The molecular and biochemical basis of non-shivering thermogenesis in an African endemic mammal, *Elephantulus myurus*

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**Abstract**  
Uncoupling protein 1 (UCP1) mediated nonshivering thermogenesis (NST) in brown adipose tissue (BAT) is an important avenue of thermoregulatory heat production in many mammalian species. Until recently, UCP1 was thought to occur exclusively in eutherians. In the light of the recent finding that UCP1 is already present in fish, it is of interest to investigate when UCP1 gained a thermogenic function in the vertebrate lineage. We elucidated the basis of NST in the rock elephant shrew, *Elephantulus myurus* (Afrotheria: Macroscelidea). We sequenced *Ucp1* and detected *Ucp1* mRNA and protein restricted to brown fat deposits. We found that cytochrome c oxidase activity was highest in these deposits when compared with liver and skeletal muscle. Consistent with a thermogenic function of UCP1 isolated BAT mitochondria showed increased state 4 respiration in the cold as well as palmitate induced, GDP sensitive proton conductance, which was absent in liver mitochondria. On the whole animal level, evidence of thermogenic function was further corroborated by an increased metabolic response to noradrenaline (NA) injection. Cold acclimation (18°C) led to an increased basal metabolic rate relative to warm acclimation (28°C) in *E. myurus* but there was no evidence of additional recruitment of NA induced NST capacity in response to cold acclimation. In summary, we showed that BAT and functional UCP1 are already present in a member of the Afrotheria, but the seasonal regulation and adaptive value of NST in Afrotherians remains to be elucidated.

**Keywords:** basal metabolic rate; uncoupling protein 1; proton leak kinetics; brown adipose tissue

**Running head:** Basis of nonshivering thermogenesis in *E. myurus*
**Introduction**

The success of early mammals is often attributed to the ability to produce heat endogenously through non-shivering thermogenesis (NST) (6; 24). In eutherian mammals NST is known to occur in brown adipose tissue (BAT) where uncoupling protein 1 (UCP1) works to generate heat instead of ATP (6; 13; 15; 36). However, the often repeated statement that all eutherians exhibit BAT is based on only a few representatives of the subclass. Until recently UCP1 was thought to be associated exclusively with NST in eutherian mammals. However, it has been demonstrated by conserved synteny that UCP1 appears in the vertebrate line as early as before the divergence of ray finned and lobe-finned fishes (17) although a thermogenic function of fish UCP1 is unlikely. This finding begs the question of where in the vertebrate lineage UCP1 gained a thermogenic function as well as the adaptive significance of UCP1 presence.

Within the Marsupialia and the Monotremata the presence and function of UCP1 remains controversial. The elephant shrews belong to the Afrotheria, a group of mammals thought to be at the base of the eutherian radiation (35). Their phylogenetic placement thus makes them an excellent model in the quest for the presence of UCP1 and BAT mediated thermogenesis. In addition, the rock elephant shrew, *Elephantulus myurus* shows a pronounced thermogenic response to noradrenalin (NA) injection (28) which is often taken as an indicator of BAT-mediated NST (5; 6). However, at present it is unclear whether this NA-induced thermogenesis observed in *E. myurus* is indicative of classical NST and whether it is of any adaptive value, i.e. can be recruited in the cold.

Classical, adaptive, cold mediated NST is accompanied by a pronounced recruitment of BAT and enhanced thermogenic capacity (6; 12; 15; 20). This adaptive thermogenic capacity is evident as changes in physiological and biochemical parameters such as high mitochondrial density and respiratory capacity in BAT, a high UCP1 concentration in BAT mitochondria of cold acclimated mammals (19; 20) as well as a higher metabolic response to NA injection when compared to warm acclimated animals. Furthermore, in rodents, UCP1 increases proton leak in the presence of free-fatty acids, in a GDP-sensitive manner (32). In addition, basal metabolic rate (BMR) is also increased.
in the cold, a response thought to be associated with increased blood flow to intestinal
organs and increased peripheral vascularization (10).

Drastic seasonal changes in thermogenic capacity are often associated with high
latitude species. However, it is becoming increasingly clear that phenotypic plasticity is
selected for, even in those species from tropical and subtropical environments because
plasticity allows organisms to match environmental variability at an ecological scale (25;
26; 31). We might therefore expect the African-endemic elephant shrews to exhibit
adaptive changes in their thermogenic properties during warm and cold acclimation.

The aim of the study was therefore two-fold. Firstly, we aimed to elucidate the
molecular and biochemical basis of nonshivering thermogenesis in rock elephant shrew,
*E. myurus*. Our approach was to investigate the occurrence of BAT and the patterns of
tissue expression of UCP1 in *E. myurus* as well as to characterize UCP1 function by
measuring the proton leak kinetics of isolated BAT mitochondria. Secondly, we
investigated whether parameters associated with thermoregulation and cold adaptation
such as BMR, NST capacity, amount of UCP1, cytochrome c oxidase activity were
regulated in response to cold and warm acclimation.

**Materials and methods**

**Study animals**

Adult *E. myurus* (n = 12) were captured on the MacKay farm, 17 km east of Estcourt
(28°56.749’S 30°00.848’E), KwaZulu-Natal, South Africa, in June 2006. They were
then flown to Germany and housed in the Department of Animal Physiology, Philipps
Universität Marburg. Within 3 weeks of arrival, 3 of the animals died, presumably due to
stress associated with capture and handling.

The animals were individually housed in large Makrolon Type IV cages and
provided with sawdust bedding and nesting boxes. They were maintained at 24°C under
a 12L: 12D photoperiod. Food and water was available ad libitum throughout the study.
The animals were fed ProNutro, a commercial high protein cereal (22% protein, 59%
carbohydrate and 6% fat) mixed with water. This diet was alternated with canned dog
food and was supplemented with fresh lettuce and cockroaches. The food was replenished
once every 24 hrs.
**Experimental sequence**

All experiments complied with the German Animal Welfare Laws and are fully compliant with the American Physiological Society’s “Guiding Principles for Research Involving Animals and Human Beings.”

The metabolic measurements were made in October – December 2006. Our approach was to first measure BMR and NST capacity of animals acclimated to 24°C, after which the animals were divided into two groups, the warm acclimated (WA) group (n = 4) at 28°C and the cold acclimated (CA) group (n = 5) at 18°C. We expected that both BMR and NST capacity would increase and decrease under cold and warm acclimation, respectively. The BMR of the animals was measured at 31°C. This temperature is within the known thermoneutral zone for this species (23). After measurement of BMR, the NST capacity of the animals was tested by measurement of the metabolic response to NA injection. The animals were then maintained at their respective acclimation temperatures for at least 3 weeks before repeat BMR and NST capacity measurements were made. After four weeks the animals were sacrificed so as to ascertain the biochemical and molecular basis of non-shivering thermogenesis in this species.

**Measurement of BMR and NST capacity**

Animals were placed in 1.8ℓ metabolic chambers inside a constant environment cabinet. Oxygen consumption (VO₂) was measured using an open-flow through system using an electrochemical analyzer (S-3A/II, Ametek). Air was pumped through the metabolic chambers at flow rates *ca.* 50ℓ hr⁻¹. The use of solenoid relay valves for each chamber allowed us to measure three animals and a control channel sequentially in 1min intervals. Measurements were therefore obtained for each animal every four minutes. During the determination of NST capacity, we measured single animals and therefore increased the resolution of measurement to two minutes. Further details of the respirometry have been described previously in (11).

All metabolic measurements were made during the daytime, the known rest phase for this species (30). On each measurement day, animals were removed from their cages around 09h00 and measurements commenced shortly thereafter. The animals were kept
in the metabolic chambers for at least 6 hours and the BMR was taken as the mean of the three lowest consecutive measurements, equivalent to 12 min, obtained during the last two hours of data measurements. The length of time spent in the metabolic chambers coupled with the fact that the animals were not fed prior to measurement ensured that the animals were post absorptive at the time of BMR determination. Animals were allowed at least 3 days of recovery from BMR measurements, after which their capacity for NST was determined through the injection of noradrenaline (NA).

The measurements of response to NA were made at 25°C, so as to avoid hyperthermia (31). We used 85% of the dose recommended by Wunder and Gettinger (1996; 0.458 mg/kg) because Mzilikazi and Lovegrove demonstrated that the maximal NA induced thermogenesis in this species can already be elicited at this dose (31). Animals were injected with NA at least 2 hours after they were initially placed in the respirometers. For each animal the NST capacity was determined as the difference between the highest NA-induced VO$_2$ and the BMR.

**Cloning of Ucp1 cDNA and phylogenetic inference**

Total RNA was isolated from selected tissues and cDNA synthesized as described previously (16). Primers were deduced from the lower hedgehog tenrec (*Echinops telfairi*) genome found at www.ensembl.org. Primers (forward 5'-GACTATGGGGGTGAAGATCTTC-3'; reverse 5'-AAAGGCCGGCAGCCCTTCCTTG-3') were used for polymerase chain reaction on cDNAs of the interscapular fat deposit of *E. myurus*. 40 cycles of denaturation at 94°C (1 min), annealing at 59°C (1 min) and elongation at 72°C (1 min) were performed. A final extension at 72°C was applied for 10 minutes and followed by rapid cooling to 4°C. The PCR product was gel-purified and ligated into a pJET1/blunt cloning vector (Fermentas). Inserts were sequenced using vector based primers.

Three plant UCPs, three bird UCPs, 22 UCP2, 16 UCP3, and 22 UCP1 sequences including the novel *E. myurus* sequence were aligned and subjected to phylogenetic analysis using Neighbour-joining method as described previously (16). For bootstrap analysis the alignment dataset was shuffled 1000 times. The oxalacetate-malate carrier (OMCP) served as the distantly related outgroup.
**Northern blot analysis**

Five micrograms of RNA were electrophoresed in a 1% denaturing agarose gel (5% formaldehyde, 0.02 MOPS, 5 mM sodium acetate, 1 mM disodium EDTA, pH 8), transferred overnight in 10X SSC to a nylon membrane (Hybond N, Amersham), and UV cross-linked. All blots were hybridized with probes corresponding to the cDNA sequences of *Elephantulus myurus* UCP1. The cDNA probes were labeled by random priming with $[^{32}\text{P}]$ dATP. Nylon membranes were prehybridized at 63°C with BSA solution (0.5 M Na$_2$HPO$_4$/NaH$_2$PO$_4$, pH 7.0, 1 mM EDTA, pH 8.0, 0.7% SDS, 1% BSA) for at least one hour and hybridized overnight at 63°C with the $^{32}$P-labelled probe. After hybridization, the blots were washed with 2X SSC/0.1% SDS for 30 min, 1X SSC/0.1% SDS for 10 min, 0.5X SSC/0.1% SDS for 10 min and 0.1X SSC/0.1% for 10 min at room temperature. Signal intensities were then monitored by exposure to a PhosphoScreen (Molecular Dynamics). The hybridized probes were then detected by phosphor imaging (Storm 860, Molecular Dynamics) and the signal intensities were quantified using ArrayVision 7.0 (Imaging Research). Signal intensities were normalized to ethidium-bromide stained 28S rRNA.

**Immunological detection of UCP1**

Samples of frozen tissues were homogenized in sample buffer (100 mM KH$_2$PO$_4$/K$_2$HPO$_4$, 2 mM EDTA pH 7.5) after which the protein concentration was determined by the Bradford method. Protein samples were then run on a SDS-polyacrylamide gel (3% stacking gel and 12.5% running gel). The protein was then transferred to a nitrocellulose membrane (Hybond-C, Amersham Biosciences). After blocking against non-specific binding with 5% Slimfast for at least 1 h at room temperature, the membrane was incubated with a rabbit polyclonal antibody to hamster UCP1 (1: 10 000) for 1 h and then treated with a second antibody, goat-anti-rabbit-IgG, HRPO-conjugate 1: 10 000) for another 1 h at room temperature. The membrane was then washed before being incubated for 2 min with ECL reagents for peroxidase detection. The signals were detected by exposing the membrane to autoradiography film for 30 sec.
Isolation of interscapular fat mitochondria

Clearly identifiable fat deposits from the interscapular region were excised from the animal and quickly transferred to an excess of ice-cold isolation medium A (250 mM Sucrose, 10 mM TES, 1 mM EDTA, 0.4% (w/v) BSA, pH 7.2). The tissue was then minced in the buffer, on ice, with fine scissors. The tissue was then transferred to a glass-teflon homogenizer. The tissue was homogenized with 6 strokes of a loose-teflon pestle, filtered through a layer of gauze and centrifuged for 10 min at 8740 g. The lipid layer was removed through aspiration, the supernatant was discarded, and the lipid remaining on the inside walls of the tube was removed using tissue paper. The mitochondria pellet was suspended in isolation medium B (250 mM Sucrose, 10 mM TES, 1 mM EGTA, 0.4% w/v BSA, pH 7.2) and centrifuged at 950 g for 10 min and the resulting supernatant was transferred to a new tube and centrifuged at 8740 g for 10 minutes. The final mitochondrial pellet was suspended in a buffer containing 100 mM KCl, 20 mM TES, 1 mM EGTA, pH 7.2). Protein concentration was determined using the Biuret method.

Isolation of liver mitochondria

The liver was removed and immediately placed in ice cold isolation medium (250 mM Sucrose, 5 mM Tris-HCl, 2 mM EGTA, pH 7.4). The tissue was minced with scissors and disrupted using a Dounce homogenizer with a medium-fitting pestle. The homogenate was centrifuged at 8500 g for 10 min at 4°C and the pellet was re-suspended in isolation medium and spun at 1047 g for 10 min. The resulting supernatant was subjected to a high speed cycle (11630 g, 10 min, 4°C). The pellet was re-suspended in medium without BSA. The high speed cycle was repeated twice and the final pellet re-suspended in a minimal volume of isolation medium. Protein concentration was determined using the Biuret method.

Proton leak kinetics: measurement of oxygen consumption and membrane potential

Oxygen consumption of BAT mitochondria was measured using a Clark-type electrode (Rank Brothers Ltd., UK) maintained at 37°C and calibrated with air-saturated medium (50 mM KCl, 5 mM TES, 2 mM MgCl₂ x 6H₂O, 4 mM KH₂PO₄, 1 mM EGTA, 0.4% (w/v) BSA, pH 7.2). For the liver mitochondria, the measuring medium contained 120 mM
KCl, 5mM KH$_2$PO$_4$, 3mM HEPES, 1mM EGTA and 0.3% (w/v) BSA, pH 7.2. The measuring medium was assumed to contain 406 nmol O/ml (33). Oxygen consumption and membrane potential were measured simultaneously using an electrode sensitive to the potential-sensitive probe, TPMP$^+$ (triphenylmethylphosphonium), a method previously described (1; 3). Briefly, the kinetics of the mitochondrial proton leak were measured by determining the dependence of the respiration rate required to drive the proton leak on membrane potential. BAT and liver mitochondria were incubated at protein levels of 0.3 mg/ml and 0.7 mg/ml, respectively in 2.5 ml of medium containing 8µM rotenone to inhibit complex I, (4µg ml$^{-1}$) oligomycin to inhibit phosphorylation of ADP and (110ng ml$^{-1}$) nigericin to abolish $\Delta$pH. The TPMP$^+$ electrode was calibrated with sequential additions up to 2.5µM TPMP$^+$. Succinate (6 mmol l$^{-1}$) was provided as the substrate for respiration. Oxygen consumption and membrane potential were titrated through sequential steady states by successive additions of malonate up to 2mM for BAT mitochondria and up to 4mM for liver mitochondria. Finally, 0.3µM FCCP was added to dissipate the membrane potential and release all the TPMP$^+$ from the mitochondria, allowing for correction of baseline drift. Respiration at each steady state was then plotted against the appropriate membrane potential so as to display the dependence of proton leak rate on potential.

We were interested in whether the presence of UCP1 in the BAT of *E. myurus* was associated with uncoupled respiration (proton leakage) representing the molecular mechanism of NST. Proton leakage catalyzed by UCP1 has two distinct characteristics: inhibition by purine nucleoside di- or triphosphates as well as activation by fatty acids (32). We therefore investigated if the proton leak kinetics in elephant shrew BAT mitochondria were affected by GDP and palmitate in CA and WA elephant shrews.

**Cytochrome c oxidase activity measurements**

Cytochrome c oxidase (COX) activity was measured polarographically (34) in BAT mitochondria, BAT, liver and skeletal muscle tissue homogenates. All tissues were weighed and were then homogenized in tissue buffer containing 100mM KH$_2$PO$_4$/K$_2$HPO$_4$, 2mM EDTA, pH 7.5. The homogenate was incubated at 25°C with 1ml air-saturated medium (79 mM K$_2$HPO$_4$ x 3H$_2$O, KH$_2$PO$_4$, 5 mM EDTA-Na$_2$, pH 7.4)
assumed to contain 479 nmol O/ml with 43μL 3mM cytochrome c, and 71μL of 0.25M
ascorbic acid added. The tissue homogenate was diluted 1:2 up to 1:6 for BAT, liver and
skeletal muscle, and 1:15 for BAT mitochondria, with polyoxyethylenether W1, 1.5%
(w/v) no longer than ten minutes before the assay.

All mean values are reported ± SEM. Differences between groups were analyzed using t-
tests, paired t- tests and ANOVAs as appropriate.

Results

Body mass
The mean ± SEM body mass of all the animals acclimated to 24°C was 63.5 ± 2.1g.
After three weeks of respective temperature acclimation the body mass of both WA and
CA had decreased to 59.2 ± 3.1 and 59.4 ± 1.8g, respectively and did not differ
significantly between the groups (t = 0.07; p > 0.05).

Basal metabolic rate and non-shivering thermogenesis
The mean BMR of all animals acclimated to 24°C was 1.23 ± 0.03 ml O₂.g⁻¹.hr⁻¹. There
was no significant difference in the BMR of the two groups before they were transferred
to their respective acclimation temperatures. However, there was a pronounced cold
acclimation effect on the BMR of the rock elephant shrews (Fig. 1). Except for one
individual, the WA animals either maintained the same BMR as at 24°C or decreased
BMR. Overall, the BMR of the WA group (1.31 ± 0.07ml O₂.g⁻¹.hr⁻¹) was not
significantly different to that measured at 24°C. In contrast, all the CA animals exhibited
a significant increase in BMR (1.64 ± 0.04 ml O₂.g⁻¹.hr⁻¹). This BMR increase was on
average 37.9 ± 1.6% and ranged from 35 – 42%. Overall, there was a significant
difference in the BMR of cold and warm acclimated animals (t = 4.65; p = 0.002).

The injection of NA caused an increase in VO₂ which was observed within 45
min of administration. These high metabolic rates following NA injection were not
associated with activity as the animals typically spread out their bellies on the metabolic
chambers, presumably to dissipate heat. The increase in VO₂ caused by NA injection
was in most cases up to 4x BMR values. The NST capacity for all animals at 24°C was
2.75 ± 0.69 ml O₂.g⁻¹.hr⁻¹ and there were no differences in the NST capacity of the animals that were placed in the respective acclimation groups (data not shown).

Surprisingly, cold acclimation did not lead to an increase in the NST capacity of rock elephant shrews (Fig. 1; 2.65 ± 0.65 ml O₂.g⁻¹.hr⁻¹). Similarly, warm acclimation did not lead to a decrease in the NST capacity (2.53 ± 0.18 ml O₂.g⁻¹.hr⁻¹). Overall there were no significant differences in NST capacity of cold and warm acclimated animals (t = -0.09; p = 0.93).

Cloning of the Ucp1 cDNA and phylogenetic inference

We amplified a 726bp cDNA fragment by RT-PCR from the interscapular fat deposit (Genbank Acc. No EF 121740) which had the characteristics of eutherian brown fat, extensive blood supply as well as abundant mitochondria, giving it a brownish colour. For classification of the deduced *E. myurus* UCP1 protein sequence and its relationship to other homologues, we performed phylogenetic analyses and generated a tree. Phylogenetic inference revealed that *E. myurus* UCP1 groups amongst other known eutherian UCP1 orthologues (Fig. 2). It is most closely related to the tenrec UCP1 sequence with 81% amino acid identity, has 76% identity to mouse UCP1, 55% identity to mouse UCP2 and 53% to mouse UCP3.

UCP1 expression patterns and regulation

We analyzed the tissue distribution of UCP1 both at the mRNA as well as the protein level in the elephant shrews. Northern blot hybridization of total RNA with a *E. myurus* UCP1 cDNA probe selectively detected a transcript in interscapular adipose tissue but not in WAT or any of the other tissues dissected from *E. myurus* (Fig. 3A). Accordingly, on Western blots our polyclonal hamster UCP1 antibody only detected a 32kD protein in the interscapular fat pad (Fig. 3B). Weak signals detected in the other tissues represent cross reactivity of the antibody with unknown proteins of different molecular mass. Cold acclimation did not increase UCP1 mRNA expression in the interscapular fat (Fig. 3C; CA = 0.7 ± 0.1, WA = 1.0 ± 0.2; t = -1.24, p = 0.25). We then analyzed the UCP1 concentration in protein extracts prepared from tissue homogenates (Fig. 3D) and in isolated mitochondria (Fig. 3E). The UCP1 protein levels in the interscapular fat of
warm and cold acclimated elephant shrews was not significantly different (CA = 1.0 ± 0.1 WA = 1.1 ± 0.1; t = -0.51, p = 0.62).

**Cytochrome c oxidase (COX) activity**

The enzyme COX is a reliable indicator of respiratory capacity in a tissue. We therefore investigated whether there were differences in COX activity in the BAT, liver and skeletal muscle of CA and WA animals. COX activity was highest in BAT, followed by skeletal muscle and the lowest enzyme activity was observed in the liver (Fig. 4A). However, none of the tissues showed increased COX activity during cold acclimation. In addition to investigating COX activity at tissue level, we also measured COX activity in BAT mitochondria. There was a tendency towards increased activity in the mitochondria of CA animals, although this did not amount to a statistically significant difference (Fig. 4B; t = -1.29, p = 0.24). We also compared state 4 respiration (in the presence of 5mM GDP) of BAT mitochondria from CA and WA animals. The state 4 respiration of CA mitochondria (151.9 ± 4.4 nmol O min⁻¹. mg⁻¹ protein) was significantly higher than that of WA animals (130.3 ± 7.1 nmol O min⁻¹. mg⁻¹ protein) (t = 2.72, p = 0.03), suggesting a higher respiratory capacity in CA animals (Fig. 4C). There were no differences in the state 4 respiration of liver mitochondria from CA (54.4 ± 6.1 nmol O min⁻¹. mg⁻¹ protein) and WA (55.9 ± 6.7 nmol O min⁻¹. mg⁻¹ protein) animals (t = -0.16, p = 0.87).

**Proton leak kinetics and functional characterization of UCP1**

Firstly, we titrated the effect of GDP on mitochondria isolated from BAT and liver of rock elephant shrews. Because proton leak is a non-linear function of membrane potential, we compared the oxygen consumption driving the proton leak at a common membrane potential of 135mV in the presence of 0, 1, 3, and 5 mM GDP. There was a clear sensitivity of proton conductance to GDP presence in BAT mitochondria whereas this sensitivity could not be discerned on liver mitochondria (Figure 5A). In the absence of GDP the oxygen consumption required to balance the proton leak in BAT mitochondria was quite substantial and decreased with increasing GDP concentration (Fig. 5B).
The addition of 100μM palmitate increased proton conductance (Fig. 6). In the absence of GDP, palmitate almost completely dissipated the membrane potential whilst stimulating respiration. We therefore used 3mM GDP in our assay. Under this condition, palmitate caused a strong increase in proton leakage as evidenced by the increased oxygen consumption and decreased membrane potential. At 3mM GDP the proton leak induced by palmitate was even higher than that observed in the absence of GDP (0mM) and palmitate. However, palmitate had no discernible effect on oxygen consumption of liver mitochondria (Fig. 6). The increased proton leakage due to palmitate combined with the observed GDP inhibition was consistent with an uncoupling function of UCP1.

There always remains the possibility that the GDP sensitive proton conductance in the BAT mitochondria was due to the adenine nucleotide translocase (ANT), a mitochondrial anion carrier that exchanges ADP for ATP across the mitochondrial inner membrane (3; 9). ANT can be potently inhibited by carboxyatractylate (CAT). As the uncoupling effect of ANT is so strong in rat liver and kidney mitochondria, Skulachev and co-workers have suggested that the recoupling effect of GDP on mitochondria cannot be considered as a specific probe for the involvement of a UCP in fatty acid-mediated uncoupling and that GDP suppresses ANT function (18). However, addition of 2.5μM CAT in the absence of GDP did not result in any observable change in proton conductance in BAT mitochondria of cold acclimated *E. myurus*. In fact, in the presence of CAT (no GDP), the BAT mitochondria were more uncoupled than when 1mM GDP was present in our assay (data not shown). This suggests that the proton leakage induced by fatty acids in this study was mediated by UCP1 and not by ANT. In addition, 5mM ADP had an effect even more potent than that of 5mM GDP, demonstrating strong purine nucleotide sensitivity.

We then compared kinetics of proton conductance in BAT mitochondria from CA and WA animals. The oxygen consumption driving the proton leak in the absence of ATP synthesis was plotted against different membrane potentials imposed by malonate titration of succinate oxidation to display the kinetic dependence of proton leak on its driving force, the membrane potential at 37°C. We then assessed similarities and differences in proton leak rate curves by the overlap or non-overlap of standard error bars as has been done in other studies (2; 4; 14). In agreement with our other results, the
proton leak curves of BAT mitochondria overlapped for cold and warm acclimated animals, indicating similar proton leak kinetics under the two acclimation treatments (Fig. 7), suggesting that BAT mitochondria from CA animals were no more uncoupled than those from WA animals. Similarly, there were no significant differences in the proton leak kinetics of liver mitochondria between groups (data not shown).

Discussion

The overall aim of this study was to elucidate the basis of nonshivering thermogenesis in the rock elephant shrew, *Elephantulus myurus* by integrating measurements at the molecular, subcellular, tissue and whole animal levels. We have demonstrated the presence of the *Ucp1* gene as well as its protein UCP1 whose expression was BAT specific in a member of the Afrotheria. The orthologue of UCP1 has already been demonstrated in fish although a thermogenic role in ectotherms is unlikely (17). It is therefore imperative that in addition to showing occurrence of UCP1, uncoupling thermogenic function should also be demonstrated if it is to be implicated in nonshivering thermogenesis. We therefore characterized the function of UCP1 and investigated proton leak kinetics and demonstrated that addition of fatty acids to isolated BAT mitochondria increases proton conductance in a GDP-sensitive manner. To fully display thermogenesis, an organ requires high oxidative capacity to oxidize metabolic substrates and a leak (such as UCP1 to release heat) to uncouple oxidative phosphorylation (20). We demonstrated that BAT of the elephant shrew displays highest oxidative capacity in comparison to skeletal muscle and liver. Taken together, our data show that in this ancient eutherian, UCP1 has a thermogenic function and that NST in the elephant shrew occurs in BAT and is UCP1 mediated.

We expected adaptive recruitment of thermogenic capacity during cold acclimation, the hallmarks of which are increases in the expression of the *Ucp1* gene and its associated UCP1, and mitochondrial biogenesis (19). We found no evidence of recruitment of cold-induced thermogenic capacity in BAT when investigating NA-induced response, *Ucp1* mRNA, mitochondrial UCP1 levels and amounts of tissue UCP1 in rock elephant shrews. We also could not find any differences in the rate of proton leakage from mitochondria of cold and warm acclimated animals. Instead, of all the
parameters considered, the principal adjustment in response to cold acclimation was only
evident in BMR and state 4 respiration rate of BAT mitochondria.

Although in highly seasonal species such as the Djungarian hamster, uncoupled
respiration has a dominating role during cold acclimation, with the total amount of UCP1
in BAT estimated to increase by a nearly 20-fold magnitude (10), it has been widely
documented that small mammals increase their BMR in response to cold acclimation (7;
22; 31). This elevated BMR is thought to improve total thermogenic capacity, as well as
extend the thermoneutral zone to lower Tₘs and is partly associated with blood
distribution to the intestinal organs, whose metabolic activities are presumably increased
(10).

The higher state 4 respiration suggests a higher respiratory capacity in the BAT
mitochondria of cold acclimated animals. Cytochrome c oxidase is a marker enzyme for
the inner mitochondrial membrane and can be used to estimate the amount of
mitochondrial protein in BAT and respiratory capacity of mitochondria (19). Increased
COX activity also points towards increased respiratory capacity and is often accompanied
by a pronounced stimulation of UCP1 gene expression, thus elevating the capacity of
BAT for NST (21). The high COX activity in BAT compared to skeletal muscle and
liver does support a thermogenic function of this tissue. In addition, although there were
no significant differences in COX activity between groups, both at tissue and
mitochondrial level, we did find a tendency towards higher respiratory capacity in the
BAT mitochondria of cold acclimated animals.

It is tempting to ask whether pronounced differences might have been observed
between the two groups had the animals in the cold acclimation group been placed at a
much lower ambient temperature or cold/warm acclimated for longer. It is true that in
most studies animals are often acclimated to ambient temperatures below 10°C and that
acclimating our animals to lower ambient temperatures for longer might have yielded
significant differences between the two groups. Nevertheless, comparable temperature
gradients such as used in this study have been shown to induce changes in thermogenic
capacity of mice. For example, mice acclimated to 21°C and then transferred to 33°C
decreased BAT mass, mitochondrial protein as well as UCP1 content within 48 hrs (8).
We are interested in the extent to which ambient temperature fluctuations influence physiological responses of animals in their natural habitats. We therefore considered that placing the animals at lower ambient temperatures may not necessarily have been ecologically relevant because even in winter in their natural habitat, the rock elephant shrews would not experience consistently low ambient temperatures. Instead, in its natural range, this species is exposed to fluctuating diel ambient temperature cycles, with cool nights (ca. 5°C) and warm days (> 20°C). We suggest that the animals may not have increased their thermogenic capacity during our cold acclimation conditions simply because NST is not an exclusive source of heat gain for this species. In fact, it has been shown previously that during winter when E. myurus use daily torpor they accrue significant energy saving by exogenous passive heating during arousal from torpor (27; 29). The apparent lack of NST recruitment may therefore be explained simply by considering the ambient temperature cycles that occur in this species’ natural habitat. In addition, Lovegrove et al. (2001) predicted that metabolic rate of E. myurus acclimated to 18°C was 3.01 ± 0.15 ml O₂.g⁻¹.hr⁻¹ at 0°C (Figure 5C, Lovegrove et al. 2001). Since there were no differences in NST capacity of cold and warm acclimated animals in this study, we pooled our results and found that the mean maximum NA-induced oxygen consumption was 4.03 ± 0.22 ml O₂.g⁻¹.hr⁻¹. This implies that at 28°C E. myurus have already acquired BAT providing them with enough NST capacity to deal with Tₘs below freezing. Since Tₘs seldom decrease below 0°C in E. myurus’ natural habitat the BAT recruited at 28°C is sufficient for survival and meets their ecological requirements. On the other hand the maintenance of functional BAT at high Tₘ (28°C) might allow them to deal with unpredictable and aseasonal cold spells known to occur in this species’ range.

In nature, ambient temperature and photoperiod often act in concert to bring about seasonal physiological adjustments. In retrospect, it remains possible that had we coupled cold and warm acclimation with short and long photoperiod, respectively, we might have observed some differences between the two groups. Nevertheless, in a study investigating phenotypic plasticity of physiological variables between species, (22) found that after controlling for latitude and phylogenetic effects, the seasonal changes in NST cannot (as yet) be reliably associated with any independent predictor variable.
In conclusion, we show that BAT and functional UCP1 are already present in the rock elephant shrew, a member of the Afrotheria and a basal eutherian mammal although they did not show any evidence of additional recruitment of NST capacity when cold acclimated. This does not necessarily mean that NST is not adaptive in this species. We suggest that what is likely to be most informative regarding the seasonal regulation and the adaptive nature of thermoregemonic parameters in any species will be to capture free-ranging individuals, during the different seasons and immediately measuring the parameters of interest without prior acclimation. An understanding of interseasonal and interannual plasticity in physiological responses is likely to be important during seasonal climate perturbations resulting from large scale climate anomalies.

Acknowledgements

This study was financed by the Alexander von Humboldt Foundation (postdoctoral research fellowship to NM), the National Research Foundation of South Africa, the Nelson Mandela Metropolitan University and a DFG grant (KL973/7) to MK. Any opinions, findings and conclusions or recommendations expressed in this material are those of the authors and the NRF does not accept any liability in regard thereto. We are grateful to the Ezemvelo KwaZulu Natal Wildlife for capture and export permits and Mr and Mrs Bruce McKay for granting permission to work and capture animals on their farm in Estcourt, South Africa. We thank Sigrid Stöhr for excellent technical assistance.
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Figure legends

Figure 1: Basal metabolic rate (BMR) and maximum oxygen consumption (VO₂ max) induced by noradrenalin (NA) injection in (A) cold acclimated and (B) warm acclimated *Elephantulus myurus*. The NST capacity was calculated as the difference between VO₂ max and BMR.

Figure 2: Phylogenetic relationships of known eutherian UCP1. A neighbour joining tree was derived from the amino acid alignment. Bootstrap values from 1000 replications are given next to the internal branches. The oxaloacetate-malate carrier served as the outgroup.

Figure 3: (A) Northern blot analysis of UCP1 expression in multiple tissues of *Elephantulus myurus*. Total RNA (5µg) isolated from multiple tissues was hybridized with ³²P-radiolabelled cDNA corresponding to a *E. myurus* UCP1 fragment. (B) Western blot analysis of UCP1 expression in multiple tissues of *E. myurus*. (C). Northern blot analysis of BAT UCP1 mRNA showing no significant differences in the levels of expression in response to cold (n = 5) and warm (n = 4) acclimation. Ten µg RNA were used in this analysis. (D) Western blot analysis of UCP1 levels in BAT homogenate and (E) BAT mitochondria of cold (n = 5) and warm (n = 4) acclimated *E. myurus*.

Figure 4: (A) Cytochrome c oxidase (COX) activity in brown adipose tissue, liver and skeletal muscle homogenate of cold and warm acclimated *E. myurus*. There were no significant differences between groups in any of the tissues measured. (B) Cytochrome c oxidase activity measured in isolated BAT mitochondria of cold and warm acclimated *E. myurus*. (C) State 4 respiration of isolated *E. myurus* BAT mitochondria respiring on succinate in the presence of 5mM GDP. Respiration was significantly higher in the mitochondria from cold acclimated animals.
Figure 5: (A) Dependence of proton leak rate (measured as the respiration rate driving proton leak) in the presence of oligomycin on membrane potential of isolated BAT (open circles) and liver (closed circles) mitochondria of cold acclimated *E. myurus* in the presence of 0, 1, 3 and 5mM GDP. Replicate measurements were performed on each mitochondrial preparation and averaged. Values are ± SEM from five independent preparations. The broken line indicates the highest common potential (135.3 mV).

(B) The relationship between proton leak rate at the highest common potential (measured as the respiration rate driving proton leak) at 135.3 mV in (A) and GDP concentration.

Figure 6: Dependence of proton leak rate (measured as the respiration rate driving proton leak) in the presence of oligomycin on membrane potential of isolated BAT (open circles) and liver (closed circles) mitochondria of cold acclimated *E. myurus* in the presence of 0, and 3 mM GDP as well as 100 μM palmitate (in the presence of 3mM GDP). Replicate measurements were performed on each mitochondrial preparation and averaged. Values are ± SEM from five independent preparations.

Figure 7: Dependence of proton leak rate (measured as the respiration rate driving proton leak) in the presence of oligomycin on membrane potential of isolated BAT mitochondria from cold (open circles) and warm (closed circles) acclimated *E. myurus* in the presence of 1, 3 and 5mM GDP. There were no observable differences in the mitochondrial proton leak kinetics of the animals from the two groups. Replicate measurements were performed on each mitochondrial preparation and averaged. Values are ± SEM from five and four independent preparations of cold and warm acclimated animals, respectively.
Figure 1

Oxygen consumption (ml O2.g⁻¹.hr⁻¹)
Bootstrap values >500 are shown in the tree

Figure 2
Figure 3
Figure 4
Figure 5

**Figure A**

Graph showing oxygen consumption (nmol O min$^{-1}$ mg protein$^{-1}$) plotted against membrane potential (mV). Lines represent data for BAT mitochondria and Liver mitochondria at different mM GDP concentrations (0mM, 1mM, 3mM, 5mM).

**Figure B**

Bar graph comparing oxygen consumption (nmol C min$^{-1}$ mg protein$^{-1}$) between BAT and Liver at various mM GDP concentrations (0, 1, 3, 5).
Figure 6
Figure 7