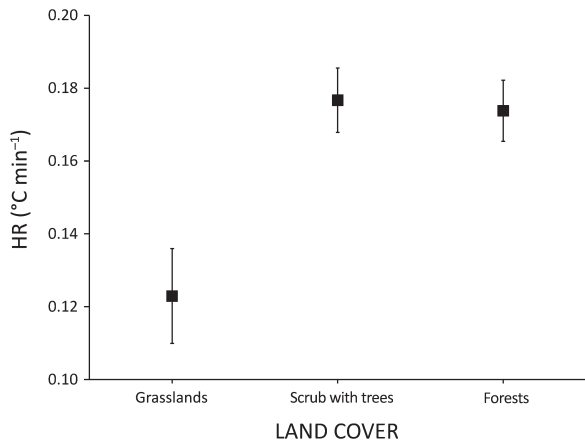
Fig. 4. Variation of the heating rate (HR) from chill coma to the beginning of activity measured under common laboratory conditions in *Thorectes lusitanicus* beetles from 12 populations of southern Spain ($F = 5.51$, d.f. = 2,9, $P = 0.027$). The *y*-axis shows the adjusted values ($\pm$95% CI) controlling for body mass of individuals and using populations as a random factor.

high water loss rates, as its respiration type is continuous (J.R. Verdú, pers. obs.), lacking a discontinuous gas-exchange respiratory type as found in other dung beetles inhabiting xeric areas (Duncan & Byrne, 2002; Scholtz *et al.*, 2009). According to our results, individuals from localities dominated by grasslands had lower heating rates during the chill coma recovery range (i.e. lower activation rates, HR) than those inhabiting more vegetated habitats, with shrubs and trees projecting shadows on to the ground. This could be attributed to the microclimatic effects of this vegetation cover, affecting the intensity of sun radiation, movement of air, and evapotranspiration (Geiger, 1965; Willott, 1997). Although tussocks of grass can act as a microclimatic refuge (Hamilton, 1975; Young, 1979), the decrease in heating rates in these open landscapes could be considered as an adaptation to the higher probability of passive conductive heat gain to avoid overheating in areas without a sun-shade mosaic (Digby, 1955; Kingsolver & Watt, 1983).Further research on the variation of heating rates under different microclimatic conditions is necessary to support this hypothesis. The relevance of HR is in agreement with former results (Turner & Tracy, 1983; Stevenson, 1985; Huey & Bennett, 1990; Gallego *et al.*, 2016) in which modifications of the heating rates during the chill coma recovery range are considered as one of the main physiological mechanisms in insects (Sinclair *et al.*, 2012), because those populations inhabiting cooler conditions recover more quickly (Hoffmann *et al.*, 2005; Sisodia & Singh, 2010). The main factors influencing the heating rate from the chill coma state are the cold exposure temperature and time (MacMillan & Sinclair, 2011), which affect the ability of individuals to remain active. We predicted that trophic quality would influence the cold response variables, considering that Verdú *et al.* (2010) found an increase in the period of activity of individuals feeding on acorns, and a diminished tolerance to cold conditions when the individuals fed only on dung, in this polyphagous species under laboratory conditions. Nevertheless, these subtle ecophysiological patterns did not emerge at the macroecological, inter-population scale.

Further fine-grained ecological studies focused on individual beetles and microhabitat characteristics are needed to elucidate the effect of the interaction between nutrition and land cover on the activation rates from cold conditions.

Our results throw light on the interaction of behavioural thermoregulation, thermal physiology, and ecology in *T. lusitanicus* as a means of coping with the conditions imposed by the Mediterranean climate. Some populations of this dung beetle species show different thermal tolerance and recovery responses, but these differences are difficult to explain considering macroenvironmental gradients. Thus, a future step would be to examine the correspondence among the differences in the thermal responses, genetic divergence, and local environmental characteristics in order to disentangle the observed physiological differences separating the evolutionary history and the influence of broad abiotic gradients (i.e. climate) from mere local contingent factors related to farming history and habitat diversity of trophic resources.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/een.12447

Table S1. Detailed information of each of the individuals used in this study.

Table S2. Thermoregulation variables derived from the heat response assay.

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Supporting Information

Table S1. Detailed information of each one of the individuals used this study

Heat Individuals			Cold Individuals		
Locality	**Date**	**Sex**	**Locality**	**Date**	**Sex**
Alcolea	December 2013	Male	Alcolea	December 2013	Male
Alcolea	December 2013	Female	Alcolea	December 2013	Female
Alcolea	December 2013	Male	Alcolea	December 2013	Female
Baza	October 2012	Male	Baza	March 2014	Female
Baza	October 2012	Male	Baza	March 2014	Male
Baza	October 2012	Male	Baza	March 2014	Female
Baza	March 2014	Male	Baza	March 2014	Female
Baza	March 2014	Male	Baza	March 2014	Female
Baza	March 2014	Male	Carratraca	February 2013	Female
Baza	March 2014	Male	Carratraca	February 2013	Male
Carratraca	February 2013	Female	Carratraca	February 2013	Male
Carratraca	February 2013	Female	Carratraca	February 2013	Female
Casares	October 2012	Male	Carratraca	February 2013	Female
Casares	October 2012	Female	Casares	February 2013	Female
Casares	October 2012	Female	Casares	February 2013	Female
Casares	October 2012	Male	Casares	February 2013	Female
Casares	October 2012	Female	Casares	February 2013	Male
Casares	February 2013	Female	Casares	October 2012	Female
Casares	February 2013	Female	Casares	October 2012	Male
Charco Redondo	February 2013	Male	Casares	October 2012	Male
Charco Redondo	February 2013	Female	Charco Redondo	February 2013	Female
Charco Redondo	February 2013	Male	Charco Redondo	February 2013	Female
Iznalloz	March 2014	Male	Charco Redondo	February 2013	Female
Iznalloz	March 2014	Female	Charco Redondo	February 2013	Male
Iznalloz	March 2014	Female	Iznalloz	January 2013	Female
Iznalloz	March 2014	Male	Iznalloz	January 2013	Male
La Contadora	March 2014	Male	Iznalloz	January 2013	Female
La Contadora	March 2014	Female	Iznalloz	March 2014	Male
La Contadora	March 2014	Male	Iznalloz	March 2014	Female
La Contadora	March 2014	Female	Iznalloz	March 2014	Male
La Contadora	March 2014	Male	Iznalloz	March 2014	Male
Las Lagunas	October 2012	Male	Iznalloz	March 2014	Female
Las Lagunas	October 2012	Male	La Contadora	March 2014	Female
Las Lagunas	October 2012	Male	La Contadora	March 2014	Female
Las Lagunas	March 2014	Male	La Contadora	March 2014	Male
Las Mimbres	October 2012	Female	La Contadora	March 2014	Female
Las Mimbres	October 2012	Female	Las Lagunas	October 2012	Female
Las Mimbres	October 2012	Male	Las Lagunas	October 2012	Female
Las Mimbres	October 2012	Female	Las Lagunas	October 2012	Female
Las Mimbres	March 2014	Male	Las Lagunas	March 2014	Male
Alhaurín de la torre	November 2014	Female	Las Lagunas	March 2014	Male
Alhaurín de la torre	November 2014	Male	Las Mimbres	January 2013	Male
Alhaurín de la torre	November 2014	Male	Las Mimbres	October 2012	Male
Alhaurín de la torre	November 2014	Female	Las Mimbres	October 2012	Male
Los Alcornocales	October 2012	Male	Las Mimbres	October 2012	Male
Los Alcornocales	October 2012	Male	Las Mimbres	March 2014	Male
Los Alcornocales	October 2012	Male	Las Mimbres	March 2014	Male
Los Alcornocales	October 2012	Male	Las Mimbres	March 2014	Male
Los Alcornocales	October 2012	Male	Las Mimbres	March 2014	Male
Sierra Mágina	May 2014	Female	Las Mimbres	March 2014	Female
Sierra Mágina	May 2014	Male	Las Mimbres	March 2014	Male

Sierra Mágina	May 2014	Male	Alhaurín de la torre	November 2014	Female
	December 2013	Male	Alhaurín de la torre	November 2014	Female
			Los Alcornocales	October 2012	Male
			Los Alcornocales	October 2012	Male
			Los Alcornocales	October 2012	Female
			Los Alcornocales	October 2012	Female
			Sierra Mágina	May 2014	Male
			Sierra Mágina	May 2014	Female
			Sierra Mágina	May 2014	Female
			Sierra Mágina	May 2014	Female

Table S2. The thermoregulation variables derived from the heat response assay

ACRONYM	DEFINITION	EXPRESSION
rSAR		$rSAR = \dfrac{SST - 25}{t1 - t0}$
rHSR	The slope of the relationship between the body temperature and time for each of the four delimited ranges	$rHSR = \dfrac{HRT - SST}{t2 - t1}$
rTR		$rTR = \dfrac{CTmax - HRT}{t3 - t2}$
rHCR		$rHCR = \dfrac{ULT - CTmax}{t4 - t3}$
iSAR		$SAR = \int_{t0}^{t1} model - \int_{t0}^{t1} individual$
iHSR	The difference in the integrals between the model and living individuals for each of the four ranges	$SR = \int_{t1}^{t2} model - \int_{t1}^{t2} individual$
iTR		$TR = \int_{t2}^{t3} model - \int_{t2}^{t3} individual$
iHCR		$HCR = \int_{t3}^{t4} model - \int_{t3}^{t4} individual$
ΔSAR		$\Delta SAR = (SSTm - 25) - (SSTi - 25)$
ΔHSR	The difference in temperature increment between the model and living individuals for each of the ranges	$\Delta HSR = (HRTm - SSTm) - (HRTi - SSTi)$
ΔTR		$\Delta TR = (CTmaxm - HRTm) - (CTmaxi - HRTi)$
ΔHCR		$\Delta HCR = (ULTm - CTmaxm) - (ULTi - CTmaxi)$