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Effect of photoperiod and acclimation temperature on nonshivering thermogenesis and GDP-binding of brown fat mitochondria in the Djungarian hamster *Phodopus s. sungorus*

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Abstract. Acclimation to short photoperiod at 23°C constant T_a caused *P. sungorus* to improve their NST capacity from 752 to 1,082 mW. Chronic cold exposure in short photoperiod further enhanced the NST capacity, reaching a maximum level of 1,573 mW at −5°C acclimation temperature. Improvements in NST capacity were always accompanied by an increase in brown fat mitochondrial mass and GDP-binding of brown fat mitochondria, in proportion with the cold load applied during temperature acclimation (23°, 15°, 5°, −5°C). Brown fat mitochondrial protein increased from 7.41 mg (23°C T_a, long photoperiod) through 21.6 mg (23°C T_a, short photoperiod) and 81.6 mg (−5°C T_a, short photoperiod). This ~10-fold increase was accompanied by a ~ 35-fold increase in GDP-binding (2.0, 7.3 and 71.6 nmol GDP bound, respectively), demonstrating that the increase in capacity for uncoupled respiration in brown fat is of primary significance for thermogenic acclimation to cold as well as to short photoperiod.

Key words: Nonshivering thermogenesis – Brown fat – Uncoupling protein – Mitochondria – Noradrenaline – Cytochrome c oxidase – Cold acclimation – Scatchard analysis

Introduction

Nonshivering thermogenesis (NST) is the dominating pathway of thermoregulatory heat production in small mammals (Heldmaier 1971; Jansky 1973). In warm acclimated adult mammals the capacity for NST is absent or only of minor significance, but it increases during acclimation to cold (Smith and Horwitz 1969; Jansky 1973; for reviews see Himms-Hagen 1986), exposure to short photoperiod (Lynch et al. 1978; Heldmaier et al. 1981, 1985) or feeding a highly palatable diet (Rothwell and Stock 1980, 1986). The increase in capacity for NST is primarily due to an increase in the brown fat capacity for mitochondrial respiration. Furthermore, heat generation in brown fat mitochondria is facilitated by the presence of a proton conductance pathway in the inner mitochondrial membrane (uncoupling protein) which allows maximum respiration without feedback inhibition by synthesis of ATP. Thus, a large portion of energy obtained from respiration is liberated directly as heat (for review see Needergaard and Cannon 1984; Nicholls and Locke 1984).

The aim of this study was to demonstrate, whether there is a uniform and graded response of NST, brown fat mitochondria and uncoupling protein to the cold load during temperature acclimation. We exposed Djungarian hamsters to 23°C T_a (thermoneutral) or a chronic cold load of 15°, 5° and −5°C T_a. After 4 weeks acclimation period we measured their in vivo capacity for NST as well as the in vitro thermogenic properties of brown fat namely cytochrome c oxidase, mitochondrial protein and uncoupling protein (GDP-binding). This comparison should reveal whether there is coincidence in the acclimation of in vivo NST and in vitro thermogenic properties of brown fat. Furthermore, we compared the effect of temperature acclimation with the effect of photoperiod acclimation at thermoneutrality, and by chronic exposure to 15°, 5° and −5°C T_a we tried to determine conditions for maximum thermogenic acclimation in Djungarian hamsters.

Material and methods

Djungarian hamsters, *Phodopus s. sungorus*, were bred and raised in the laboratory as described previously (Heldmaier and Steinlechner 1981). Adult animals, born in the summer, were investigated during the following winter between December and February. Four groups with 9–19 animals each were temperature acclimated under natural (short) photoperiod for at least 4 weeks (December L:D 8:16 through February L:D 10:14). The animals were exposed to 23°C (thermoneutral), 15°, 5° and −5°C ± 1°C in air conditioned rooms. A further group of hamsters was maintained in long photoperiod (L:D 16:8) at 23°C.

Basal metabolic rate and noradrenaline dependent NST were measured as described previously (Heldmaier et al. 1982a; Böckler et al. 1982). Maximum NST was evoked by an injection of noradrenaline [Arterenol (Hoechst AG, Frankfurt, FRG), 0.6 mg/kg body weight^{-1}, s.c., 15°C T_a]. NST capacity was obtained by subtracting basal metabolic rate from maximum NST. Metabolic rate was measured by an open system using a paramagnetic oxygen analyzer (Oxytest-S, Hartmann and Braun, Frankfurt, FRG) as described previously (Heldmaier and Steinlechner 1981).

Two days after measurement of NST, the hamsters were killed and all sites of brown fat were removed and weighed.

Abbreviations: Cox, cytochrome c oxidase; GDP, guanosine-5-diphosphate; NST, nonshivering thermogenesis: K-Tes, potassium salt of N-tris-(hydroxymethyl)-methyl-2-amino-ethane-sulfonic-acid; T_a, ambient temperatures; U, units of enzyme activity (μmol min^{-1} at 25°C)

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Axillary and intrathoracic brown fat (approximately 60% of total) was pooled and homogenized in a glass/teflon homogenizer in 30 ml isolation medium containing 250 mM sucrose, 5 mM K-Tes, and 16 μM bovine serum albumin [Sigma (St. Louis, MO, USA), No. A7030, essentially fatty acid free], pH 7.2. The homogenate was filtered through two layers of gauze and centrifuged at $8,500 \times g_{max}$ for 10 min. The pellet was resuspended in the same medium and centrifugated at $600 \times g_{max}$ to remove larger cell fragments. The supernatant was centrifuged at $8,500 \times g_{max}$. The pellet was resuspended in 30 ml 250 mM sucrose, 5 mM K-Tes pH 7.2 and again centrifuged at $8,500 \times g_{max}$ (two times).

Cytochrome c oxidase activity (EC 1.9.3.1) in brown fat was assayed polarographically in a Hansa Tech electrode (Bachhofer, Reutlingen, FRG) as described by Rafael (1983) and Rafael et al. (1985a). A piece (about 50 mg) of the axillary brown fat pad was mechanically disintegrated with a glass/glass homogenizer in 1 ml of buffer solution containing 100 mM potassium phosphate, 2 mM EDTA, 10 mM glutathion and 1 mM ADP, pH 7.5. An aliquot was displaced with 1.5% (w/v) polyoxyethylen-ether W1 to solubilize the remaining vesicles. After 10 min an appropriate amount of the homogenate was incubated at 25° C in air saturated potassium phosphate buffer (100 mM, pH 7.4) containing 470 natom 0 ml^{-1}, 100 mM ascorbic-acid, 2 mM cytochrome c and 5 mM EDTA. The cytochrome c oxidase activity in isolated mitochondria was determined as described above.

Total mitochondrial protein was calculated by using the cytochrome c oxidase as a marker enzyme in tissue and mitochondria homogenates [mg mitochondrial protein = (Cox/brown fat)/(Cox/mg mitochondrial protein)]. Mitochondrial protein was determined with a modified biuret where the lipids were extracted with diethylether after protein denaturion with TCA (Rafael et al. 1985a).

Rial and Nicholls (1984) could clearly demonstrate a correlation between GDP-binding sites and the nucleotide sensitive proton conductance pathway in BAT mitochondria. Therefore, we quantified the uncoupling protein by GDP-binding according to Rial and Nicholls (1983, 1984) with minor modifications. The mitochondria, final concentration about 0.1 mg/ml, were added to 500 μl incubation medium containing 100 mM sucrose, 20 mM K-Tes, 1 mM EDTA and 2 μM rotenone (pH 7.1) with ^{14}C-sucrose (NEN, Dreieich, FRG) (0.1 μCi/ml) and ^{3}H-GDP (Amersham Buchler, Braunschweig, FRG) (0.3 μCi/ml) at seven different concentrations of GDP (0.125, 0.25, 0.5, 1, 2.5, 5 and 10 μM). After 10 min incubation at room temperature and continuous agitation the mitochondria were centrifuged for 60 s in a Beckman Microfuge B. Supernatants were removed by aspiration and the pellets were dissolved in 500 μl of 5% (w/v) sodium dodecylsulphate and radioactivity was counted in a liquid scintillation counter. ^{14}C-sucrose was used to trace the presence of unbound ^{3}H-GDP in aqueous solution in the pellet water. For each individual animal a Scatchard analysis for GDP-binding was performed, B_{max} and K_d were obtained by using a linear regression program (least square method) on a Tektronix desk computer.

All data are expressed as means ± 1 standard error of the mean (x ± SEM). The temperature effects in long photoperiod were compared by analysis of variance (SPSSx on Sperry Univac Computer) and simple contrasts were analysed by Tukey-tests. Level of significance was set at $p < 0.05$. In cases where variances were not found to be homogenous (brown fat weight, mitochondrial protein, total GDP bound) a parameter free analysis was performed (Kruskal-Wallis and Mann-Whithney *U*-test. The effect of photoperiod was compared by Student *t*-test (one sided, significance level $p < 0.01$ or $p < 0.05$ as indicated).

Table 1. Effect of photoperiod and temperature acclimation on NST and brown fat in *Phodopus sungorus*

	Long photoperiod	Short photoperiod			
Acclimation temperature (°C)	23	23	15	5	−5
n	14	19	10	14	5****
Body weight (g)	39.9 ±1.5*	27.4 ±0.9	25.5 ±0.8	26.3 ±1.0	27.7 ±0.7
BMR (ml/g · h)	1.29 ±0.08*	1.87 ±0.06	1.64 ±0.07	2.01 ±0.09	2.04 ±0.08
NST capacity VO_2 (ml/g · h)	3.46 ±0.34*	7.1 ±0.2	9.3 ±0.2***	9.4 ±0.4***	10.2 ±0.5***
NST capacity (mW)	752 ±62*	1,082 ±32	1,298 ±56***	1,327 ±51***	1,573 ±89***
Brown fat weight (mg)	1,347 ±101**	1,154 ±63	1,057 ±42	1,007 ±56	968 ±56
BAT Cox activity (U/mg mitochondrial protein)	6.39 ±0.33**	5.54 ±0.26	5.55 ±0.33	4.97 ±0.26	4.34 ±0.57
Mitochondrial protein (mg)	7.41 ±0.8*	21.6 ±2.4	48.6 ±5.3***	61.0 ±7.8***	81.6 ±10.5***
GDP bound (pmol/mg mitochondrial protein)	259 ±22**	320 ±23	535 ±47***	639 ±39***	881 ±62***
K_d (μM)	0.81 ±0.1**	1.02 ±0.06	0.96 ±0.08	0.84 ±0.05	0.87 ±0.05

* Short photoperiod 23° C significantly different from long photoperiod 23° C ($p < 0.01$)
** As * but level of significance $p < 0.05$
*** Significantly different from 23° C (short photoperiod)
**** Originally 9 hamsters were acclimated to −5° C but 4 of them showed extended hemorrhagia in their brown fat after the NA-test and were therefore omitted

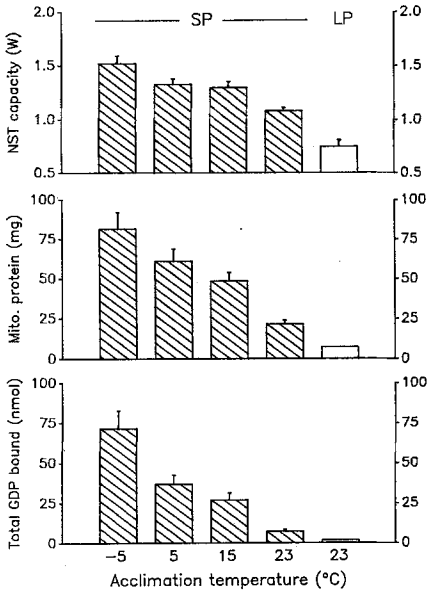

Fig. 1. Effect of photoperiod and cold acclimation on NST, brown fat, mitochondrial protein and GDP-binding. Djungarian hamsters were kept in long (*LP*) or short photoperiod (*SP*) either at 23° C (thermoneutral) or acclimatized to 15°, 5° or −5° C T_a. For statistic see Table 1

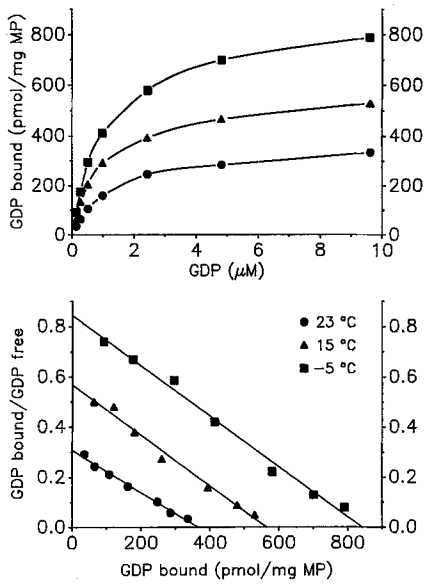

Fig. 2. Saturation curves and Scatchard plots for GDP-binding of brown fat mitochondria of Djungarian hamsters. We prepared mitochondria from each hamsters brown fat separately, and performed a Scatchard plot to calculate the number of binding sites (3-fold measurement for each point). Three plots for individual hamsters acclimated to 23°, 15° and −5° C (short photoperiod) are shown

Results

The lowest level of NST (752 mW) was observed in hamsters living in long photoperiod at 23° C acclimation temperature (Fig. 1, Table 1). When hamsters were living in short photoperiod at 23° C, their NST capacity was elevated to 1,082 mW. Cold acclimation in short photoperiod caused an increase in NST capacity in proportion with the cold load applied during the period of accumulation. Maximum NST of 1,573 mW was found after acclimation to −5° C. Lower acclimation temperatures could not be applied to Djungarian hamsters since chronic exposure caused a rapid and lethal weight loss within several days. In the range of temperatures studied here (23°, 15°, 5°, −5° C) chronic exposure for several weeks had no major effect on body weight (Table 1), however, it was largely reduced by the exposure to short photoperiod as described before (Figala et al. 1973; Steinlechner et al. 1983).

The amount of mitochondrial protein in brown fat showed similar responses to photoperiod and acclimation temperature (Fig. 1, Table 1). Hamsters living in long photoperiod had only 7.41 mg mitochondrial protein in their total brown fat and this was nearly tripled in short photoperiod (21.6 mg). Cold acclimation caused a further increase in mitochondrial protein which was rather linearly related to the cold load during the period of acclimation. The maximum equipment of brown fat with 81.6 mg mitochondria was about 4 times above the mitochondrial protein content at 23° C (thermoneutral) and more than 10 times above its corresponding value from hamsters living in long photoperiod. This indicates that relative changes in mitochondrial protein content due to photoperiod and temperature acclimation are far more exaggerated than the rather moderate changes of in vivo NST capacity (total increase by factor 2).

GDP-binding of mitochondria was lowest in hamsters living in long photoperiod at 23° C. We observed only a small increase in specific GDP-binding due to short photoperiod acclimation (Table 1), whereas cold acclimation provoked a nearly 3-fold increase in specific GDP-binding of mitochondria (Fig. 1). The elevated GDP-binding was due to an increase in the number of binding sites as may be seen from saturation curves and Scatchard plots (Fig. 2). No major changes in affinity to binding sites were observed since K_d values showed only small variations between 0.81 through 1.02 μM (Fig. 2, Table 1). To compare total changes in GDP-binding it should be kept in mind that the amount of mitochondria was nearly tripled in short photoperiod and increased ~ 10-fold during chronic cold exposure. Thus, the total amount of uncoupling protein as measured by GDP-binding increased about 35-fold during chronic exposure to −5° C (2.0 through 71.6 nmol GDP bound, Fig. 1).

Discussion

Acclimation to short photoperiod caused a 44% increase in total NST capacity. Maximum cold acclimation in short photoperiod elevated total NST capacity by another 60%. These percentages are similar to previous studies on the role of photoperiod and T_a during seasonal acclimation in *P. sungorus* (Heldmaier et al. 1982a, b). The significance of NST for seasonal acclimation by far exceeds this 104% increase of total NST capacity since acclimation to short photoperiod causes *P. sungorus* to reduce their body weight from 40 to 26 g which could have been associated with a reduction in NST capacity (Heldmaier 1971). Instead, a smaller body is equipped with a greater capacity for NST during winter, and changes in weight specific NST are much larger (photoperiod +105%, −5° C chronic cold exposure another +90%, Table 1). This increase in NST capacity does extend the cold tolerance of Djungarian hamsters from

$\sim -20°C$ (long photoperiod, 23°C) to $\sim -70°C$ (short photoperiod, −5°C acclimation temperature) (Heldmaier et al. 1982a).

Improvements of in vivo NST during acclimation to either short photoperiod or cold were always accompanied by an increase in brown fat mitochondrial mass and GDP-binding. However, the magnitude of these adaptive changes of in vitro thermogenic properties appeared much more prominent than the adaptive modification of in vivo NST itself. Comparing hamsters from long photoperiod at 23°C with hamsters acclimated to short photoperiod at −5°C we found a doubling of NST, but the total amount of mitochondrial protein in brown fat increased by a factor of 10.5, and the total amount of GDP-binding increased by a factor of 35. In preliminary investigations we did not find any evidence for an unmasking phenomenon of GDP-binding in these mitochondria, suggesting that the number of GDP-binding sites is equivalent to the amount of UCP. The large changes of brown fat thermogenic properties are in accordance with similar observations in mice where acclimation temperatures from 33° to −2°C caused a ~9-fold increase in Cox and an ~80-fold increase in GDP-binding (Ashwell et al. 1983). In rats an ~11-fold increase in Cox and a ~100-fold increase in GDP-binding as well as uncoupling protein detected by radioimmunoassay was found between 29° and 4°C T_a (Trayhurn et al. 1987). A response of similar magnitude was observed for GDP-binding in rats acclimated between 30° and 5°C (Sundin 1981). Golden hamsters were found to elevate their total brown fat Cox ~ 5-fold (total GDP-binding ~ 12-fold and uncoupling protein ~ 17-fold) during acclimation between 30° and 4°C (Trayhurn et al. 1983).

In a previous study with *P. sungorus* cold acclimation (5°C) in long photoperiod caused an ~3-fold rise in brown fat Cox and a 9-fold increase in GDP-binding (Rafael and Vsiansky 1985). Thereby smaller levels of Cox and GDP-binding were obtained as compared to the present study where hamsters were kept in short photoperiod. However, such a difference is in accordance with previous findings on the predominance of the photoperiod for seasonal acclimation, showing that cold acclimation in short photoperiod caused greater improvements of NST and brown fat respiration as compared to the effect of cold acclimation in long photoperiod (Heldmaier et al. 1982b; Rafael et al. 1985a, b).

The large increase in brown fat respiratory and thermogenic capacity indicates that brown fat is a predominant organ site for thermogenic acclimation. In rats and in the Djungarian hamster it has been shown that improvements of in vivo NST capacity during cold acclimation are primarily occurring in brown fat (Foster and Frydman 1978; Heldmaier and Buchberger 1985; Rafael et al. 1985b). In warm acclimated hamsters brown fat delivers 25–30% of all NST, and is contribution increase to 66–80% in cold acclimated hamsters (5°C, long photoperiod) (Heldmaier and Buchberger 1985; Puchalski et al. 1987). Since brown fat is only a fraction of the entire body mass, its respiratory and thermogenic properties are exaggerated to carry the burden of thermogenic acclimation for the entire body.

The biochemical nature of uncoupled respiration in brown fat was clarified in recent years (Nicholls et al. 1986; Ricquier and Bouillaud 1986). However, its role and its quantitative contribution to total NST in vivo are still unknown. There are only few studies where total NST capacity in vivo and uncoupling thermogenic properties of brown fat in vitro have been compared. Rafael and Vsiansky (1985) ascribe only a minor contribution of uncoupled respiration to total NST. Our present study allows a maximum estimate of heat delivered by uncoupled respiration during in vivo NST. Assuming that GDP-binding represents the number of proton conductance pathways (Trayhurn et al. 1987), and further assuming that all NST in brown fat of −5°C acclimated hamsters is due to uncoupled respiration (between 66% and 100% of total in vivo NST), this would predict a heat generating capability of uncoupling protein between 14.5 and 22 W μmol^{-1} per GDP-binding site[1].

This maximum estimate for in vivo heat generation due to uncoupled respiration cannot be exceeded. If we use this figure to calculate the contribution of uncoupling to NST in warm-acclimated mammals it shows that only 4%[2] may be due to uncoupled respiration, indicating that in warm-acclimated hamsters the uncoupling thermogenesis is almost negligible. This underlines the paradigm that thermogenic acclimation to cold and short photoperiod are primarily based upon an enhancement of the brown fat potential for thermogenesis by uncoupled respiration.

Nicholls (1977) estimated the proton conductance of the uncoupling protein and found values of 20 H^+ per minute, mV and GDP binding site. Assuming an electrochemical potential difference for protons (uncoupled conditions) of about 160 mV, the proton conductance would be about 3,200 H^+ per minute and GDP binding site. With this figure we are able to calculate the energy changes during uncoupled respiration[3]. Per unit GDP binding site the predicted value is $1.4 \cdot 10^{-18}$ W. However, this is one magnitude less than the value calculated from our data ($2.4 \cdot 10^{-17}$ W). The reason for this difference is not known. It could suggest that the contribution of uncoupling protein-dependent heat generation during NST is less than expected (66 through 100% of total NST), or alternatively, the proton conductance of brown fat mitochondria in vivo is higher than the previously published values obtained from in vitro studies (Nicholls 1977).

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[1] 1,573 mW of NST originate from 71.6 nmol GDP-binding sites. The amount of heat per nmol GDP-binding site was calculated:

$$\frac{1{,}573\,[\text{mW}]}{71.6\,[\text{nmol}]} \sim 22\,[\text{W}\,\mu\text{mol}^{-1}] \quad (1)$$

(100% of NST by uncoupled respiration)

$$21.97\,[\text{mW}\,\text{nmol}^{-1}] \cdot 0.66 = \sim 14.5\,[\text{W}\,\mu\text{mol}] \quad (2)$$

(66% of NST by uncoupled respiration).

To obtain the amount of heat produced per proton conductance pathway the results of (1) and (2) were divided by the Avogadro constant $6.022 \cdot 10^{23}$ which calculates $3.65 \cdot 10^{-17}$ or $2.41 \cdot 10^{-17}$ W per GDP-binding site

[2] 14.5 [W mol^{-1}] multiplied by the total number of GDP-binding sites found in 23°C long photoperiod acclimated hamsters (2.0 nmol, Table 1) calculates total uncoupling heat production of 29.1 mW, i.e. 4% of total NST (752 mW, Table 1)

[3] To convert the membrane potential in terms of energy the membrane potential (mV) was multiplied by the Faraday constant (Nicholls 1982):

$$160\,\text{mV} \cdot 0.0965\,\text{kJ}\,\text{mol}^{-1}\,\text{mV}^{-1} = 15.44\,\text{kJ per mol}\,H^+. \quad (3)$$

To obtain the amount of maximal heat production the result (3) was converted per unit GDP binding site (3,200 H^+ min^{-1}) and expressed in Watt:

$$\frac{15.44\,[\text{kJ}] \cdot 3{,}200\,H^+\,\text{min}^{-1} \cdot 1{,}000}{6.022 \cdot 10^{23} \cdot 60} \quad (4)$$

$\sim 1.4 \cdot 10^{-18}$ W per unit GDP binding site

Maximum NST capacity was developed when hamsters were exposed to −5° C chronic cold load at short photoperiod. This 1,573 mW capacity was similar to the maximum obtained during seasonal acclimatization in hamsters living outside during winter (Heldmaier et al. 1982a; Rafael and Vsiansky 1985). We were unable to expose hamsters to acclimation temperatures below −5° C. They rapidly lost weight and died within several days. Obviously, the supply of energy by feeding is limited, and these hamsters ran into a negative energy balance at chronic cold exposure below −5° C T_a. This is confirmed by a study of energy assimilation rates in Djungarian hamsters, showing that maximum energy assimilation rate was reached at T_a's between 0 and −5° C (Weiner 1987). These measurements were performed in hamsters during the reproductive season in long photoperiod, but our present result suggest that even in short photoperiod energy requirements for thermoregulation at −5° C will be at the maximum of their capacity for energy assimilation, and will be the limit for chronic cold exposure.

This is in contrast to the impressive cold tolerance of Djungarian hamsters during acute exposure, which enables them to thermoregulate and survive at −70° C for several hours (Heldmaier et al. 1982a, 1986). This shows that energy requirements for thermogenesis is brown fat may in fact exceed the hamsters capability for energy supply from feeding. Brown fat has to rely on substrate import even for moderate thermogenesis (Trayhurn 1980; Heldmaier and Seidl 1985), and during sustained thermogenesis brown fat will consume a considerable amount of energy stores from other tissues in the body. The discrepancy between acute and chronic cold tolerance further demonstrates that Djungarian hamsters living in the Siberian steppe during winter can only be exposed to severe cold load during short periods of activity outside their burrows. Inside their burrows they have to be exposed to more comfortable conditions, i.e. nest and burrow temperatures have to be well above 0° C or close to thermoneutrality to allow for compensation of energy deficits from thermoregulation outside the burrow.

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