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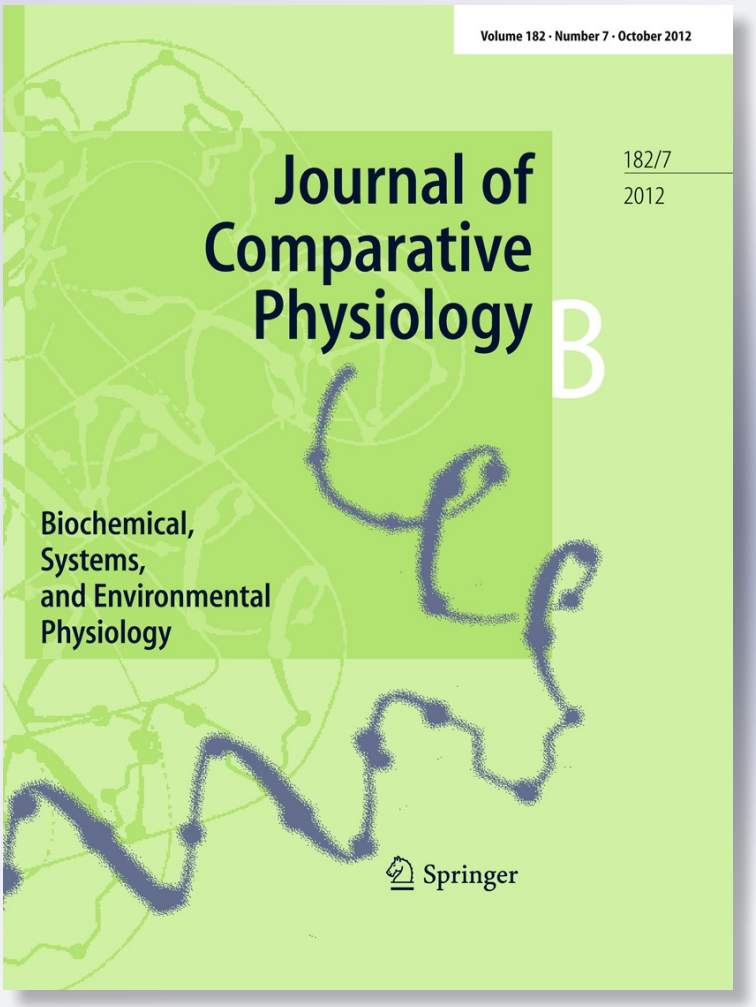

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ORIGINAL PAPER

Maximal thermogenic capacity and non-shivering thermogenesis in the South American subterranean rodent *Ctenomys talarum*

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Abstract Subterranean rodents inhabit closed tunnel systems that are hypoxic and hypercapnic and buffer aboveground ambient temperature. In contrast to other strictly subterranean rodents, *Ctenomys talarum* exhibits activity on the surface during foraging and dispersion and hence, is exposed also to the aboveground environment. In this context, this species is a valuable model to explore how the interplay between underground and aboveground use affects the relationship among basal metabolic rate (BMR), cold-induced maximum metabolic rate (MMR), shivering (ST), and non-shivering thermogenesis (NST). In this work, we provide the first evidence of the presence of NST, including the expression of uncoupling proteins in brown adipose tissue (BAT), and shivering thermogenesis in *Ctenomys talarum*, a species belonging to the most numerous subterranean genus, endemic to South America. Our results show no differences in BMR, cold-induced MMR, and NST between cold- (15 °C) and warm- (25 °C) acclimated individuals. Furthermore, thermal acclimation had no effect on the expression of mitochondrial uncoupling protein 1 (UCP1) in BAT. Only cytochrome *c* oxidase (COX) content and activity increased during cold acclimation. When interscapular BAT was removed, NST decreased more than 30 %, whereas cold-induced MMR remained unchanged. All together, these data suggest that cold-induced MMR reaches a maximum in warm-acclimated individuals and so a probable ceiling in NST and UCP1 expression in BAT. Possible thermogenic mechanisms explaining the increase in the oxidative capacity, mediated by COX in BAT of cold-acclimated individuals and the role of ST in subterranean life habits are proposed.

Keywords Aerobic capacity · Cold-induced maximum metabolic rate · Non-shivering thermogenesis · Brown adipose tissue · Uncoupling proteins

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Introduction

Assessing how energy is processed to maintain a constant body temperature is one of the most important issues in the study of energetics of endotherms. Maintenance of body temperature in fluctuating thermal environments is among the essential traits of mammals and birds. The emergence of endothermy allowed individuals to cope with challenging environments (Bennett and Ruben 1979), and the expansion of their distribution.

The aerobic capacity model proposes that thermoregulatory capacity arose in different groups of vertebrates as a side effect of selection for increased energy metabolism and not as a main selective factor (Bennett and Ruben

1979). A key point of this hypothesis is that aerobic metabolism appears to be linked to basal metabolism (Sadowska et al. 2005, but see Gębczyński and Konarzewski 2009). As a result of the relationship between MMR and BMR, an increment in the sustained aerobic capacity would have lead to high BMRs, allowing physiological thermoregulation and, hence endothermy (Hayes and Garland 1995). Therefore, an essential point to be considered to evaluate the aerobic capacity model of evolution of endothermy (Bennett and Ruben 1979) is to understand the relationship between maximum metabolic rate (MMR) and basal metabolic rate (BMR). Although the precise relationship between BMR and MMR is not fully understood, some cellular and sub-cellular mechanisms (e.g. membrane-related processes in both cell and mitochondria membrane), seem to link both variables (Hulbert and Else 1999; Else and Hulbert 2003; see also Hulbert and Else 2005, for a review). In this regard, Wunder and Gettinger (1996) defined MMR as the addition of BMR, shivering (ST), and non-shivering thermogenesis (NST). Jansky (1973) suggested that ST is less efficient than NST, because heat is produced by the muscles at the periphery of the body, where conductance is higher. On the other hand, NST has been proposed to be a more plastic element in the machinery of heat production (Cannon and Nedergaard 2004), responding rapidly to cold acclimation (Jansky 1973). For placental mammals, there is evidence that NST occurs in brown adipose tissue (BAT; Cannon and Nedergaard 2004). BAT is a highly vascularized tissue showing a high number of mitochondria and respiratory enzymes and almost destined exclusively to metabolic heat production.

The activation of BAT depends on the presence of norepinephrine (NE), which is released by sympathetic nervous system. NE produces a fall in mitochondrial membrane potential mediated by uncoupling proteins (UCP). Particularly, UCP1 short-circuits the electron transport chain circuit producing heat, alternatively to the storage of energy in the form of ATP (Valle et al. 2005). Indeed, different studies were conducted to assess the relationship between seasonality or thermal acclimation and the regulation of thermogenic capacity (Feist and Morrison 1981; Nespolo et al. 2001a; Moshkin et al. 2001; Wang et al. 2006a; Zhang and Wang 2007a; Zhao et al. 2010; see Heldmaier 1989; Lovegrove 2005). However, none of them was conducted in a solitary subterranean species that live in relatively thermally stable environments, but face also aboveground environment conditions.

Subterranean rodents live in tunnel systems parallel to the soil surface. Underground habitats buffer fluctuations in ambient temperature (T_a) and protects against predation, but are also challenging because they are humid, dark, hypoxic and hypercapnic (see Nevo 1999, but see Burda et al. 2007 for a review in burrow atmospheric conditions). This particular environment resulted in selection pressures that led to convergent morphophysiological characteristics shared by most of subterranean species (i.e. low BMR compared to surface-dwelling counterparts McNab 1966, 1979; White 2003).

Ctenomys talarum (tuco-tucos) is a subterranean rodent endemic to South America (Woods 1984) that inhabits coastal grasslands of Buenos Aires Province (Argentina). Individuals of *C. talarum* build and maintain extensive and relatively complex tunnels systems (Antinuchi and Busch 1992). Morphophysiological characteristics of this species match with most of other subterranean rodents (Antinuchi et al. 2007). However, contrary to strictly subterranean species that rarely venture outside the burrows (Nevo 1999), individuals of *C. talarum* are exposed to contrasting T_as during their bouts to forage on the surface.

The aim of this study is to ascertain the magnitude of MMR during cold acclimation. Particularly, we proposed to establish the role of ST and NST, and also the relationship between NST and UCPs expression in BAT. Considering the stability of ambient temperature within the burrows and the constraints imposed by the burrow environment on metabolic rates (and consequently the low capacity of heat dissipation), we hypothesized that *C. talarum* shows low variability in thermogenic variables, compared to those observed in surface dwelling rodents. On the other hand, we hypothesized also that this species shows a higher plasticity in thermogenic variables than strictly subterranean rodents because of its bouts to forage and disperse aboveground.

Materials and methods

Animal capture and thermal acclimation

Animals were live-trapped in Mar de Cobo (37 °45′S, 57 °56′W, Buenos Aires Province, Argentina), during the austral summer. Throughout the year, individuals experience a wide range of surface T_as (range ~2 °C to ~25 °C; mean T_a min = 13.6 °C, and mean T_a max = 24.8 °C during summer months; mean T_a min = 1.8 °C, and mean T_a max = 12.7 °C during winter months; Luna F., personal observation; Argentine National Forecast Service, http://www.smn.gov.ar). In this species, temperature fluctuations within the burrow show a similar annual pattern to T_a, but with lower variation (Cutrera and Antinuchi 2004). Therefore, we used T_as as a proxy of burrow temperatures, as used by Luna et al. (2009) to evaluate BMR variation within the genus *Ctenomys*.

Once individuals were captured, they were taken to the laboratory and housed in individual cages

(0.30 × 0.40 × 0.25 m) with wood shavings as nesting material. The animal room was kept at 25 ± 1 °C, and photoperiod was LD 12:12 (lights on at 7.00 a.m.). Animals were fed with mixed native grasses, carrots, lettuce, corn, alfalfa and sunflower seeds ad lib.

To assess the effect of ambient temperature regime on thermogenic variables, after 7–10 days of laboratory habituation, randomly chosen animals were acclimated to 25 ± 1 °C for 25 days (25 °C group). The remaining animals were acclimated to 15 ± 1 °C for the same time period (15 °C group). Acclimation time was chosen according to the standard procedure used by Nespolo et al. (2001a). The selected temperature for cold acclimation was chosen because it resembles T_as during winter and individuals could be subjected to it without experiencing changes in body mass (see "Results"). Acclimation was followed by physiological measurements.

Basal metabolic rate and non-shivering thermogenesis

Oxygen consumption was measured using a computerized positive pressure open-flow respirometry system (Sable System, Las Vegas, NE, USA). A cube-shaped chamber (volume 1.8 l) was used to estimate O_2 consumption during resting. The chamber consisted on a double wall of aluminum with polyurethane in the middle. The chamber has a 20-mm acrylic window door that allows the observation of the animals, with an inlet and outlet air port. Chamber temperature was controlled by two Peltier elements (model CP-1.4-127-061, Melcor, Cleveland, OH, USA) connected to a PC and controlled by a specially designed software (Laboratorio de Instrumentación y Control, Universidad Nacional de Mar del Plata). T_a inside the chamber was maintained with an accuracy of 0.1 °C. The chamber received dry and CO_2 free air at 1.5 l min^{-1} from a mass flowmeter (Side-Trak Sierra model 830/840, Sierra Instruments, Monterey, CA, USA), which was enough to ensure 90 % equilibration of the air in the chamber within 3 min (Lasiewski et al. 1966; Withers 1977). Air passed through a CO_2 absorbent (self-indicating soda lime, Laboratories IQB, Quilmes, BA, Argentina), and water scrubber (Silica Gel, Industrias Kubo, Mar del Plata, BA, Argentina) before and after passing through the chamber. Excurrent air from the chamber was sub-sampled at 150 ± 10 ml min^{-1} and oxygen consumption was obtained from an Oxygen Analyzer FC-1B every 1 s by a Datacan V—PC program (Sable System, Las Vegas, NE, USA). The chamber was maintained at T_a of 25 ± 0.1 °C, which is within the thermoneutral zone for this species (Busch 1989). Rates of oxygen consumption were calculated using the equation 4a of Withers (1977),

$$\dot{V}O_2 = \dot{V}(FIO_2 - FEO_2/1 - FIO_2)$$

where $\dot{V}$ is the flow rate through the system, FIO_2 and FEO_2 are the fractional O_2 concentration in the incurrent and the excurrent air, respectively (FIO_2 was 0.2095). *C. talarum* showed no circadian patterns of activity and O_2 consumption (Luna et al. 2000; Meroi 2008), which allows recordings of lowest O_2 consumption, independent of the time of the day (Meroi 2008). So, all metabolic trials were performed between 9:00 and 17:00 h (see below for individual total time trials). All individuals were adults, non-reproductive and post-absorptive, fulfilling BMR criteria (see Antinuchi et al. 2007).

Body mass of individuals (M) was measured using an electronic balance (model FX-3000, ±0.01 g, A&D Company Limited, San Jose, CA, USA) at the beginning of each trial and rectal temperature was recorded at the end of each experiment with a YSI probe (model 93k73545-402) connected to a Cole-Parmer thermistor meter (model 8402-10, ±0.1 °C, Cole-Parmer Instrument Company, Vernon Hills, IL, USA).

BMR and NST were measured using the protocol described by Nespolo et al. (2001a) for a subterranean species (*Spalacupus cyanus*). The protocol was as follows: (1) after a period of habituation in the chamber (~30 min), oxygen consumption was recorded for 1 h at rest. This total time for sampling was chosen because it allows a 5–10 min lowest steady-state of O_2 consumption (Busch 1989; Antinuchi et al. 2007). BMR estimated using this procedure were similar to those found with longer periods of experimentation (Luna and Antinuchi 2007), ensuring a reliable estimation of minimum metabolic rate (Antinuchi et al. 2007). (2) 30 min of O_2 consumption was recorded after an intramuscular injection of saline solution. (3) 30 min record after an intramuscular injection of norepinephrine (NE, the same volume as saline solution). In eutherians, the oxygen consumption in response to NE occurs 10 min after the injection and lasts for at least 5–10 min (Feist and Rosenmann 1976; Richardson et al. 1994). Doses of NE were estimated according to Wunder and Gettinger (1996), described as NE (mg kg^{-1}) doses = 2.53 $M^{-0.4}$. During this period, the maximum 10-min steady-state oxygen consumption after the injection of NE was considered to be NST_{max}, which includes both BMR and thermoregulatory NST (Wunder and Gettinger 1996).

Cold-induced maximum metabolic rate

Maximum thermogenic metabolism was estimated in a He-rich atmosphere, according to the procedure described by Rosenmann and Morrison (1974), using a positive pressure open-flow respirometry system (Sable System, Las Vegas, NE, USA). A mixture of He (79 %) and O_2 (21 %) was passed through a mass flowmeter before entering the chamber (Side-Trak Sierra model 830/840, Sierra Instruments, Monterey, CA, USA). Before MMR estimation, flow rate was corrected for the He-O_2 gas mix (K factor

relative to N_2 = 1.454, 2.1 l min^{-1}). As in the case of BMR, the mixture was passed through a CO_2-absorbent and water scrubber before and after passing through the chamber. After a period of habituation in the chamber (~20 min), oxygen consumption was recorded for 1 h at an ambient temperature of 5 ± 0.1 °C. This T_a was chosen based on previous trials that determined the lowest T_a in which individuals reach maximum metabolism (data not shown; Rosenmann and Morrison 1974), and produce a desirable effect (hypothermia; see Almeida and Cruz-Neto 2011).

Shivering thermogenesis

Shivering thermogenesis (ST) was estimated for each acclimation treatment according to the equation proposed for eutherian mammals (MMR = BMR + NST + ST, Jansky 1973; Wunder and Gettinger 1996).

Surgical procedure for IBAT extraction

Interscapular BAT of cold- (15 °C), and warm-acclimated individuals (25 °C) was removed by surgery. Each individual was anesthetized by an intramuscular injection of ketamine hydrochloride (40 mg kg^{-1}) and xylazine (2 mg kg^{-1}). After shaving, the back of the animal (3 × 4 cm, approximately), a 2-cm longitudinal incision was made between the scapulae through the epidermal layer, the skin was carefully opened out and interscapular BAT was completely removed. An hypodermic injection of antibiotic (Dipenisol®) was given to the animals after suturing the incision. After surgery, they were closely monitored until the incision healed to assure that there was no infection.

Measurement of UCP1 and COXII content, and COX activity

Extracted interscapular brown adipose tissue was homogenized in Tris/sucrose buffer (250 mM sucrose, 5 mM Tris–HCl, 2 mM EDTA, pH 7.2) in a Teflon/glass homogenizer. Total protein content was determined by the Bradford method (Bradford 1976). Samples of BAT were denatured and 30 μg of proteins per line were loaded and run in a SDS-PAGE (3 % stacking gel and 12 % running gel) according to Laemmli (1970), and electrotransferred onto a nitrocellulose filter, as described by Puigserver (1991). Samples were incubated with UCP1 and COXII antibody (Alpha Diagnostics). To validate the results, a sample of warm-acclimated rat BAT tissue that has previously been identified to express UCP1 and COXII (Quevedo et al. 1998; Rodriguez-Cuenca et al. 2002) was included in the Western blot. Bands on films were analyzed by scanner photodensitometry and quantified using Kodak 1D Image Analysis Software. Different aliquots of the obtained homogenates were used to measure COX activity using spectrophotometry (Wharton and Tzagoloff 1967; Chrzanowska-Lightowlers et al. 1993).

Effect of IBAT removal

Warm-acclimated individuals were used to assess the effect of IBAT removal on T_b and metabolic variables. Following the same period of acclimation mentioned earlier, individuals were divided into three groups for the measurement of T_b, BMR, NST and MMR: (1) unmanipulated individuals (+IBAT group), (2) interscapular BAT removal individuals (−IBAT group) and (3) surgery control individuals, which were subjected to surgery, but without carrying out the interscapular BAT removal (SHAM group). For surgically treated groups, a period of 10 days was waited before variables were estimated to allow the recovery of the animals. All individuals subjected to surgery were released at the capture site when the experiments ended up.

Data set and rodent phylogeny for interspecific MMR and NST analyses

To compare MMR and NST among species, we used both conventional and phylogenetically independent contrasts (Felsenstein 1985). Data of MMR (ml O_2 h^{-1}) were sourced from the literature (Moshkin et al. 2001; Rezende et al. 2004; White et al. 2008; this study). Values were used only when MMR were obtained using He–O_2 procedures. As proposed by Rezende et al. (2004), we included values of MMR when studies of thermal acclimation used T_as that closely reaches the range of T_as that individuals experience in the field during summer, or when they were obtained during seasons other than winter.

NST (ml O_2 h^{-1}) data were also obtained from the literature (Bao et al. 2001; Li et al. 2001; Moshkin et al. 2001; Perrin and Richardson 2005; Scantlebury et al. 2005; Rodriguez-Serrano and Bozinovic 2009; this study). As in the case of MMR, NST values included in the analysis were those reported during seasons other than winter (see Rodriguez-Serrano and Bozinovic 2009). We excluded values from studies in which dates of animal captures and measurements were not specified (see Mzilikazi and Lovegrove 2006). In some cases, we recalculated NST values from the literature. The phylogeny of rodents was derived from trees described in Rezende et al. (2004), and in Rodriguez-Serrano and Bozinovic (2009). Additional literature was used to build the tree (Michaux et al. 2001; Spotorno et al. 2004; Jansa and Weksler 2004; White 2003). In both cases, we subtracted 1 *df* each time we used two populations of the same species or two data from the same species reported in different studies (Purvis and Garland 1993; Garland and Diaz-Uriarte 1999). In the case of MMR, 3 *df* were subtracted due to unresolved branch, plus 7 *df* resulted

from different populations of the same species. In the case of NST, the obtained tree was completely dichotomous, although we subtracted 6 *df* because data belonged to different populations of the same species. Because the subtraction of *df* in each case did not change our results, our analyses are presented including all the data. The arbitrary branch length transformation of Pagel (1992) was used to standardize branch lengths in the tree.

Statistical analyses

All data are presented as mean ± SEM. A paired *t* test was used to compare body mass before and after the acclimation period within each group. ANCOVA was used to compare BMR, NST, ST or cold-induced MMR between acclimation temperatures. Body mass was used as a covariate when ANCOVA was performed. *t* test was used to assess for differences in T_b after cold exposure or after NE injection, and IBAT mass between temperature acclimation. ANCOVA was used to evaluate the effect of acclimation temperature on UCP1 content, COXII content and COX activity. Body mass was used as a covariate. UCP1 and COXII contents were $\log_{10}$ transformed before the ANCOVA analysis. Normality and homoscedasticity were tested before the analysis (Zar 2010). Mann–Whitney test was used only when assumptions were not meet.

ANCOVA was used to test the null hypothesis of no differences in metabolic variables among intact (+IBAT), sham-operated (SHAM) and surgically IBAT removed individuals (−IBAT). In the same way, body mass was used as a covariate. ANOVA was used to compare T_b, after cold exposure or after NE injection among +IBAT, SHAM and −IBAT groups. As described earlier, normality and homoscedasticity were tested before the analysis (Zar 2010).

Conventional allometric equations were estimated by least squares linear regression. For phylogenetically informed (PI) regression, independent contrasts were computed using PDAP:PDTREE module (Midford et al. 2003) of Mesquite (ver. 1.12; Maddison and Maddison 2006). Before any analysis was performed, MMR, NST, and body mass were $\log_{10}$ transformed. We performed conventional or phylogenetic ANCOVA to test for differences in NST between subterranean and strictly subterranean rodents, using $\log_{10}$-transformed body mass as a covariate. Finally, Mann–Whitney test was used to assess for differences in the percentage of variation of NST between cold and warm acclimation between surface dwelling and subterranean rodent species.

Results

Effect of thermal acclimation

Acclimation period did not affect body mass (cold-acclimated, paired *t* test, $t_5 = 1.34$, $P = 0.24$, warm-acclimated, paired *t* test, $t_5 = 0.62$, $P = 0.56$). No differences were found in BMR (ANCOVA, $F_{1,9} = 1.74$, $P = 0.22$), NST (ANCOVA, $F_{1,9} = 0.78$, $P = 0.44$), or cold-induced MMR (ANCOVA, $F_{1,9} = 0.81$, $P = 0.39$), or ST (ANCOVA, $F_{1,9} = 0.61$, $P = 0.46$) between individuals exposed to different acclimation conditions (Table 1). All the animals were normothermic after NE injections (36.9 ± 0.2 °C) and hypothermic after He–O_2 exposure (32.7 ± 0.4 °C). No differences were observed in T_b after NE injection (Mann–Whitney, $T = 45.5$, $P = 0.31$, Table 2) or after He–O_2 exposure (*t* test, $t_{10} = 0.22$, $P = 0.83$, Table 2) between 15 and 25 °C treatments.

In the same way, IBAT mass related to body mass did not differ between groups (pooled data of IBAT mass over $M^{-1} = 3.74 \pm 0.37$ mg g^{-1}; $t_{10} = 2.08$, $P = 0.07$, Table 3). When UCP1 protein content in IBAT was analyzed using ANCOVA, an outlier was found when assumptions were tested (Studentized residual = 18.41; Durbin–Watson test, $D = 2.26$). Because, UCP1 content can vary with age (Florez-Duquet and McDonald 1998), and we were not able to determine the age of individuals accurately, we attributed this outlier to differences in longevity of individuals. Longevity of *Ctenomys talarum* is relatively high for a rodent of this size, exceeding 2 years in the wild, and some individuals have survived at least 3 years (Malizia 1998). After excluding this individual from the ANCOVA analysis, no differences between

Table 1 Body mass (*M*) and metabolic variables in individuals of *Ctenomys talarum* in different experimental conditions

	Experimental condition		P^a
	15 °C ($n = 6$)	25 °C ($n = 6$)	
M (g) before BMR	175.19 ± 5.70	181.95 ± 19.14	
BMR (ml O_2 h^{-1})	166.54 ± 5.65	162.91 ± 9.65	0.22
NST (ml O_2 h^{-1})	221.25 ± 18.76	245.07 ± 10.10	0.44
ST (ml O_2 h^{-1})	332.12 ± 32.42	378.05 ± 22.35	0.46
M (g) before MMR	166.42 ± 6.19	180.01 ± 6.19	
MMR (ml O_2 h^{-1})	719.90 ± 21.36	786.03 ± 30.65	0.39

[a] Probability for ANCOVA test performed in each variable between temperature acclimation. Body mass was used as a covariate

Table 2 Body temperature (T_b) after HELOX exposure and after norepinephrine (NE) injection in individuals of *Ctenomys talarum* in different experimental condition

	Experimental condition		P^a
	15 °C	25 °C	
T_b after HELOX exposure (°C)	32.8 ± 0.7	32.6 ± 0.5	0.83
T_b after NE injection (°C)	36.8 ± 0.2	37.0 ± 0.2	0.31

[a] Probability for *t* test performed in each variable between temperature acclimation. T_b of *C. talarum* individuals is 36.5 ± 0.9 °C (Luna et al. 2009)

acclimation groups was found (ANCOVA, $F_{1,8} = 0.08$, $P = 0.78$). Both COXII content in IBAT (ANCOVA, $F_{1,9} = 8.36$, $P = 0.02$) and COX activity (ANCOVA, $F_{1,9} = 6.34$, $P = 0.03$, Table 3) were higher in cold-acclimated individuals.

Effect of IBAT removal

No differences were observed in BMR (ANCOVA, $F_{2,13} = 0.14$, $P = 0.87$), cold-induced MMR (ANCOVA, $F_{2,13} = 2.75$, $P = 0.10$), or ST (ANCOVA, $F_{2,13} = 1.60$, $P = 0.24$) among experimental groups (Table 4). When IBAT was removed, NST of −IBAT was lower (ANCOVA, $F_{2,13} = 14.61$, $P < 0.01$, Table 4) than sham-operated (Scheffé, $P < 0.001$) or +IBAT individuals (Scheffé, $P = 0.048$), whereas no differences were observed between +IBAT and sham-operated individuals (Scheffé, $P = 0.25$). After He–O_2 exposure at 5 °C, T_b decreased drastically in −IBAT (ANOVA, $F_{2,14} = 40.37$, $P < 0.01$, Table 5) compared to +IBAT (Tukey, $P < 0.001$) or sham-operated individuals (Tukey, $P < 0.001$). No differences were observed in T_b between +IBAT and sham-operated individuals (Tukey, $P = 0.562$). Contrary, T_b after NE injections at 25 °C was similar among experimental groups (ANOVA, $F_{2,14} = 3.00$, $P = 0.09$, Table 5).

Interspecific MMR and NST analyses

To evaluate whether *C. talarum* displays a lower cold-induced MMR or lower NST in relation to other rodent species, we compared MMR and NST values of this species with those obtained from the literature. Allometric equations calculated for studied rodent species using conventional analysis were:

Table 3 UCP1 and COXII content and COX activity in individuals of *Ctenomys talarum* in different experimental condition

	Experimental condition		P^a
	15 °C	25 °C	
IBAT mass M^{-1} (mg g^{-1})	4.42 ± 0.42	3.06 ± 0.50	0.07
UCP1 content (au g $IBAT^{-1}$)	54.98 ± 24.23[b]	23.06 ± 9.98	0.20
COXII content (au g $IBAT^{-1}$)	231.49 ± 52.19	55.56 ± 14.02	0.02*
COX activity (nKat g $IBAT^{-1}$)	39.30 ± 16.17	28.93 ± 5.91	0.03*

au arbitrary units, *nKat (nanokatal)* defined as a unit of catalytic activity

[a] Probability for ANCOVA test performed in each variable between temperature acclimation. Body mass was used as a covariate

[b] $n = 5$ (see "Results")

* Significant differences between experimental conditions

Table 4 Body mass (*M*) and metabolic variables in individuals of *Ctenomys talarum* in different experimental conditions

	Experimental condition			$P^\dagger$
	+IBAT ($n = 6$)	SHAM ($n = 5$)	−IBAT ($n = 6$)	
M (g) before BMR	184.28 ± 3.69	153.77 ± 9.12	156.52 ± 5.17	
BMR (ml O_2 h^{-1})	153.74 ± 7.07	135.12 ± 10.22	134.67 ± 5.92	0.87
NST (ml O_2 h^{-1})	247.74 ± 11.07[a]	235.52 ± 14.01[a]	135.71 ± 40.76[b]	<0.01*
ST (ml O_2 h^{-1})	267.84 ± 50.08	347.93 ± 53.20	341.83 ± 29.53	0.24
M (g) before MMR	183.86 ± 3.69	148.74 ± 11.14	155.09 ± 5.21	
MMR (ml O_2 h^{-1})	669.32 ± 43.59	691.92 ± 46.43	612.21 ± 40.37	0.10

Small letters represent pairwise comparisons (Scheffé's test) among groups

[†] Probability for ANCOVA test performed in each variable between experimental conditions. Body mass was used as a covariate

* Significant differences among experimental conditions

Table 5 Body temperature (T_b) after cold exposure and after norepinephine (NE) injection in individuals of *Ctenomys talarum* in different experimental condition

	Experimental condition			$P^{\dagger}$
	+IBAT	SHAM	−IBAT	
T_b after cold exposure (°C)	33.4 ± 0.1[a]	34.3 ± 0.3[a]	27.1 ± 0.8[b]	<0.01*
T_b after NE injection (°C)	36.8 ± 0.2	36.1 ± 0.4	35.7 ± 0.3	0.09

Small letters represent pairwise comparisons (Tukey test) among groups

† Probability for *t* test performed in each variable between experimental conditions

* Significant differences among experimental conditions

$$\text{MMR}\left(\text{ml O}_2\text{h}^{-1}\right) = 29.58M^{0.65\pm0.004}$$
$$\left(R^2 = 0.89, F_{1,59} = 464.54, P < 0.001\right)$$
$$\text{NST}\left(\text{ml O}_2\text{h}^{-1}\right) = 20.39M^{0.54\pm0.013}$$
$$\left(R^2 = 0.38, F_{1,53} = 32.25, P < 0.001\right)$$

where M is the body mass in grams. Slopes of the regressions were similar to those previously reported for cold-induced MMR ($M^{0.662}$, Rezende et al. 2004) or NST for different rodent species ($M^{0.503}$, Rodriguez-Serrano and Bozinovic 2009). Using conventional regression equation, data of MMR (Fig. 1a) and NST (Fig. 2a) of *C. talarum* fall within the 95 % prediction limits for the obtained equations (Zar 2010).

When evaluating metabolic variables corrected for the effect of phylogeny to render data independent, we obtained the following equations

$$\text{MMR}\left(\text{ml O}_2\text{h}^{-1}\right) = 32.01M^{0.63\pm0.003}$$
$$\left(R^2 = 0.94, F_{1,57} = 877.38, P < 0.001\right)$$
$$\text{NST}\left(\text{ml O}_2\text{h}^{-1}\right) = 25.72M^{0.49\pm0.009}$$
$$\left(R^2 = 0.53, F_{1,51} = 55.66, P < 0.001\right)$$

where M is the body mass in grams. Similar to the pattern observed using conventional analysis, when regression equations were corrected by phylogeny, data of MMR (Fig. 1b), and NST (Fig. 2b) of *C. talarum* fall within the 95 % prediction limits for the obtained equations for rodent species (Zar 2010).

Overall, the 95 % confidence intervals for slopes of the regression between cold-induced MMR and body mass (M), using conventional statistics (CS) or phylogenetically informed (PI), overlapped (95 % CI for CS 0.59–0.71; for PI 0.59–0.67). In the same way, the slopes of the relationship between NST and body mass for both types of analyses were similar (95 % CI for CS 0.36–0.75; for PI 0.36–0.61).

No differences were found among rodent species with different commitment to subterranean life (ANCOVA, conventional, $F_{1,5} = 3.94$, $P = 0.11$, phylogenetically informed, $F_{1,4} = 0.34$, $P = 0.59$, Table 6). The percentage of variation in NST between acclimation regimes were lower in subterranean species than those observed in surface dwelling species (Mann–Whitney, $T = 5.73$, $P = 0.02$, Table 7). Moreover, the distribution of the

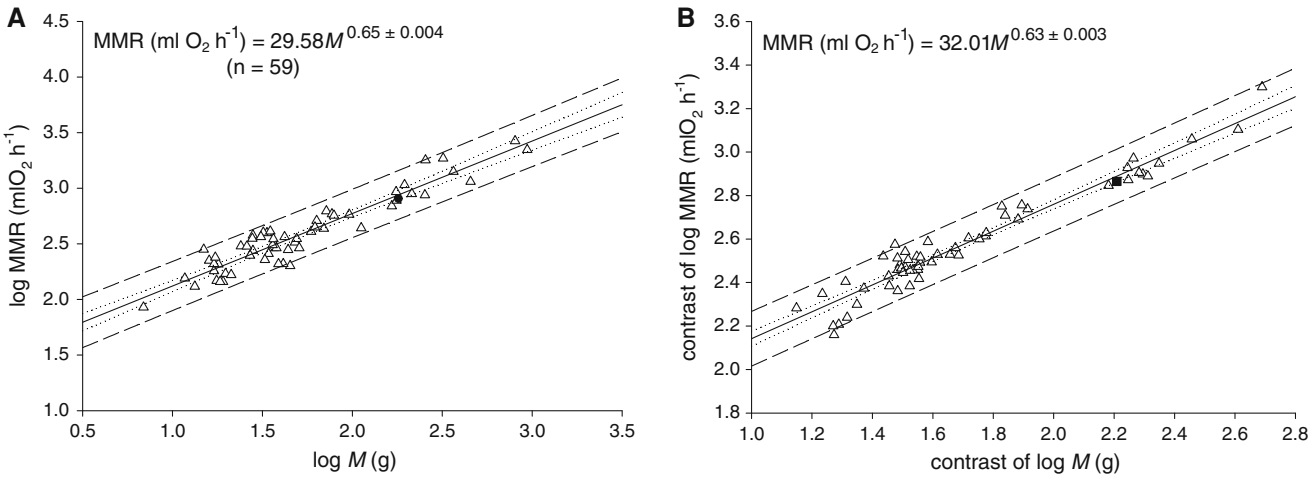

Fig. 1 Relationship between $\log_{10}$ MMR and body mass (M) for rodent species (*open triangles*) and *C. talarum* (*filled square*) using conventional (**a**) or phylogenetically informed (**b**) analyses. *Solid line* represents the allometric equation (see "Results"). *Inner dotted lines* represent the 95 % confidence interval of this regression; *outer broken lines* represent the 95 % prediction interval

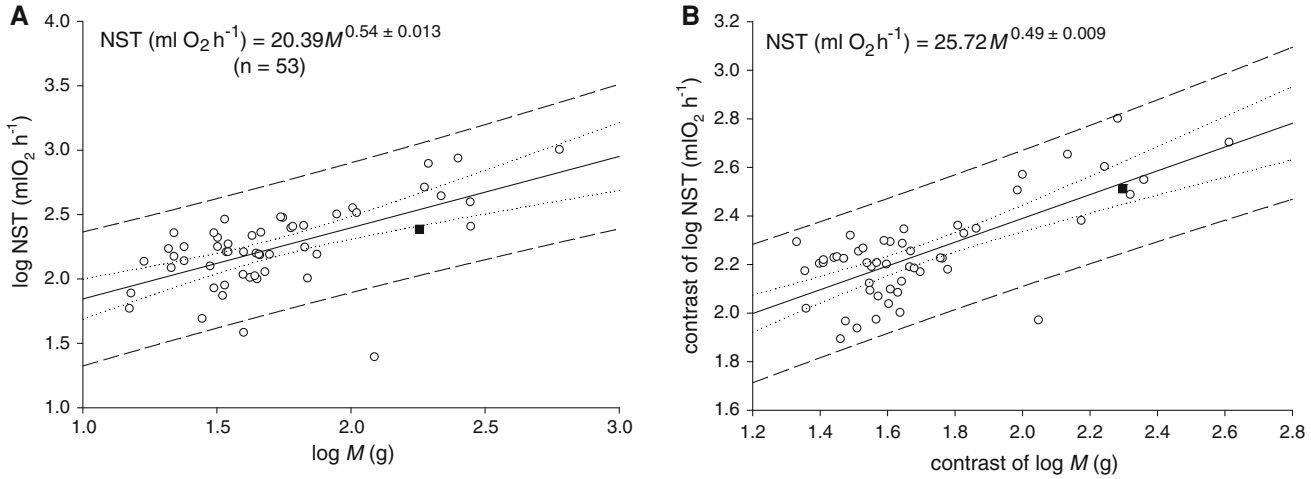

Fig. 2 Relationship between $\log_{10}$ NST and body mass (M) for rodent species (*open triangles*) and *C. talarum* (*filled square*) using conventional (**a**) or phylogenetically informed (**b**) analyses. *Solid line* represents the allometric equation (see "Results"). *Inner dotted lines* represent the 95 % confidence interval of this regression; *outer broken lines* represent the 95 % prediction interval

Table 6 Mean body mass (M), non-shivering thermogenesis (NST), and predicted NST (%) in different subterranean or strictly subterranean rodent species

Species	M (g)	NST (ml O_2 h^{-1})	%[a,b]	Life habits	References
Ellobius talpinus	45	153.67	95.8	Strictly subterranean	Moshkin et al. (2001)
Fukomys damarensis	123	55.35	20.2	Strictly subterranean	Hislop and Buffenstein (1994)
Cryptomys hottentotus	102	176.46	71.2	Strictly subterranean	Haim and Fairall (1986)
Heterocephalus glaber	36	54.72	38.8	Strictly subterranean	Woodley and Buffenstein (2002)
Ctenomys magellanicus	280	392	91.7	Subtterranean	Rodriguez-Serrano and Bozinovic (2009)
Ctenomys opimus	218	436	116.8	Subtterranean	Rodriguez-Serrano and Bozinovic (2009)
Spalacopus cyanus	88	268.36	117.5	Subtterranean	Nespolo et al. (2001a)
Ctenomys talarum	182	245.07	72.2	Subtterranean	This study

[a] Percentage of predicted NST by curve for rodents: NST (ml O_2 h^{-1}) = 20.39 $M^{0.54}$ (This study)

[b] No differences between subterranean and strictly subterranean species (see "Results")

Table 7 Non-shivering thermogenesis (NST) in several rodent species under two different T_a acclimation regimes

Species	NST (ml O_2 g^{-1} h^{-1})		%[a,b]	Life habits	References
	Warm acclimation	Cold acclimation			
Spalacopus cyanus	3.06	3.54	15.7	Subtterranean	Nespolo et al. (2001a)
Ctenomys talarum	1.37	1.26	8.7*	Subtterranean	This study
Heterocephalus glaber	1.52	1.73	13.8*	Strictly subterranean	Woodley and Buffenstein (2002)
Octodon degus	1.99	2.70	35.3	Surface	Nespolo et al. (2001b)
Phyllotis xanthopygus	2.24	5.77	158.2	Surface	Nespolo et al. (2001b)
Phyllotis darwini	1.61	5.58	247.4	Surface	Nespolo et al. (2001b)
Eothenomys miletus	4.99	7.88	57.8	Surface	Li et al. (2001)
Microtus brandi	3.52	6.81	93.3	Surface	Li et al. (2001)
Meriones unguiculatus	1.71	2.85	66.5	Surface	Li et al. (2001)
Spermophilus dauricus	0.90	1.66	84.8	Surface	Li et al. (2001)

[a] Percentage of variation between T_a acclimation regimes

[b] Significant differences between subterranean and surface species (see "Results")

* No differences between warm and cold acclimation

variable did not overlap between groups, as could be inferred from mean and SEM values (12.73 ± 2.09 % for subterranean species vs. 106.19 ± 27.69 % for surface dwelling species, Table 7).

Discussion

Energetics: components of MMR

Subterranean rodents show low BMR when compared with the predictions based on the standard allometric equation of surface-dwelling mammals (Kleiber 1961; McNab 1966; Vleck 1979). In this study, values of BMR match with those previously reported for *C. talarum* (see Antinuchi et al. 2007, for a review) and show no variation after cold acclimation (Table 1).

In the same way, MMR was similar between experimental groups, and was at least 4.3 times higher than BMR (Table 1). It is interesting to note that the observed value for cold-induced MMR was ~92 % of the predicted by the allometric equation for maximal thermogenic metabolism based on the different rodent species using conventional analysis (Fig. 1a) or phylogenetic contrast (Fig. 1b).

Similarly, NST did not change after thermal acclimation (Table 1). Although a few species of *Ctenomys* had been studied to assess NST, the observed value of *C. talarum* was similar to those reported for *C. opimus*, *C. magellanicus* (Rodriguez-Serrano and Bozinovic 2009), *C. porteusi* (Luna F., unpublished data), and other subterranean or strictly subterranean rodents (Table 6). NST in *Fukomys damarensis* differs considerably from other subterranean rodents (Table 6). Raw or contrast data of NST in *C. talarum* fall inside the 95 % prediction limits of the regression for rodent species, using conventional or phylogenetically informed equations, suggesting that this species displays a non-shivering thermogenic capacity within the range of rodents. However, NST was lower than the expected (~72 %) for a rodent of the body mass of *C. talarum* (Fig. 2a, b). Interestingly, NST of subterranean or strictly subterranean rodents did not differ, or has a small variation, when individuals are subjected to different T_a acclimation regimes in the laboratory compared to surface rodents (Table 7).

As we hypothesized before, species that live exclusively within burrows could have low variability in thermogenic capacity (see Table 7). For the naked mole rat *Heterocephalus glaber*, a thermoconforming eusocial rodent, this is a deductive assumption because burrow temperatures are within thermoneutral zone and heat dissipation is further limited. In fact, for this species, burrow temperature varies between 31 and 34 °C throughout the year (Buffenstein and Yahav 1991), and individuals are rarely exposed to temperatures outside this range, thus having low need for cold tolerance (Woodley and Buffenstein 2002). However, *Ctenomys* species differentiate from *H. glaber* in this regard. In the case of *C. talarum*, as in *C. fulvus* (Cortés et al. 2000), T_as within burrows are below the thermoneutral zone (i.e., 12.9 ± 2.2 °C during winter; 22.6 ± 1.5 °C during summer, Cutrera and Antinuchi 2004). Although fluctuations in T_a within the burrows are buffered in relation to surface T_as (9.5 ± 2.9 °C during winter, 29.1 ± 2.7 °C during summer; Cutrera and Antinuchi 2004), individuals are also exposed to aboveground T_a variation, since surface exploration is common during their regular, but short, bouts to gather food near the burrow′s openings (see Luna and Antinuchi 2003; Antinuchi et al. 2007), or during dispersal periods (Malizia et al. 1995). Therefore, the inability to increase both cold-induced MMR and NST in *C. talarum* after cold acclimation suggest that extreme T_as do not represent a challenge for individuals when they are aboveground, allowing an arrhythmic pattern of activity outside the burrows throughout the day (Luna et al. 2000; Cutrera et al. 2006).

On the other hand, maximal thermoregulatory capacity could be limited by external factors other than T_a, and linked to limitations on other physiological systems. Burrows are characterized by atmospheres with low O_2 and high CO_2 concentrations (Nevo 1999), and thus, can be stressful in terms of an adequate O_2 supply to metabolic active organs (Weibel and Hoppeler 2005). *C. talarum* face low T_as even inside the burrows during winters (Cutrera and Antinuchi 2004). In this regard, an increase in O_2 consumption is expected to compensate for heat loss, thus, the low O_2 content within the burrows could prevent animals to elevate MMR, due to hypoxia risk. In this scenario cold-induced MMR would not be affected by thermal acclimation.

If burrow's atmosphere imposes limitation on respiratory or cardiovascular systems [central limitation or symmorphosis hypotheses, see Bacigalupe and Bozinovic (2002) for a review] individuals could show similar maximal thermogenic metabolism under different ambient temperature regimes. In this context, cold-induced MMR and exercise-induced MMR should also be similar. Our data on cold-induced MMR in warm-acclimated individuals were rather similar to those estimated using untrained individuals running in a motorized treadmill at high speeds (exercise-induced MMR, F. Luna unpublished data), and during digging (Luna and Antinuchi 2006).

Because of cold-induced MMR in *C. talarum* is unchanged during cold acclimation, O_2 supply to the active thermogenic organs could restrict the increase in NST [e.g. lung limitation to O_2 changes (Maina et al. 2001), or O_2 provision for cardiovascular work (Weibel et al. 1991; see Suarez 1998)]. Therefore, it is possible that NST in warm-

acclimated individuals may already be maximal, precluding a further increase in cold-acclimated individuals. Ambient temperature used in this study is the minimum temperature to elicit a desirable thermal effect (Table 2). At this temperature, thermal unbalance was exacerbated when interscapular BAT was removed (Table 2). In this case, NST decreased more than 30 % with a consequent T_b fall from ~33 °C to ~27 °C after surgery (Table 2). This finding emphasized the importance of IBAT in NST capacity.

NST is not the exclusive source of heat in *C. talarum*. Besides *Heliophobius argenteocinereus* were observed to shiver during cold acclimation (Šumbera et al. 2007), to our knowledge, this is the first work that suggests, using respirometric technique, the presence and importance of ST in a subterranean mammal. Shivering is the major component of total heat production (~61 % total thermogenesis), and is not affected by thermal acclimation. Although ST was proposed to be inefficient (Jansky 1973) for a subterranean rodent, muscular work that individuals perform during digging could be exploited also as source of heat, as was hypothesized by Luna and Antinuchi (2007).

Molecular correlations

Several surface-dwelling species were observed to change NST and UCP1 content depending on the season or thermal acclimation (e.g. *Acomys russatus*, Kronfeld-Schor et al. 2000; *Microtus oeconomus*, Wang et al. 2006a; *Ochotona curzoniae*, Wang et al. 2006b; *Meriones unguiculatus,* Zhang and Wang 2007a; *Lasiopodomys brandtii*, Zhang and Wang 2007b). Our data demonstrate the presence of UCP1 in BAT of a subterranean rodent species. Although UCP1 content was variable (see "Results"), the content of uncoupling proteins in BAT was similar in cold- and warm-acclimated individuals (Table 3). Besides the leak to uncouple oxidative phosphorylation to produce heat, BAT could have a high oxidative capacity compared to other tissues to ensure NST (Klingenspor 2003; Mzilikazi et al. 2007). Cytocrome *c* oxidase (COX) is a marker enzyme for the mitochondrial membrane and, particularly COXII, is commonly used to estimate respiratory capacity in BAT mitochondria (Klaus et al. 1988; Klingenspor et al. 1996). In our study, UCP1 content was not different between experimental conditions, but COXII content and COX activity were augmented in cold-acclimated individuals (Table 3).

The reason for the increased COXII content and COX activity in cold-acclimated individuals remains to be clarified. As described before, extraction of IBAT induces a fall in NST (more than 30 %). We would assume that ST is unchanged during recovery time after surgery (10 days), which in fact was similar (see Table 1), because any change in ST implies transformation of muscle fiber types (Egginton et al. 2001). In this context, we would expect the same fall (~36 %) in cold-induced MMR. However, cold-induced MMR in IBAT-removed individuals was similar (Table 4). Therefore, values of MMR and ST of IBAT-removed individuals (see Table 4) could disguise another unknown thermogenic mechanism.

Conclusions

Thermal acclimation in *C. talarum* had no effect on metabolic variables such as cold-induced MMR, BMR, and thermogenic capacity. However, we provide the first evidence of the presence of BAT in a subterranean genus endemic of South America. Consistently UCP1 content was independent of thermal acclimation, but COXII content and COX activity was higher in cold-acclimated individuals. As blueprint, we propose that cold-induced MMR in warm-acclimated individuals reaches a maximal, being ST the main thermogenic mechanism. NST could also have a ceiling in warm-acclimated individuals, due to physiological restrictions in O_2 intake or delivery systems. Furthermore, the expression of UCP1 could also be limited in BAT tissue of warm-acclimated individuals. Although this assertion must be taken with caution, the increment in the oxidative capacity, mediated by COX in BAT in cold-acclimated individuals, might be related to a different thermogenic mechanism.

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