Energetics and thermal adaptation in semifossorial pine-voles *Microtus lusitanicus* and *Microtus duodecimcostatus*

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Abstract

Rodents colonizing subterranean environments have developed several morphological, physiological and behaviour traits that promote the success of individuals in such demanding conditions.

Resting metabolic rate, thermoregulation capacity and daily energy expenditure were analysed in two fossorial pine-vole species *Microtus lusitanicus* and *M.duodecimcostatus* inhabiting distinct areas of the Iberian Peninsula. Individuals were captured in locations with different habitat and soil features, allowing the comparison of energetic parameters with ecological characteristics, that can help explain the use of the subterranean environment and dependence of the burrow system. Results showed that *M. duodecimcostatus* has lower mass independent resting metabolic rate when compared with *M. lusitanicus,* which may be a response to environmental features of their habitat, such as dryer soils and lower water availability. Thermal conductance increased with body mass and was dependent on the ambient temperature. No significant differences were observed in the DEE, but water economy data demonstrated the influence of the water available on the habitat on the energetics of voles. These may rely on behavioural adaptations and seasonal use of burrows to cope which thermal challenges of subterranean activity and soil constraints. We found strong evidence that *M.lusitanicus* is able to use torpor as response to low ambient temperatures which is a new observation among Arvicolines.

Introduction

Energy metabolism has been extensively studied as a method to approach physiological adaptations to environmental variations (Mueller and Diamond 2001; Lovegrove 2003; Rezende et al 2004; Bozinovic et al 2007), as it represents the summed result of all functions simultaneously occurring in the whole animal, is ecologically relevant to define food requirements (Speakman 2000) and potential for heat stress (Speakman and Król 2010). The resting metabolic rate (RMR) represents a large portion of an animals' energetic requirement comprising 20 to 60% of the daily energetic demands (Ricklefs et al 1996; Hammond and Diamond 1997; Speakman 2000; Westerterp and Speakman

2008), therefore can be highly informative about the adaptive strategies of different species (Morgan and Price 1992; Bennett et al 1994) and populations (Bozinovic et al 2007; Castellanos-Frías et al 2015).

In this study we compared the energetics of two sister species the Lusitanian pine vole (*Microtus lusitanicus* Gerbe,1879) and the Mediterranean pine vole (*Microtus duodecimcostatus* de Selys-Longchamps, 1839).

The *M.lusitanicus* occurs in the northern most side of the Iberian peninsula, while *M. duodecimcostatus* inhabits the south eastern part of the Iberian Peninsula and southern France, the species share a small portion of their distribution area across the central area of the Iberian peninsula (Aulagnier et al 2008; Aulagnier and Palomo 2008). Although genetically closely related (Jaarola et al 2004), overall *M. lusitanicus* has smaller size than *M. duodecimcostatus* (*Microtus lusitanicus*, body mass 14-19g; *M. duodecimcostatus*, body mass 19-32g). Broadly, pine voles can be found in meadows, pastures, agricultural areas and orchards where they can often cause severe damage (Vinhas 1993; Cotilla and Palomo 2007; Mira and Mathias 2007). However, at the micro scale species exhibit different ecological preferences (Borghi et al 1994; Santos et al 2011) and clear differences in their burrowing behaviour (Giannoni et al 1993), *M.lusitanicus* pushes earth from burrows mostly using the hindlegs and *M.duodecimcostatus* uses the incisors to break the soil and pushes it outwards using the head. Soil properties have been reported as a key factor in shaping the morphology of subterranean rodents (Stein 2000) and influencing the energetic demands of burrowing (Luna and Antinuchi 2006). Therefore, we analysed soil properties and compare them with energetic parameters to analyse the response of each species to their environmental conditions. We hypothesised that *M.duodecimcostatus* exhibits reduced RMR compared with *M.lusitanicus* given that inhabits warmer and dryer areas which influences the properties of the inhabited soil. Thus, soils with higher water content facilitate heat dissipation allowing animals to sustain elevated metabolic rates. Moreover, we measured the energy expenditure of free-living voles and compare it with captive voles to evaluate the costs of inhabit a subterranean environment.

Material and methods

Field sampling and maintenance of voles

Field work was carried out across the species ranges from the north to the south of Portugal (N 37º00 - 41º50; W 6º30 – 9º20). The selection of trapping sites was dependent of the presence of fresh mounds, holes or burrows, mainly in agricultural areas, road verges and meadows. Voles were captured using modified tube traps to include a nesting box, partially filled with local grass and bated with apple. The number of traps per site varied in accordance with the extension of the colony, estimated by the number of signs such as mounds and tunnel entrances. Trapping was successful in 18 sites within the range of *M. lusitanicus* and in 30 sites within the range of *M. duodecimcostatus*. Soil samples were collected in the 48 sites where voles were captured. Samples were collected in a radius of 1 meter from the trapping point. Approximately 1 dm^3 of soil was removed and kept in a closed plastic bag until being processed.

Although a larger number of voles have been trapped, only 46 non-reproductive adults (include split by species) were used in the present study. Experimental animals were housed in small individual cages (255 x 220 mm) partially filled with soil collected in capture sites. The majority of voles (40 out of 46) were taken to the laboratory where they were kept under controlled light (12L:12D) and room temperature ($\approx$ 25ºC), with free access to water and fed with carrots, apples and grass *ad libitum* The remaining 6 individuals were used in field measurements of energy expenditure (see below).

Respirometry

A total of 24 voles (*M. lusitanicus*: 5 females, body mass 17.1g ± 0.2g and 6 males, body mass 16.4g ± 0.4g; *M. duodecimcostatus*: 7 females, body mass 24.5g ± 0.3g and 6 males, body mass 25.5 g ± 0.4 g), were used to assess energy expenditure and thermoregulatory abilities. Energetic measurements were carried out after a 4 week period for laboratory acclimation. Oxygen consumption (VO2) was measured using an open-circuit respirometry system (Servomex, series 1100), as previously described by Duarte (2010). Animals were measured in a cylindrical chamber (approximately 1000 ml capacity), and dried atmospheric air was pumped into the chamber at a flow rate of 500ml/min.

Carbon dioxide was not removed to minimise error in the conversion of oxygen consumption to energy expenditure (Koteja 1996). Each vole was monitored twice, over two consecutive days, for 2 hours each day, in different periods, to avoid potential effects of daily metabolic cycle (Halle and Stenseth 1994). The average of the two measurements was use in the data analysis. Voles were not fasted prior to entering the chamber, but no food or water were available during the 2h-experiments. Consequently at the end of the runs voles had not been fed for at least 2 hours.

Measurements of oxygen consumption were recorded at 15s intervals, using the *Labtech* data acquisition and process control software. This procedure was repeated at different ambient temperatures: 5ºC, 15ºC, 20ºC, 25ºC, 30ºC, 32.5ºC, 35ºC, 37.5ºC and 40ºC. A total of 358 measurement runs were performed, but 10 of these were excluded from analysis because the voles did not settle in the chamber. At higher ambient temperature, if animals showed signs of distress, they were removed from the chamber and the temperature registered as the upper survival limit. To correct for machine drift baseline, values of atmospheric oxygen were measured for 15 minutes before and after each trial.

At each ambient temperature, resting metabolic rate was estimated as the average value of the ten lowest consecutive readings (equivalent to 2min30s in the chamber) (Hayes et al 1992). At the beginning and end of each run, animals were weighed and body temperature measured rectally (at depth around 2.5 cm), with a thermocouple K probe (MI-K-Miniz-1.0-100) connected to a *Digitron* thermometer (2088T, Sifam Instruments Limited).

Oxygen consumption was calculated after Depocas and Hart (1957) as $VO_2=V_2 (F_1O_2-F_2O_2)/(1-F_1O_2)$, where V_2 is the flow rate measured after the metabolic chamber, and F_1O_2 and F_2O_2 are the oxygen concentrations before and after the metabolic chamber. All VO_2 measurements were corrected to standard temperature and pressure (STPD).

Thermal conductance was calculated as $C=VO_2/(T_b-T_a)$, where C is conductance, T_b is the body temperature of the animal and T_a the ambient temperature (McNab 1970).

Doubly Labelled Water

The Doubly Labelled Water technique (DLW) (Butler et al 2004) was used to determine the Daily Energy Expenditure (DEE) and the Water Turnover (WTO), estimating how hard animals are working through the calculation of sustained metabolic scope (SusMs = DEE/RMR). WTO is considered as a balance between internal fluids and the input and output of water from the external environment (Speakman and Racey 1988).

A total of 22 animals (13 females and 9 males) were used in the DLW experiments. Three groups of voles were injected with doubly labelled water (30% ^{18}O, ^{2}H). The first group included six *M. lusitanicus* (4 females and 2 males), captured during the summer period in an apple orchard and released in their natural environment in the field. We failed to successfully recapture any individuals of *M. duodecimcostatus* and hence have no field metabolic rate measurements for this species. Accordingly we compared the DEE of two further groups measured in captive conditions. The second group included eight *M. lusitanicus* (5 females and 3 males) captured in the same site where the field measurements had been made, but taken to the laboratory, housed in individual cages, kept under natural light conditions and fed *ad libitum* with a mixture of fresh grass and carrots. The third group included eight *M. duodecimcostatus* (4 females and 4 males), captured in an orange orchard and kept under the same laboratory conditions.

One hour after the injection of the DLW, a blood sample was taken by retro orbital bleeding, to estimate the initial isotope enrichment of ^{2}H and ^{18}O. Blood samples were flame sealed in glass capillaries immediately after being collected. After the blood collection, the individuals from the first group were released in the same place of capture. The individuals from the second and third groups were kept in the laboratory, in individual cages, as described above. After 24h, a second blood sample was taken, to evaluate the final isotope enrichment (Speakman and Racey 1988). In the field, the blood samples were collected from 6 recaptured animals (from a total of 8 previously captured and injected). To assess the correct amount of injected isotope, the syringes were weighed before and after the administration of the water (0.0001g, Sartorius 4-figure balance).

The capillaries were vacuum distilled (Nagy 1983) and water from the resulting distillate was used to produce CO_2 and H_2 (methods in Speakman et al. (1990)and Speakman and

Król (2005). The isotope ratios $^{2}H:^{1}H$ and $^{18}O:^{16}O$ were then analysed using gas source isotope ratio mass spectrometry. The elimination constants (K_o and K_d) and the dilution spaces (N_o and N_d) of the ratios ^{2}H and ^{18}O injected were determined. Initial and final pools were calculated by the plateau method (Speakman et al 1993). This method assumes that the initial blood sample was taken when the administered isotope has equilibrated with water pool of the animal's body, and reached a maximal value before the isotope has been washed out from the body, according with the following equation (Król and Speakman 1999): $N_i = M_{inj} (E_{peak} - E_{inj})/(E_{bg} - E_{peak})$, where N_i (mol) is the dilution space for deuterium, oxygen-18 or tritium; M_{inj} is the amount of injectate (mol) injected; E_{peak} is the initial isotope enrichment (ppm) of body water; E_{inj} is the enrichment (ppm) of the deuterium/oxygen-18 or tritium injectate; E_{bg} is the background isotope enrichment (ppm) of body water. CO2 production was estimated according with equation $rCO2 = (N/2.078) \times (K_o - K_d) - 0.0062 K_d N$ (Speakman, 1997, eq. 7.17), where rCO2 is the CO2 production, N (mol) is the size of body water pool. The rate of C02 production was converted to DEE assuming a respiratory quotient of 0.85 and oxygen equivalent to $20.1 kJ.L^{-1}$.

The WTO (ml/day) values were calculated using the deuterium elimination rates (K_d per day), and deuterium dilution spaces (N_d, ml), WTO = $K_d . N_d.F$, where F is the fractionation factor of the isotope (0.941; Speakman 1997). The Water Economy Index (WEI) was calculated was WTO/DEE. Sustained metabolic scope was calculated as SusMs=DEE/RMR (Peterson et al 1990).

All experimental procedures were conducted in the University of Lisbon facilities by an expert in laboratory animal science accredited by the Portuguese Veterinary Authority (1005/92, DGV-Portugal, following FELASA category C recommendations), according to the European guidelines (86/609/EEC).

Data analysis

Resting metabolic rates and thermal conductance were analysed using a mixed model procedure, setting species and ambient temperature as factors, body mass as covariate

and individual id as random factor to account for repeated measures (methods in Tschöp et al (2011)). Thermal conductance (C) calculated from the individual measurements of oxygen consumption using the equation $C=VO_2/(T_b-T_a)$ (Mcnab 1980). Limits of thermoneutrality were determined for each species using a segmented regression model through SegReg software (www.waterlog.info). A General linear model was used to analyse DEE, WTO, WEI and SusMs data, setting species and environment (field vs lab) as fixed factors and body mass as covariate. All values were expressed as mean ± S.E. and $p < 0.05$ was taken as statistically significant, data were analysed using SPSS v19 for windows.

Soil samples were analysed for soil texture, percentage of organic matter, and water availability. The mineral contents of the soil were separated according with their sizes, and classified into three major classes: clay, silt and sand. Texture designates the relative proportion of each class in a sample; USDA soil textural classes were adopted (Soil Survey Division Staff 1993). The samples from the three main texture categories, were processed to the determine water availability and percentage of organic matter in the soil. Water availability was assessed by the difference between field capacity and permanent willing point (assessed by pF 2.14 and pF 4.2) (Richards 1947). Differences between sites were then compared using One-way ANOVA, setting species as fixed factor.

**Results**

Resting Metabolic Rate and Thermoregulation

Mean body temperature of *M.lusitanicus* ranged from 35.8±0.39ºC at Ta=5ºC to 40.1±0.51ºC at Ta=37.5ºC. At lower ambient temperatures some animals reduced their body temperature: six individuals had body temperatures between 30 and 33 ºC and one individual had a body temperature of 26 ºC (Figure 2A). The mean body temperature for *M. duodecimcostatus* ranged from 38.1±0.36ºC at Ta=5ºC to 42.3±0.55ºC at Ta=40ºC (Figure 2B). Upper survival limit was registered at 37.5ºC for *M. lusitanicus* and at 40ºC for *M. duodecimcostatus.*

Resting metabolic rate variation was explained by the body mass ($F_{1,21}$ =29.944; p<0.001), ambient temperature ($F_{8,21}$ =49.860; p<0.001) and the interaction between ambient temperature and species ($F_{8,21}$ =4.172; p<0.001). Overall, *M.duodecimcostatus* had lower metabolic rates than *M. lusitanicus*, except when ambient temperature was 5ºC. At 5ºC some *M.lusitanicus* reduced their metabolic rate by 68% when comparing all the individuals at 5ºC and body temperature fell to between 26 and 33 ^{o}C (see above), suggesting the induction of a shallow torpor.

The thermoneutral zone was calculated for *M.duodecimcostatus* to be between 31.6ºC and 37.5ºC, for *M. lusitanicus* lower critical point was calculated as 30.7ºC, and due to reduced data upper critical temperature was estimated 35 – 37.5ºC.

Below thermoneutrality thermal conductance was fairly stable at 0.101 $mlO_2.min^{-1}.ºC^{-1}$ for *M.lusitanicus* and 0.131 $mlO_2.min^{-1}.ºC^{-1}$ *M.duodecimcostatus*. Above lower critical point thermal conductance increased about 186% to 0.287 $mlO_2.min^{-1}.ºC^{-1}$ in *M.lusitanicus* and to 0.430 $mlO_2.min^{-1}.ºC^{-1}$ (227%) in *M.duodecimcostatus.* Thermal conductance was mostly influenced by the ambient temperature ($F_{8,20}$ =33.102; p<0.001) and the body mass ($F_{1,20}$ =12.396; p=0.001).
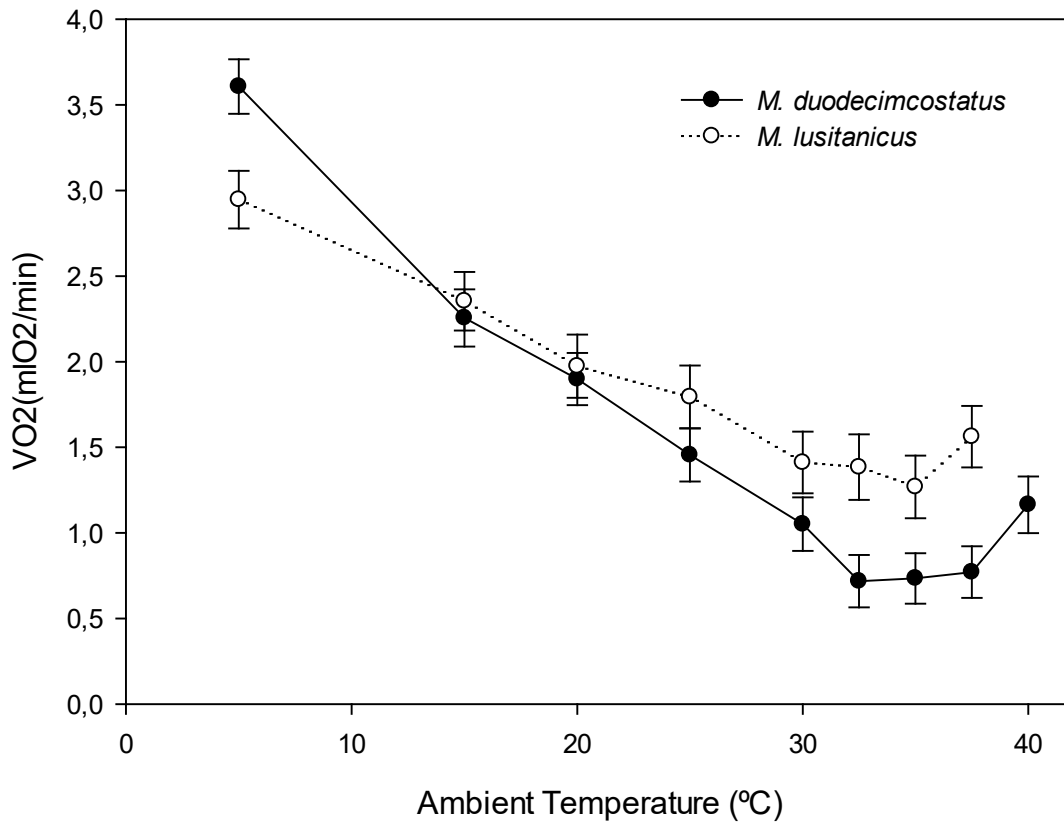
Figure 1 – Oxygen consumption of *Microtus duodecimcostatus* and *M. lusitanicus* across ambient temperature. (Adjusted values for BM= 21,53g).
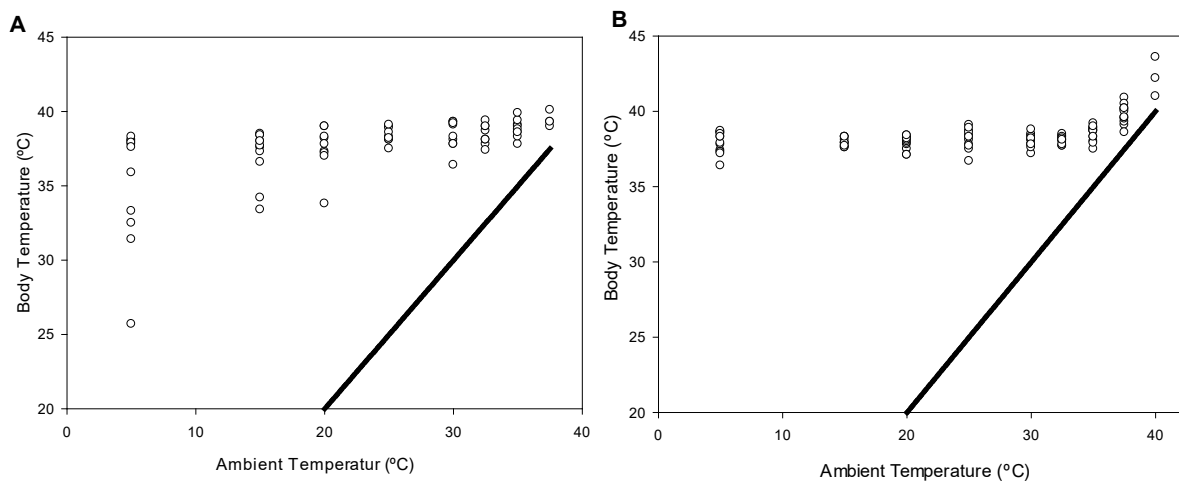
Figure 2 - Individual values of body temperature (T_b) in relation to ambient temperature (T_a) (— $T_{b} = T_{a}$). **A** – *Microtus lusitanicus*; **B** – *Microtus duodecimcostatus.*

Soil characteristics

The analysis of soil samples revealed that *M.lusitanicus* was mostly found on sandy-loam (41%), silty-loam (23%), loam (18%) and clay loam soils (14%), whereas *M. duodecimcostatus* occupied mainly loam (35%), sandy-loam (28%), silty-loam soils (17%), and other residual classes (20%). Figure 3 summarises data on water availability and % of organic matter on the three texture classes more abundant on both species. The soil from locations inhabited by *M.lusitanicus* have significant higher organic matter content (ANOVA $F_{2,39}$=35.283; p<0.001) and water available (ANOVA $F_{2,38}$=45.390; p<0.001) then those inhabited by *M.duodecimcostatus*.
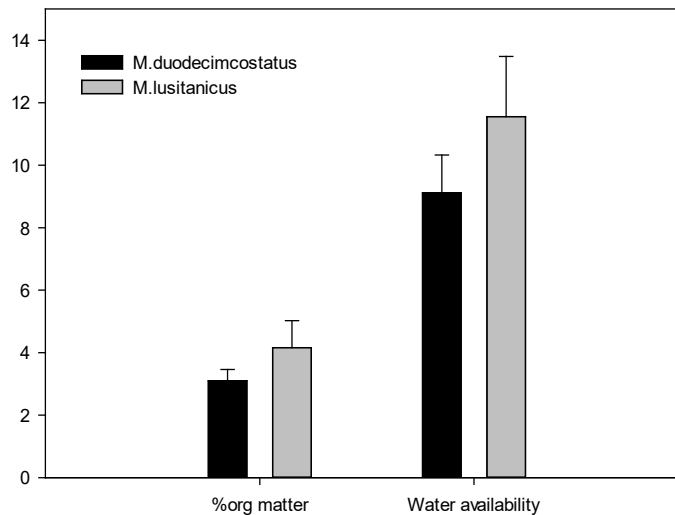

Figure 3 - % of organic matter and water availability on the sandy-loam, silty loam and loam soils, in sites inhabited by *M. duodecimcostatus* (dark) and *M.lusitanicus* (light).

Daily Energy Expenditure and Water Flux

All the variables examined had no significant effects on the DEE and SusMs. The data are summarised in Table I.

The WTO (ml. day^{-1}) was significantly higher in the field animals ($F_{1,17}$=18.256; p=0.001). The WEI was also significantly different between animals from the field and from the lab ($F_{1,17}$=22.264; p<0.001).

Table I – Mean ± standard error of the mean for Water turnover (WTO), daily energy expenditure (DEE), Sustained Metabolic Scope (SusMS) and Water economy index (WEI) (all estimated for body mass = 20.34g).

	M.lusitanicus		*M.duodecimcostatus*
	Field	Laboratory	Laboratory
N	6	7	8
WTO (ml.day^{-1})	3.04 ± 0.31	1.57 ± 0.25	1.52 ± 0.32
DEE (kJ.day^{-1})	57.0 ± 6.47	55.2 ± 5.37	52.9 ± 6.82
WEI (ml. kJ^{-1})	0.97 ± 0.07	0.58 ± 0.06	0.55 ± 0.07
SusMS	2.10 ± 0.23	1.87 ± 0.19	2.28 ± 0.24

Discussion

The extreme demands of subterranean life have resulted in unique specialisations that allow them to cope with the burrow environment. Reduced metabolic rates has been described as one of the features that allows subterranean species to avoid overheating due to the elevated costs of burrowing activity (Vleck 1979; Bozinovic et al 2005).

The measurements of oxygen consumption showed clear differences between the metabolic rates of the two studied species, *M.duodecimcostatus* had reduced resting metabolic rates, comparing with *M. lusitanicus.* According with hypothesised such variation can be interpreted as response to environmental features, such us soil dryness and availability of water.

Our data show evidence for the occurrence of torpor periods in the *M. lusitanicus.* Torpor is a common strategy in several rodent species as the house mouse *Mus musculus* (Overton and Williams 2004)*,* deermice *Peromyscus sp.* (Tannenbaum and Pivorun 1987) and the Djungarian hamster *Phodopus sungorus* (Ruf et al 1991). However, torpor have never been reported in Arvicoline rodents (McNab 1992; Nieminen et al 2013). Voles rely on constant food availability, and are not adapted to prolonged periods of fasting, perishing after 6 to 26 hours without food (Mustonen et al 2008). Thus is unlikely that torpor in *M. lusitanicus* is induced by the scarcity of

resources. However, the expression of torpor occurred only in individuals with reduced body mass, observations on mice (Rikke et al 2003; Mitchell et al 2015) suggested that body composition may be involved in the mechanisms regulating torpor induction and body temperature.

On this study, torpor occurs as response to the exposure to reduced temperatures, hence possibly voles developed such strategy to save energy and cope with winter temperatures. Our study did not consider the circadian variation of body temperature (Refinetti and Menaker 1992), however the measured range of body temperature variation (30%) gives us confidence to suggest the occurrence of torpor bouts. Other Microtine species also reduced their body temperature in response to cold acclimation, but the variation range is reduced, about 5% on the *M. cabrerae* (Mathias et al 2003) and *M. arvalis* (Ishii et al 1996), not entering in a torpid state

Several studies (Mcnab 1979; Lovegrove 1986) have suggested that the adaptations towards a life more dependent on the subterranean environment includes a reduction of resting metabolic rates due to overheating risk and elevated costs of subterranean foraging. Moreover, previous studies comparing digging behaviour (Giannoni et al 1993) and morphology (Mathias 1990; Mathias 1996) of *M.duodecimcostatus* and *M.lusitanicus* suggested that *M.duodecimcostatus* developed features that are in line with an adaptation to subterranean environment whilst *M.lusitanicus* showed adaptive traits towards surface dwelling.

Following Ebensperger and Bozinovic (2000) and Lovegrove (1989) average metabolic rates of species digging in dry, harder soils are expected to be greater than those of species digging in moist, softer soils due to the higher cost of burrowing. In fact, the soil texture, water content, organic matter and tillage are parameters that also highly constrain the thermal conductivity of the soil (Abu-Hamdeh and Reeder 2000; Abu-Hamdeh 2003). Soil thermal conductivity increases with the soil water content, thus soils with higher water content also increase the capacity of heat dispersion.

This supports the hypothesis that high metabolic rates are more sustainable in environments that facilitate heat dispersion, potentially explaining the higher RMR of *M.lusitanicus* which inhabits moister soils than *M.duodecimcostatus.* Moreover, the percentage of organic matter also facilitates the water retention on soil (Farley et al

2004). This may partly explain why the abundance of pine voles is usually higher in agricultural areas (Vinhas 1993; Mira and Mathias 1994; Miñarro et al 2012). In fact, the permanent watering of soil, through dripping, is a common agronomic practice that facilitates the expansion of vole populations (Bertolino et al 2015), and in some cases it has been suggested to be a key factor explaining rodent outbreaks (Jareno et al 2015) and continuous reproduction throughout the year (Ventura et al 2010).

The importance of water as the driver of vole's behaviour was also made evident by the measured WTO rates. Some studies suggested that reduced water turnover is a strategy to save energy (Rubal et al 1995; Scantlebury et al 2003) in desert environments, moreover WTO is linked to the energy expenditure when water is not available for drinking.

Voles under field conditions had higher values of WTO and WEI, which are indicative of the higher water intake than captive voles. Generally, subterranean species, as many other species, do not drink free water (Buffenstein 2000), thus our data suggests that free-living voles may have access to water sources, such as food. Water economy data also suggests some phenotypic plasticity that should be investigated in further studies.

The absence of differences in DEE between the field group and the housed group were unexpected, it may be explained by the abundance of resources in the field area (agricultural area with dripping watering and vegetative cover), and an absence of intensive burrow digging during the measurement period.

Considering the present results, our data supports previous observations proposing seasonal fluctuation of burrowing activity, during the summer animals are less active (Guedon et al 1992) as during winter grass cover is abundant, animals move mainly on the surface, where food is available and can be stored for scarcity periods (Mira and Mathias 1994). The climate in the burrows is highly buffered, however the atmosphere inside the tunnels can be influenced by the environment on the surface above (Burda et al 2007). Thus underground activity (excluding active burrowing) may be preferred during the summer, because helps avoiding elevated temperatures of the dry season.

Deeper soil layers maintain constant levels of moisture which may contribute to heat dispersal (Kinlaw 1999; Burda et al 2007). Seasonal and daily variations in burrowing

activity were already reported in other species, *e.g* semifossorial *Octodon degus* (Ebensperger and Bozinovic 2000) and in *Spalacopus cyanus* (Rezende et al 2003; Urrejola et al 2005). Even though subterranean rodents can modify their digging behaviour according with soil texture, using only the forelimbs to dig in loose soils, and including the use of the incisors when help in required to break some rocks (Lessa and Thaeler Jr. 1989). Shifting the activity patterns according to the ambient temperatures, creating a seasonal routine, may allow voles to cope with the thermal constrains of burrowing during the dry season.

Conclusion

The data obtained sustained our initial hypothesis that mass-independent RMR is reduced in the *M.duodecimcostatus* (considering ambient temperatures above 15ºC) which is consistent with the occurrence of the species in drier and warmer environments, typical of the south of the Iberian Peninsula. On the other hand, *M.lusitanicus* may have developed strategies to cope with low temperatures through torpor in line with the colonisation of the north of the Iberian Peninsula.

These are new insights on the species their energetic demands which provides interesting information about their biology and the complex speciation process between the two species.

Acknowledgements

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