The Journal of Experimental Biology 216, 3811-3817

doi:10.1242/jeb.089433

RESEARCH ARTICLE

Nonshivering thermogenesis in the African lesser bushbaby, *Galago moholi*

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SUMMARY

The capacity for nonshivering thermogenesis (NST) plays an important role during arousal from torpid states. Recent data on heterotherms inhabiting warmer regions, however, suggest that passive rewarming reduces the need of metabolic heat production during arousal significantly, leading to the question: to what extent do subtropical or tropical heterotherms depend on NST? The African lesser bushbaby, *Galago moholi*, enters torpid states as an emergency response only, but otherwise stays normothermic throughout the cold and dry winter season. In addition, this species shows unusual rewarming difficulties during arousal from torpor on cold days. We therefore examined the seasonal adjustments of the capacity for NST of naturally acclimatized *G. moholi* by stimulation with noradrenaline (NA) injection. Dissection of two adult female bushbabies revealed that *G. moholi* possesses brown adipose tissue, and NA treatment (0.5 mg kg^{-1}, s.c.) induced a significant elevation in oxygen consumption compared with control (saline) injection. However, the increase in oxygen consumption following injection of NA was not significantly different between winter and summer. Our results show that the ability to produce heat *via* NST seems to be available throughout the year and that *G. moholi* is able to change NST capacity within a very short time frame in response to cold spells. Together with results from studies on other (Afro-)tropical heterotherms, which also indicate low or even absent seasonal difference in NST capacity, this raises the question of whether the definition of NST needs to be refined for (Afro-)tropical mammals.

Key words: nonshivering thermogenesis, primate, rewarming difficulties, (Afro-)tropical heterotherms, NST definition.

Received 10 April 2013; Accepted 26 June 2013

INTRODUCTION

Endothermic animals are able to maintain a constant body temperature regardless of ambient conditions, which is presumed to enable them to inhabit a variety of habitats and stay active during changing environmental conditions (McNab, 1978). Behavioral adaptations such as change of resting site (Entwistle et al., 1997) or body posture (Øritsland, 1970), huddling in groups (Gilbert et al., 2010) or generating muscle heat through locomotion (Zerba and Walsberg, 1992) help endothermic animals to deal with moderate cold. However, during acute cold exposure, endotherms have to increase the generation of metabolic heat to defend their constant high body temperature (Scholander et al., 1950). Small mammals that possess brown adipose tissue (BAT) respond to cold exposure with nonshivering thermogenesis (NST) and only minimal muscular activity (e.g. Héroux and Wright, 1962). NST occurs in BAT of mammals where the uncoupling protein 1 (UCP1) alters proton conductance in the inner mitochondrial membrane and leads to heat generation instead of ATP production (Hansen and Knudsen, 1986; Heaton et al., 1978). The capacity for NST increases during cold exposure or by seasonal acclimatization with photoperiod (Heldmaier et al., 1982; Heldmaier et al., 1981; Heldmaier et al., 1989; Nedergaard and Cannon, 1985). Heat production through NST is of fundamental importance in the thermoregulation of small mammals and has also been described as an important mechanism during arousal from hibernation and torpid states (reviewed in Janský, 1973).

However, the necessity to utilize NST seems to become less indispensible with decreasing latitude. Recent data on heterotherms inhabiting warm regions in Africa or Australia suggest that passive rewarming, by the increase of ambient temperature (T_a), by basking in the sun or by social thermoregulation, significantly reduces the need of metabolic heat production during arousal from torpor (reviewed in Geiser et al., 2004). Passive rewarming seems to play an especially prominent role in Australian marsupials where the presence or absence of adaptive NST is still a subject of ongoing discussion (Polymeropoulos et al., 2012). On the African continent, the NST capacity of several South African rodent species (e.g. Haim and Izhaki, 1993; Hislop and Buffenstein, 1994; Lovegrove et al., 1991; Richter et al., 1997) as well as the NST capacity of the smaller members of the Afrotheria (Mzilikazi et al., 2007; Mzilikazi and Lovegrove, 2006; Oelkrug et al., 2012; Scantlebury et al., 2008) has been intensively studied, leading to ambiguous results concerning the extent of the capacity for NST that was found: whereas some species display an adaptive NST of the expected extent [e.g. *Amblysomus hottentotus longiceps* (Scantlebury et al., 2008)], no seasonal difference in the capacity for NST was found for other species [e.g. *Elephantulus myurus* (Mzilikazi et al., 2007)].

The African lesser bushbaby (*Galago moholi* Smith 1836) is a small (~200 g), nocturnal primate that inhabits highly seasonal habitats in southern Africa and only enters torpid states as an emergency response to acute food shortage during pronounced cold periods (Nowack et al., 2010). In this species, heating rates during

arousal from torpor were found to be almost 50% lower than expected on the basis of body mass (Nowack et al., 2013a) and individuals with body temperatures below a threshold of approximately 19°C during torpor showed substantial difficulties with rewarming. Arousal rates in the short-beaked echidna (*Tachyglossus aculeatus*), a monotreme that is known to lack classical NST, or in UCP1-ablated mice showed similarly reduced rewarming rates (Nicol et al., 2009; Oelkrug et al., 2011). This raises the question of whether *G. moholi* possesses BAT (and concomitantly UCP1) and the capacity for NST. A lack of NST in a tropical, heterothermic placental mammal would represent a radical deviation from the 'classical' thermoregulation of small-sized, non-marsupial heterotherms. We therefore examined the NST capacity of naturally winter- and summer-acclimatized *G. moholi* by stimulation with noradrenaline (NA) injection to determine whether rewarming difficulties in this species could be due to a lack of the ability to perform NST.

MATERIALS AND METHODS

Study site and ambient conditions

We conducted our fieldwork at the Nylsvley Nature Reserve (South Africa, Limpopo, 24°38.802′S, 28°40.095′E, altitude: 1100 m), which is a semi-arid (mean annual rainfall: 700 mm; Limpopo Parks data for Nylsvley), mixed bushveld habitat with the hot-wet season from October to March, and the cool-dry season from April to September. Monthly mean daily minimum temperature ranges from –1°C (July) to 17°C (January; J.N., personal observation in 2009–2011). We recorded T_a during the field season every 30 min within the known home ranges of our study animals using humidity and temperature loggers (resolution 0.0625°C; Hygrochron iButton/DS1923, Dallas Semiconductor, Maxim Integrated, San Jose, CA, USA).

Capture and handling

We captured *G. moholi* during summer 2010–2011 and winter 2011 using self-made walk-in live traps, baited with banana, honey and peanut butter. All individuals were sexed, measured, weighed, classified in age and reproductive status, and individually marked with subcutaneously injected passive identification transponders (ID100 Trovan, EURO I.D. Usling GmbH, Weilerswist, Germany) (for details, see Nowack et al., 2010). No female individuals were used in experiments (because of the high numbers of pregnant females throughout the year) and we only worked with reproductively active (developed testes) males with a body mass of at least 160 g (summer: N=7, winter: N=10). Captured individuals were held in an outside enclosure (180.5×61.5×193.5 cm) for <1 week (one at a time). The enclosure was equipped with a wooden nest box and branches, and galagos were fed with bananas, gum (when available) and water *ad libitum*. All measurements were conducted in a field laboratory. We released individuals at the exact capture location after measurements.

All procedures in this study complied with the National Institutes of Health 'Principles for animal care' (publication no. 86–23, revised 1986) and the 'Code of ethics for animal experimentation' manual adopted by the Nelson Mandela Metropolitan University (animal ethics clearance no. A09-SCI-ZOO-001). Animals were captured under permit no. CPM-002-00003, issued by the Department of Economic Development, Environment and Tourism.

Determination of NST

The NST capacity of *G. moholi* was obtained in winter- and summer-acclimatized animals through measurement of the increase in the rate of O_2 uptake ($\dot{V}_{O_2}$; as a proxy for metabolic rate, see below) following NA injection (Janský, 1973; Wunder and Gettinger, 1996). On each day of measurement, we removed food from the enclosure at least 3 h prior to the commencement of the experiment to ensure that the animals were post-absorptive. All measurements were conducted during the rest phase at daytime between 10:00 and 15:00 h. $\dot{V}_{O_2}$ was measured for 2 h before NA (1 mg ml^{-1}) or saline solution (0.9% NaCl; control) was injected subcutaneously. Because of high body mass loss in captivity (~10% in 4 days, data from previous experiments), we did not establish a dose–response curve to calculate the optimal dose of NA. Instead we used 0.5 mg kg^{-1} NA or control solution, respectively.

We continued $\dot{V}_{O_2}$ measurements for 2 h after each NA injection and for 1 h after each control injection. Each individual was subjected to a NA and control injection on two consecutive days.

Measurement of $\dot{V}_{O_2}$

Animals were individually placed in an 8 liter respirometry chamber located in a portable thermal cabinet (35×66×44 cm) with a transparent part in the lid for behavioral observations. T_a inside the thermal cabinet was maintained using a self-built water bath, driven by an aquarium heater, pump and processor fan. The water was pumped from the water bath through copper pipes inside the chamber, thereby ensuring that T_a during measurements was within the lower range of the thermoneutral zone [28–35°C (Dobler, 1978)] to guarantee basal energy expenditure (mean T_a: 31.7±1.1°C). T_a inside the thermal cabinet was monitored with a thermometer inserted into the box and a temperature logger (iButton). Oxygen consumption was determined by open flow-through respirometry using a portable oxygen analyzer (FoxBoxC, Sable Systems International, Las Vegas, NV, USA). The metabolic chamber was connected to the oxygen analyzer with airtight tubes and air was pumped through the system (pull mode). CO_2 and water vapor were scrubbed prior to analysis of $\dot{V}_{O_2}$ using soda lime and silica gel. We measured sample air for 25 min alternating with 5 min reference air (baseline). For each measurement bout, the mean $\dot{V}_{O_2}$ was calculated from ~90% of the values (sampling frequency every 10 s) which represented the most stable readings in the cycle, using the data acquisition program Expedata (Sable Systems International) (for details, see Nowack et al., 2010). For each individual, we determined the mean basal metabolic rate (BMR) as the mean value of the consecutive $\dot{V}_{O_2}$ values of 23 min. We used the lowest mean value of both measurement days (control and NA trials) for further analyses. Control and NA responses were measured as the maximum mean $\dot{V}_{O_2}$ response (15 min of the highest peak) within the first hour of injection.

During all measurements of $\dot{V}_{O_2}$, skin temperature (T_{skin}) of individuals was measured simultaneously with temperature loggers (Weetag collars, 2.6 g; Alpha Mach, Mont St-Hilaire, Canada, resolution 0.0625°C; for improved precision, calibration curves provided by the manufacturer were used) attached under the chin on a collar, which were programmed to measure T_{skin} every 10 min.

We compared the highest T_{skin} values after treatments (control/NA; timespan of 30 min) with the average T_{skin} values during BMR.

Evaluation of brown adipose tissue deposits

To evaluate whether adult *G. moholi* do possess BAT, we dissected two adult female *G. moholi* that had died naturally at Ithumela Primate Sanctuary (South Africa Gauteng, 25°35.149′S, 28°20.099′E, altitude: 1220 m). Both individuals were free-ranging and had died in May and August 2012, respectively. Individuals

were frozen until dissection. We examined BAT occurrence between the shoulders, around the kidneys and around the gonads.

Data analyses

Statistical analyses were performed with SPSS (PASW Statistics 18, IBM, Armonk, NY, USA). All values are presented as means ± s.d. Data were analyzed with non-parametrical tests because of skewness of distributions. Seasonal differences in BMR, reaction to control injection and NST capacity were tested with Mann–Whitney *U*-test for independent variables. To compare the different measurements obtained for the same animals ($\dot{V}_{O_2}$ BMR, $\dot{V}_{O_2}$ NA, $\dot{V}_{O_2}$ control), we conducted Friedman tests for repeated measures. Multiple pairwise comparisons were made using Wilcoxon tests as *post hoc* analyses. The metabolic reaction to control saline injection, as well as the NST capacity, was calculated as the net increase in metabolic rate caused by injection ($\dot{V}_{O_2}$ NA/control – $\dot{V}_{O_2}$ BMR). We further compared the increase of $\dot{V}_{O_2}$ after control and NA injections to levels of BMR. This comparison was done with absolute values including BMR ($\dot{V}_{O_2}$ NA/control + $\dot{V}_{O_2}$ BMR). Correlation between BMR and body mass and between response to NA injection and T_a was tested with the Spearman rank correlation test.

RESULTS

Ambient temperature

T_a at the study site showed a high daily amplitude (monthly average daily amplitude ranged from 10 to 26°C), as is typical for an austral, Afrotropical habitat. Between December and February (austral summer), minimum T_a mostly stayed above 15°C at night (mean for 2010–2011: 16.3±0.8°C) and mean maximum T_a was usually around 30°C (mean for 2010–2011: 31.5±3.6°C). However, cool days (maximum T_a<25°C) can occur also in summer (1 and 9% of the days in 2010 and 2011, respectively), and 12 and 33% of the nights in 2010 and 2011, respectively, were colder than 15°C. Continuous cold phases (T_a<1°C) were typically confined to short time periods, usually a couple of days (3–20 days) during mid-winter (June–August). The coldest month was June in 2010 (monthly mean: 1.5±3.2°C) and July in 2011 (monthly mean: −0.5±2.1°C) and daily minumum T_a decreased as low as –5°C (June 2010). In 2011, 17% of the winter nights had a minimum T_a warmer than 5°C and 50% of the days were warmer than 25°C.

BMR and body mass

The mean body mass of all individuals during summer was 181±18 g (*N*=7) and did not differ from body mass during winter (*N*=10; Table 1). Mean BMR was 0.5 ml O_2 g^{-1} h^{-1} and did not differ significantly between seasons (*N*=7/10; Table 1). BMR was not correlated with body mass (Spearman rank correlation: *N*=17, coefficient: 0.318, *P*=0.214) and we therefore report our data as mass-specific values (ml O_2 g^{-1} h^{-1}).

Response to injections: T_{skin} and behavior

All individuals showed a behavioral response to NA injection, regardless of season. Individuals did not settle down into a resting position after injection, but showed a markedly increased activity. Maximum reaction to injection was reached after 20–40 min (Fig. 1). For most individuals the following behaviors were noted: legs stretched out, lying down on back, fast breathing, panting, sweating and salivation. All animals increased T_{skin} after NA injection by more than 1°C in both seasons (summer: 1.2±0.7°C, winter: 1.5±0.6°C). After control injection, animals curled up again and did not show any visible behavioral reaction to injection. T_{skin} after control injection was only marginally increased (mean increase <0.3°C).

Table 1. Seasonal comparison of *Galago moholi* body mass, basal metabolic rate (BMR), $\dot{V}_{O_2}$ response to control saline (SA) injection and nonshivering thermogenesis (NST) capacity for all measurements, controlled for acclimatization temperature

			Statistic	
	Summer (*N*=7)	Winter (*N*=10)	*Z*	*P*
Body mass (g)	181±18	182±17	−0.19	0.89
BMR (ml O_2 g^{-1} h^{-1})	0.48±0.06	0.49±0.05	−0.05	0.97
SA (ml O_2 g^{-1} h^{-1})	0.43±0.11	0.48±0.15	−0.64	0.54
NST (ml O_2 g^{-1} h^{-1})	1.22±0.29	1.28±0.22	−0.73	0.48

Results of Mann–Whitney *U*-tests for independent variables are given as *Z*- and *P*-values.

Capacity for NST

Increase in $\dot{V}_{O_2}$ did not differ significantly between seasons for either control treatment or injection of NA (*N*=7/10; Table 1). As neither BMR (see above) nor the metabolic reaction to any of the treatments (control/NA) showed seasonal differences, we pooled both seasons to test differences between all three responses (BMR and NA, SA and BMR, and NA and SA; Fig. 2). The Friedman test revealed significant differences between these parameters (*N*=17, χ^2=34.000, d.f.=2, *P*<0.001). Pairwise Wilcoxon tests showed that handling/injection had a significant effect on $\dot{V}_{O_2}$, as metabolic response to control injections differed significantly from BMR (*Z*=–3.623, *P*<0.001). However, NA injections led to significantly higher metabolic responses compared with BMR (*Z*=–3.622, *P*<0.001; Fig. 2) and the control treatment, i.e. was still significantly higher than the reaction caused by handling alone (*Z*=–3.621, *P*<0.001; Fig. 2). The NST capacity of 1.26 ml O_2 g^{-1} h^{-1} showed an approximately fourfold increase compared with BMR and was very close (95%) to that predicted on the basis of body mass of 1.32 ml O_2 g^{-1} h^{-1} (Mzilikazi and Lovegrove, 2006). Considering that handling itself elicited a significant reaction, this value has to be treated cautiously and could actually be somewhat lower (i.e. between 61 and 95% of the predicted value; calculated by distraction of $\dot{V}_{O_2}$ measured for control injection). However, because effects of NA injections are clearly very pronounced, we cannot ascertain to what extent these override handling effects.

Acclimatization temperature

For determination of NST capacity in the laboratory, animals are usually acclimated at two distinct temperature regimes that differ by at least 10°C (e.g. Nespolo et al., 2001). In the present study, however, we examined the capacity for NST in naturally acclimatized animals. When we plotted the maximum, mean and minimum T_a values of the 3 days before the NA injections (Fig. 3), we found that these acclimatization T_a values were not clearly distinct between the summer and winter measurements. Spearman rank correlation revealed a significant correlation between mean acclimatization temperature and capacity for NST in winter (*N*=10, r_S=−0.689, *P*=0.028), but not for summer (*N*=7, r_S=−0.234, *P*=0.613).

Determination of BAT

Dissection of adult females revealed deposits of BAT between the shoulder blades (Fig. 4) in both individuals. No BAT was found around the kidneys or around the gonads.

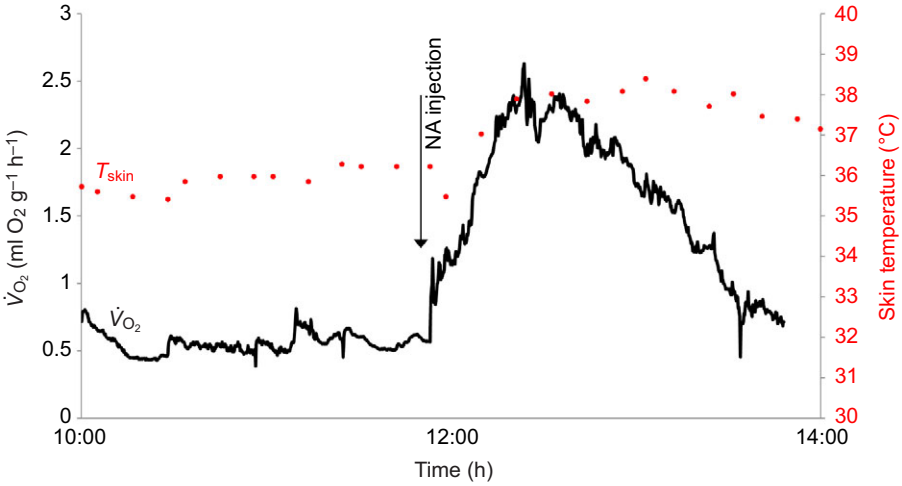

Fig. 1. Metabolic rate ($\dot{V}_{O_2}$) and skin temperature (T_{skin}) before and after injection of noradrenaline (NA) for a male *Galago moholi*.

DISCUSSION

Our study shows that *G. moholi* does possess BAT and responds to injection of NA with an increase in $\dot{V}_{O_2}$ and corresponding behavior to dissipate heat. Naturally acclimatized animals showed a fourfold increase of $\dot{V}_{O_2}$ in comparison to BMR, and NST capacity was consistent with that predicted on the basis of body mass (Mzilikazi and Lovegrove, 2006). The occurrence of BAT and the marked metabolic and behavioral response to the NA injection indicates that *G. moholi* does possess the capacity for NST and that this result is not due to the activation of NST-unrelated receptors. However, we could not detect a seasonal acclimatization effect on NST capacity. Neither BMR nor NST showed a significant seasonal variation, even though winter measurements revealed that *G. moholi* can upregulate the capacity for NST on especially cold days. We can therefore conclude that the low rewarming rates and the severe difficulties experienced by some individuals in rewarming from body temperatures below 19°C are not due to a lack of the capacity for NST. Instead we suggest that this condition is probably due to depleted energy reserves of food-deprived individuals (Nowack et al., 2013a).

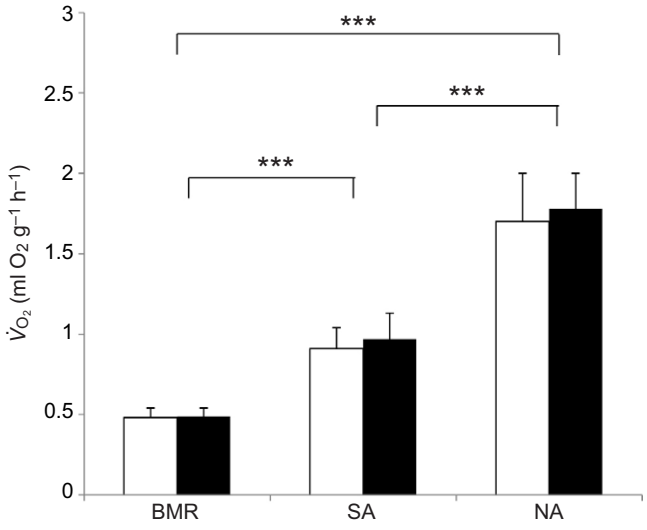

Fig. 2. Oxygen consumption ($\dot{V}_{O_2}$) of the basal metabolic rate (BMR), and in response to control (saline) and noradrenaline (NA) injection during summer (white bars) and winter (black bars). $\dot{V}_{O_2}$ increase after saline and NA injection is significantly different to BMR (***P<0.001).

The knowledge on the NST capacity of primate species is very limited. The only studies available so far were conducted in the laboratory under constant conditions and therefore might not be indicative of the natural physiological response in species inhabiting subtropical or tropical habitats. BAT and a $\dot{V}_{O_2}$ increase following NA injection have been found in captive rhesus monkeys, *Macaca mulatta* (Chaffee and Allen, 1973; Chaffee et al., 1975), and in captive common marmosets, *Callithrix jacchus* (Rothwell and Stock, 1985). The only study examining this topic in more detail was a study on captive grey mouse lemurs, *Microcebus murinus*, which found not only a winter increase in BAT and NST, but also a related increase in UCP1 (Génin et al., 2003). Most other studies investigating the capacity for NST have been performed on small temperate or arctic mammal species that are exposed to comparatively constant temperatures within a season and high seasonal differences in their natural habitats. Individuals of those species generally express adaptive NST, with a low capacity for NST in summer and an increase in capacity in winter [e.g. *Sorex cinereus* (Merritt, 1995)]. Small subtropical or tropical mammals, such as *G. moholi*, however, are often exposed to variable temperatures with smaller seasonal differences in temperature in their natural habitats, but with high day-to-day variations and daily amplitudes in T_a as high as 35°C (Dausmann et al., 2012). And indeed, the expression of seasonally changing NST in these species seems to be equivocal. Some species, such as *M. murinus* (Génin et al., 2003), *Thallomys paedulcus* (Lovegrove et al., 1991), *Rhabdomys pumilio*, *Lemniscomys griselda* (Haim, 1982), *Saccostomus campestris* (Haim et al., 1991), *Elephantulus rupestris* (Oelkrug et al., 2012) and *Amblysomus hottentotus longiceps* (Scantlebury et al., 2008), show adaptive NST adjustments between summer and winter seasons, although less pronounced than in temperate or arctic species. In contrast, other studies on Afrotropical species did not find any seasonal differences in NST capacity, as for example in *Aethomys namaquensis* (Rodentia) (Lovegrove et al., 1991) and *Elephantulus myurus* (Afrotheria) (Mzilikazi et al., 2007). The lack of seasonal differences in NST capacity, however, does not necessarily imply a lack of NST. The presence of functional BAT has been at least demonstrated for *Elephantulus myurus*, leading to a discussion about the presence of a fixed/constitutive NST capacity (Mzilikazi et al., 2007). It was further suggested that the lack of seasonal adaptive thermogenesis in basal Afrotheria could be linked to adaptive heterothermy associated with unpredictable environments (Oelkrug et al., 2012).

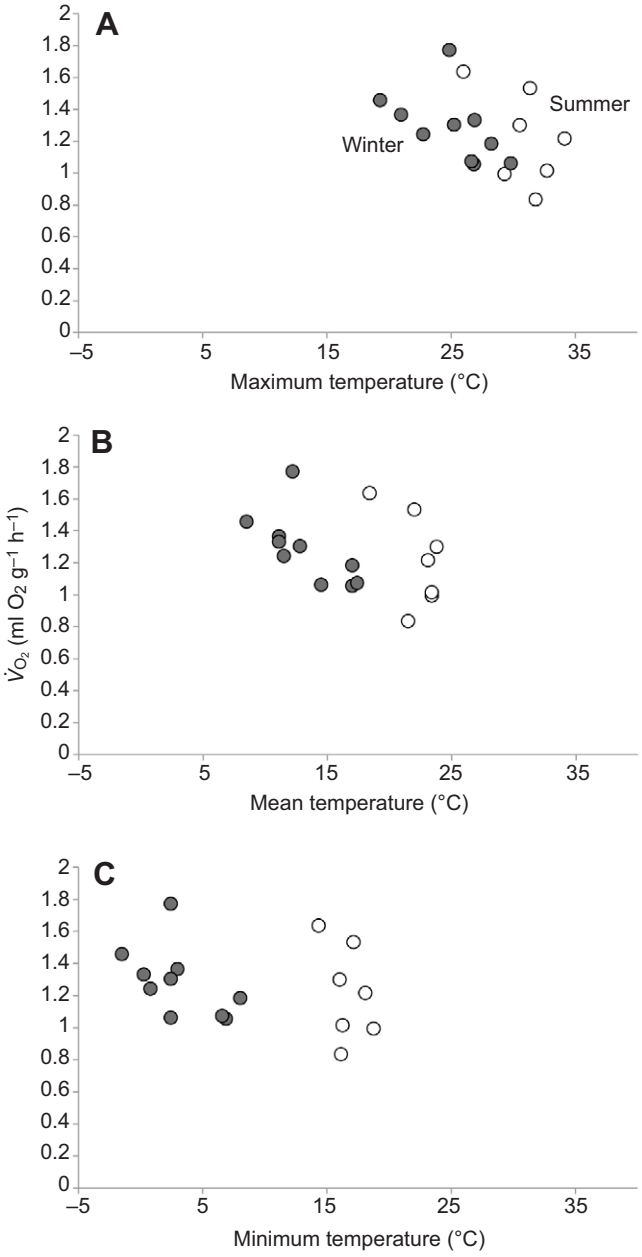

Fig. 3. Acclimatization temperature. Nonshivering thermogenesis capacity ($\dot{V}_{O_2}$) in relation to mean acclimatization temperature during the 3 days before the experiment for (A) maximum temperature, (B) mean temperature and (C) minimum temperature.

Fig. 4. Interscapular deposit of brown adipose tissue in an adult female *Galago moholi* during winter.

Species might possess the mechanism for NST, but because of climatic conditions in their habitats do not necessarily show seasonal differences in the capacity for NST when measured under natural acclimatization. A field study examining the capacity for NST in the four-striped field mouse, *Rhabdomys pumilio*, under natural conditions, found that the NST capacity in this species is extremely sensitive to the acclimatization temperature of the last 3 days before the measurement (Welman, 2013). Similarly, our study showed that in winter the capacity for NST in *G. moholi* is negatively correlated to the acclimatization temperature of the same period. Other than *R. pumilio*, *G. moholi* obviously does not need to increase the capacity for NST on most winter days, but nevertheless possesses the ability for rapid adaptation to cold spells. Winter ambient conditions in the habitat of *G. moholi* show only very short pronounced cold periods and various authors have stated the biological importance of short activation times for NST of free-ranging animals (Janský, 1973; Wunder, 1985). The average NST capacity of *G. moholi* is in the range of what would be expected on the basis of body mass. As we did not find a seasonal difference in the capacity for NST, this suggests that the NST capacity in summer is comparably high. *Galago moholi* uses heterothermy as an emergency solution in response to acute food shortage only, not as a regular seasonal strategy (Nowack et al., 2010). So far, torpid states in *G. moholi* have only been observed during the cold-dry period, when food availability is naturally low, but it is conceivable that emergency torpor bouts can be entered at any time of the year. As NST is needed for rewarming from torpor (Janský, 1973; Jefimow et al., 2004), this could explain the elevated capacity for NST (and the subsequent low seasonal difference) throughout the year.

Recent data on heterotherms inhabiting tropical to subtropical habitats suggest that passive rewarming plays an important role in decreasing the energetic costs of arousal from torpor or hibernation (Dausmann et al., 2009; Geiser and Drury, 2003; Mzilikazi et al., 2002). Passive rewarming can be achieved by following the increase of surrounding temperature, by basking in the sun or by social thermoregulation (reviewed in Geiser et al., 2004). Ambient conditions change between seasons within the habitat of *G. moholi*; however, daily maximum temperatures during the winter generally still rise to between 20 and 25°C. For a short period of the year, nightly temperatures may drop as low as −5°C (Nowack et al., 2010) during early winter mornings, but increased fur insulation (J.N., personal observation), reduced activity times and increased huddling behavior (Bearder and Martin, 1980; Nowack et al., 2013b) might render further elevated NST unnecessary. An earlier study found a pronounced seasonal NST capacity in the South African pouched mouse, *Saccostomus campestris*, living within the same temperature regime as *G. moholi* (Haim et al., 1991). This species regularly enters torpid states, and lives solitarily, thus forgoing advantages of communal thermoregulation, which might lead to a higher necessitation of increased NST during winter. At least two studies also found no significant difference between summer and winter NST in non-tropical, but highly social species: *Discrostonyx groenlandicus* (Maier and Feist, 1991) and *Ochotona curzoniae* (Wang et al., 1999). Both species live in groups in burrow systems with reduced seasonal

variation. Southern flying squirrels, *Glaucomys volans*, nesting individually experience a longer period of elevated winter NST and show an overall increase in mean NST throughout than squirrels nesting socially (Merritt et al., 2001). Indeed, most species lacking seasonal adaptations in NST capacity (*G. moholi*, *D. groenlandicus*, *O. curzoniae* and *A. namaquensis*) live in groups (at least during the cold winter), except *E. myurus*.

Another factor influencing the necessitation for NST in subtropical or tropical heterothermic species is sleeping site use. Species inhabiting stable burrows or nests live in a buffered environment with reduced possibilities of passive rewarming and a higher need for metabolic heat production during arousals. *Galago moholi* was found to show an enhanced preference for buffered, insulated sleeping sites in winter, such as tree holes, but also open-top nests, allowing access to exogenous passive heating. Additionally, some individuals were found sleeping on open trees throughout the year (Nowack et al., 2013b).

The problem with defining classical NST

Injection of NA does not only lead to classical NST, but can also lead to activation of NST-unrelated receptors in species lacking BAT, such as birds (Cannon and Nedergaard, 2004). Studies in rock doves, *Columba livia*, for example, found an increased metabolic response to NA injection. Interestingly, and in contrast to adaptive classical NST, which should increase during cold periods, metabolic reaction to NA injection was significantly higher in warm-acclimated birds than in cold-acclimated birds (Hissa et al., 1975). Consistent with this, it was argued that adaptive UCP1-dependent NST can only be demonstrated by a comparably higher increase in thermogenesis in cold-acclimated animals (i.e. seasonal adaptive thermogenesis), a method used in numerous studies (Cannon and Nedergaard, 2004). This approach, however, also has its constraints. A study on NST capacity of young pigs, for example, found functional BAT and a seasonal increase in NST capacity (Heldmaier, 1974), but later studies could not detect functional UCP1 (Berg et al., 2006). An earlier study determining the involvement of BAT and muscular tissue in NST revealed that BAT is indeed the dominant site of NST (Foster and Frydman, 1978), but is not the only tissue involved, leading to the question of the presence of an UCP1-independent mechanism of NST. Similarly, the presence or absence of NST in non-placental mammals is still controversial. Studies on NST capacity of marsupials mostly failed to report any metabolic response to NA injection (e.g. Nicol et al., 1997; Opazo et al., 1999). However, UCP1-independent NST capacity as reaction to NA or β3-adrenergic agonist was found in some Tasmanian species of the order Macropodoidae and one species of Dasyuridae, but only in cold-acclimated individuals (Kabat et al., 2003; Nicol, 1978; Rose et al., 1999). This is deviant from the classical NST response, which occurs not only in cold-acclimated, but also in warm-acclimated individuals, albeit to a lesser extent. In the fat-tailed dunnart, *Sminthopsis crassicaudata*, an orthologue of UCP1 has been recently identified that is upregulated during cold exposure resembling adaptive molecular adjustments of BAT found in placental mammals. Injection of NA during warm and cold acclimation, however, did not lead to adaptive NST (Polymeropoulos et al., 2012), suggesting the presence of a fixed/constitutive NST capacity as already proposed for *Elephantulus myurus* (Mzilikazi et al., 2007; Oelkrug et al., 2012).

Studies on species inhabiting warmer habitats are increasingly questioning the original definition of classical NST and we therefore recommend a refinement. Functional uncoupling proteins are found not only in mammals, but have been detected in ray- and lobe-finned fish (Jastroch et al., 2005) as well, and it is conceivable that evolution acted on such a fundamental mechanism leading to more than one manifestation of NST in mammals (e.g. adaptive NST, constitutive NST and a UCP1-independent mechanism of NST). Furthermore, studies on Afrotropical species in particular indicate that defining the capacity for NST only on the basis of seasonal differences in response to NA injection is not necessarily a sufficient method. Comparison of the reaction to NA injection in warm- *versus* cold-acclimatized animals does reveal information not only about classical NST, but also about seasonal adaptations, and these two variables might not always be separable. Comparison of seasonal NST determined under a natural temperature and light regime provides information about NST plasticity rather than defining adaptive NST. In the case of species living in areas without distinct seasonal temperature differences, but with high daily amplitudes in T_a, the results obtained from naturally acclimatized animals may even lead to incorrect conclusions. However, strictly laboratory-based studies performed under constant light and temperature regimes will reveal whether a species is able to alter its NST capacity in response to temperature changes, but will not allow for the description of the natural seasonal plasticity of the NST capacity of the species. A study on UCP1-ablated mice showed that individuals without functional UCP1 do not statistically differ in their reaction to control or NA treatment (Golozoubova et al., 2001), suggesting that NST capacity can also be defined on the basis of a comparison between reaction to control (or BMR) and NA injection. Therefore, we suggest that NST capacity in mammals should rather be defined by comparing the reaction between control and NA injection for both warm- and cold-acclimatized animals, preferably in their natural habitat. Classical NST will elicit a significant increase in $\dot{V}_{O_2}$ in both acclimatization groups, whereas animals without classical NST will either not show any reaction at all, or only for one of the acclimatizations (as seen in Tasmanian marsupials).

LIST OF SYMBOLS AND ABBREVIATIONS

BAT	brown adipose tissue
BMR	basal metabolic rate
NA	noradrenaline
NST	nonshivering thermogenesis
SA	saline
T_a	ambient temperature
T_{skin}	skin temperature
UCP1	uncoupling protein 1

ACKNOWLEDGEMENTS

We thank the Limpopo Province Department of Agriculture and Environment for their hospitality and permission to work at Nylsvley Nature Reserve. Nangamso Thole, Lebogang Johanna Selomo and Lucas Mamanyane provided invaluable assistance in the field. We thank Dr Martie Koen and Tom van Niekerk for providing individuals for dissection, and Juan Scheun and Irene Kotze for performing the procedure.

AUTHOR CONTRIBUTIONS

The work presented here was carried out in collaboration between all authors. J.N., K.H.D. and N.M. conceived and designed the experiments. J.N. performed the experiments and analyzed the data. Reagents, materials and analysis tools were provided by K.H.D. and N.M. All authors have contributed to, seen and approved the manuscript.

COMPETING INTERESTS

No competing interests declared.

FUNDING

Financial support was received from the Deutscher Akademischer Austauschdienst and the Deutsche Forschungsgemeinschaft (DA 1013/3-1/2).

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