Non-shivering thermogenesis in a carnivorous marsupial, *Sarcophilus harrisii*, in the absence of UCP1

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Abstract

The presence of, and the physiological mechanisms behind, non-shivering thermogenesis (NST) were investigated in the Tasmanian Devil (*Sarcophilus harrisii*, Boitard 1841). This was performed by measuring metabolic rate in response to cold exposure with and without an injection of norepinephrine. We also attempted to identify the presence of uncoupling protein 1 (UCP1) and its involvement (if any) in NST in *S. harrisii*, prior to and after cold exposure. UCP1 is a specialized protein channel found within the mitochondria of brown adipose tissue that is known to be involved in eutherian NST. Increase in resting oxygen consumption (VO\(_2\)), were measured under constant conditions at 20°C after various stimuli; long-term cold exposure (14 days at 2–3°C), and with or without subsequent norepinephrine injection was used to indicate the presence of NST. Concurrent with the functional studies, molecular level studies including Western blots and RT-PCR were used to identify the expression of UCP1. We found that injection of norepinephrine significantly increased NST after cold exposure in *S. harrisii*. The post-acclimation resting (VO\(_2\)) increased by 11%, whereas the combination of cold exposure and injection of NE elicited an approximately 30% increase in metabolic rate. However, expression of UCP1 in *S. harrisii* was not identified by the molecular techniques employed, in either the pre- or post-cold-acclimated tissues. These data suggest that *S. harrisii* shows NST ability and that it is accomplished in the absence of UCP1.

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1. Introduction

In general, mammals have the ability to maintain a relatively constant body temperature over a wide range of ambient temperatures. When a mammal has been exposed to a short-term cold stressor, it adopts an acute strategy of thermogenesis: shivering. This is accomplished via rapid skeletal muscle contractions, producing mechanical heat. Conversely, when many mammals are exposed to a relatively extended duration of cold stress, the shivering response is progressively replaced by non-shivering thermogenesis (NST: Foster and Frydman, 1979; Tidemann, 1982; Rothwell and Stock, 1985a,b; Carneheim et al., 1988; Rose et al., 1999). Typically, NST is associated with brown adipose tissue (BAT), which has been estimated to be responsible for up to 60% of the heat produced by cold-acclimated rats (Foster and Frydman, 1979). It is believed that thermogenesis is the sole purpose of BAT. It is highly vascularised, has a large number of mitochondria, has a high content of mitochondrial respiratory enzymes (notably cytochromes), and is usually located in discrete pads at the back of the neck and as interscapular bodies. BAT deposits also increase in size as an animal acclimates to a cold environment (Nedergaard and Cannon, 1992; Ricquier and Bouillaud, 2000). Activation of thermogenesis in brown fat is accomplished by...
the release of norepinephrine (NE) from the sympathetic system, which causes the mitochondrial membrane potential to be lowered via a “leaky” protein channel called uncoupling protein 1 (UCP1). UCP1 short-circuits the electron transport chain to produce heat as an alternative to storing energy as adenosine triphosphate (Eckert et al., 1988).

Previous work has shown that \( \beta \)-agonists such as NE, BRL37344, ICID7114 or BRL35135 can induce NST in members of the marsupial kangaroo family (Potorous tridactylus, Nicol, 1978b; Macropus rufogriseus, Loudon et al., 1985; Bettongia gaimardi, Ye et al., 1995, 1996; Rose et al., 1998, 1999). However, other research has shown that other families of marsupials do not have a NST response to \( \beta \)-agonists (Planigale gilesi and Dasyurides byrne. Dawson and Dawson, 1982; Trichosurus vulpecula, and Monodelphis domestica. Nicol et al., 1997; Didelphis virginiana, B. Wunder, pers. comm.).

Despite numerous attempts, establishing the existence of BAT and/or UCP1 in marsupials has been elusive and hence controversial. Only two papers have described BAT in marsupials: Loudon et al. (1985) detected small amounts of BAT in pouch young \( M. \) rufogriseus but not in adults, and Hope et al. (1997) identified BAT and UCP1 in Sminthopsis crassicaudata a member of the dasyurid family. However, the amount of BAT that Hope et al. (1997) identified was in such small quantities that it is unlikely to be of thermogenic biological importance (C. Daniels, pers. comm.). Rose et al. (1999) showed that the macropod \( B. \) gaimardi does not possess BAT or UCP1 yet has a strong NST response to \( \beta \)-agonists. The consensus of these works has led Rose et al. (1999) to suggest that there may be a phylogenetic difference within marsupials in their NST abilities.

The Tasmanian devil (Sarcophilus harrisii) a member of the dasyurid family is the largest extant carnivorous marsupial. Once found throughout Australia, it is now found only in the island state of Tasmania. The average maximum/minimum temperature in summer on the island ranges from 12\(^\circ\)C to 20\(^\circ\)C, whereas winter ranges from 5.5\(^\circ\)C to 13\(^\circ\)C, with yearly maximums in the 40\(^\circ\)C and minimums below 0\(^\circ\)C (as reported by the Australian Bureau of Meteorology). This requires that Tasmanian animals must be able to endure a wide range of temperatures at any time of the year. It has been shown that \( S. \) harrisii are efficient thermoregulators (Robinson and Morrison, 1957; Morrison, 1965; Hulbert and Rose, 1972; Guiler and Heddle, 1974; Nicol, 1978a; Nicol and Maskrey, 1980; Guiler and Heddle, 1987). Nicol (1982) reported that cold conditions stimulate an increase in heat production by an increase in shivering response, although no evidence of NST was seen. Green (1967) reported that wild \( S. \) harrisii had a subcutaneous fat layer that could measure up to 3 cm in thickness, and possessed visceral adipose deposits that completely covered the intestines and kidneys. Hayward and Lisson (1992) investigated the presence of BAT in the majority of marsupials, including a pouch young \( S. \) harrisii, by gross morphology, light, fluorescence, and electron microscopy, and did not identify BAT in this species. The present study attempted to identify the presence of NST after an extended period of cold exposure and by injection of the \( \beta \)-agonist NE, as well as endeavoured to ascertain the presence of UCP1 at both the expressional and molecular levels.

2. Methods

2.1. Housing and maintenance

Five male Sarcophilus harrisii were obtained from a wild population from southeastern Tasmania. The animals were housed in the University of Tasmania’s Central Animal House outdoor devil enclosures for at least 1 week prior to experimental procedure. Enclosure temperatures ranged from 14\(^\circ\)C to 23\(^\circ\)C. All experiments were performed between January and February (the hotter summer months in Tasmania). No more than two animals were housed together in one enclosure. Animals were held on a natural day/night cycle with free access to food and water. Diet consisted of culled or roadkilled whole wallaby, possum, and rabbit, as well as lamb and chicken off-cuts from a local butcher. Six male adult hooded Wistar rats were used as controls, and were housed at 22\(^\circ\)C on a 12L:12D cycle. These animals were fed with a commercial diet containing 20.4% protein, 4.6% lipid, 69% carbohydrate, and 6% crude fibre with added vitamins and minerals (Gibson, Hobart), and had free access to water. During cold exposure, all animals were individually housed in the School of Zoology’s controlled environment chamber. Food and water was provided as above, and minimum bedding was provided to ensure health but minimise behavioural adaptions. All experiments were approved by the University’s Animal Ethics Committee (Permit: A5705) and Tasmanian Parks and Wildlife Service (Permit: FA99291) and were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.2. Metabolic measurements

Resting (\( \text{VO}_2 \)) was measured for \( S. \) harrisii via open circuit indirect calorimetry. The animals were tested between the hours of 1100 and 1500 h, which were within the normal hours of sleep and/or quiescent phase. All metabolic readings were taken at an ambient temperature of 20\(^\circ\)C (within the thermoneutral zone as described by MacMillen and Nelson (1969)). Air was pumped through the system at a rate of 61 min\(^{-1}\). The egress-air was scrubbed for water and \( \text{CO}_2 \), and the
difference in O$_2$ concentration between the pumped air and expired air was measured by an Ametek Instrument S-3A/II oxygen analyser and N-37M oxygen sensor. Once the trial had commenced, the animals were not disturbed and were left in the apparatus until (VO$_2$) stabilised; this usually took at least 10–15 min. Metabolic rate was calculated using the formula given by Withers (2001) for open circuit measurements, and is presented as ml g$^{-1}$ h$^{-1}$.

Intramuscular NE injections were given as a bolus (0.4 mg kg$^{-1}$ as described by Song et al. (1996), Ye et al. (1995, 1996) and Hosoda et al. (1999)). Baseline basal metabolic rate (BMR) with a sham injection of sterile 0.9% saline and (VO$_2$) response to NE were recorded at the commencement of the study. Recording of O$_2$ consumption was commenced immediately upon resealing of the experimental chamber. However, S. harrisii are excitable and aggressive animals and required approximately 10–15 min post-injection in order to settle and avoid handling stress influences on (VO$_2$) calculations, while still capturing the effects of the NE injection. In eutherians peak (VO$_2$) response of NE has been reported to occur between 10 and 20 min, and lasts at least 5–10 min (Heldmaier, 1971; Feist and Rosenmann, 1976; Richardson et al., 1994). However, in marsupials this technique has also been shown to elicit a peak (VO$_2$) response to NE that can last up to 30 min post-injection (Ye et al., 1995, 1996; Clements et al., 1998; Rose et al., 1998).

All S. harrisii and three of the rats were subsequently exposed to temperatures of 3–5°C for 2 weeks with a 12L:12D light cycle. The remaining three rats were housed as previously described above for non-cold exposure. Two weeks has been shown to elicit non-shivering thermogenic responses in other mammals including marsupials (Cousin and Bowler, 1987; May, 1996; Wunder and Gettinger, 1996; Rose et al., 1999). This temperature is approximately the mean minimum for the area from which these S. harrisii were trapped as reported by the Australian Bureau of Meteorology. Post-cold exposure, the S. harrisii’s sham-injection and NE-elicited (VO$_2$) responses were again measured.

2.3. Surgical procedures

Although complete dissection of roadkilled S. harrisii presented with no samples that grossly appeared to be BAT under unstained microscopic evaluation (unpublished data). This region was determined to be the most likely candidate to possess BAT, as it has been identified from samples taken from this region in Sminthopsis crassicaudata (Hope et al., 1997) another dasyurid marsupial. Before and after cold-acclimation, two small tissue biopsies were taken from each of the S. harrisii to determine the expression of UCP1. Each animal was given an intramuscular injection of Ketamine hydrochloride (5 mg kg$^{-1}$) followed by isofluorane and oxygen gas. A 1 cm incision was cut across the left side of the interscapular region through the epidermal layer, and approximately 1 g samples of both fat and muscle were excised. Each biopsy was flash frozen in liquid nitrogen and stored at −80°C until processing. S. harrisii were given a 0.1 ml bolus injection of the antibiotic Moxylan (Jurox Pty. Ltd., Australia) directly after surgery and were closely monitored for several days until the incisions were fused and clear of infection. Animals were allowed to recover for 1 week after surgery prior to the commencement of cold exposure. The above surgery was repeated after cold exposure using the right side of the animal. The animals were monitored for 1 week after the final experiment and re-released at site of capture. The rats were euthanased by an overdose of pentobarbital and BAT, muscle, and liver tissue samples were harvested for control analysis of UCP1 expression.

2.4. Western blot

Western blotting was used to detect expression of UCP1 in S. harrisii. Muscle and adipose tissues from S. harrisii, non-cold exposed rats (negative control, n = 3) and the cold-acclimated rats (positive control, n = 3) were isolated using a modified technique outlined by Yoshida et al. (1998). In brief, tissues were homogenised in 10 volumes of 10 mM Tris(hydroxymethyl)aminomethane HCL and 1 mM EDTA (pH 7.4) for 30 s. Samples were centrifuged at 1200g for 10 min at 4°C, the supernatant was decanted and saved, and the pellet was resuspended and centrifuged as above. The two supernatants were combined and centrifuged at 9000g for 10 min at 4°C. The resulting supernatant was discarded and the pellet was again resuspended and centrifuged at 100,000g for 90 min at 4°C. The resulting pellet was resuspended and used for Western blotting. The Bradford assay was used to assay protein concentration. Samples were diluted and denatured, then approximately 10 μg of protein per lane were loaded and run on a 4–20% Tri-glycine gel in a Novex mini-cell$^{	ext{®}}$ at 120 V for 90 min, and subsequently transferred to a PVDF membrane. The membrane was blocked with 5% skim milk powder in 1M PBS-Tween for 5–6 h. Samples were incubated with UCP1 antibody (Research Diagnostic Inc., USA) at a concentration of 1:500 in PBS-Tween overnight at 4°C, and labelled by chemiluminescence via a rabbit anti-goat secondary antibody for 1.5 h and exposed to autoradiography film. This technique was validated using non-cold-acclimated and cold-acclimated rat tissues that have been identified in the literature to express UCP1 (Carneheim et al., 1988; Scarpace et al., 1998). In support of this approach, the use of Western blotting as a means of identifying UCP1 expression using a similar technique and antibody has been shown to produce positive results in another dasyurid marsupial (Hope et al., 1997).
2.5. Reverse transcriptase—polymerase chain reaction

RNA was extracted from the tissues as described by Beattie et al. (2000). The resulting mRNA was used in a one-step RT-PCR as described by Genelamp®. Synthetic oligonucleotides were designed to conserved regions of several species of mammals (humans, rats, mice) known to possess UCP1. This was accomplished using the CloneTech® Primer Detective V1.01 and ClustalW Multiple Sequence Alignment programs (courtesy of the BCM Search Launcher). Consensus primers were obtained from Sigma® Genosys, Australia (Sense: AACTGTACAGCGGTCTGCCT; Antisense: ATCC-GAGTCGCAGAAAAGAA). Thermal cycling was performed using a Corbett Research CR-PC960 Cycler. All cycling involved 35 cycles of denaturation at 95°C (1 min), annealing at 54°C (1 min) and extension at 72°C (1 min). Cycling finished with a final extension time of 10 min at 72°C followed by a rapid ramp to a 4°C hold. The RT-PCR reactions were visualized by gel electrophoresis containing ethidium bromide. For comparison, rat tissues known to express UCP1, and several species of mammals (humans, rats, mice) known to possess UCP1. This was accomplished using the CloneTech® Primer Detective V1.01 and ClustalW Multiple Sequence Alignment programs (courtesy of the BCM Search Launcher). Consensus primers were obtained from Sigma® Genosys, Australia (Sense: AACTGTACAGCGGTCTGCCT; Antisense: ATCC-GAGTCGCAGAAAAGAA). Thermal cycling was performed using a Corbett Research CR-PC960 Cycler. All cycling involved 35 cycles of denaturation at 95°C (1 min), annealing at 54°C (1 min) and extension at 72°C (1 min). Cycling finished with a final extension time of 10 min at 72°C followed by a rapid ramp to a 4°C hold. The RT-PCR reactions were visualized by gel electrophoresis containing ethidium bromide. For comparison, rat tissues known to express UCP1, and β-actin primers were used with all mRNA samples to demonstrate positive RT-PCR. RT-PCR products were sequenced to ensure amplification of the desired cDNA.

2.6. Statistical analysis

All data analyses were performed using the statistical package SPSS for Windows® 10.0.1. Data are presented as mean ± standard error (SE). Student’s t-test or repeated-measures ANCOVA were used to determine significance, where P < 0.05 was regarded as significant. Fisher’s PSLD post hoc tests were used to identify differences within groups.

3. Results

3.1. Metabolic rates

An investigation of body condition before and after cold-acclimation found that during cold exposure, all S. harrisii lost a small but significant amount of mass (Table 1; Student’s t-test; P < 0.001, n = 5). Although there was a mean loss of approximately 8% of mass, this had no impact on the level of significance when the metabolic rate data were analysed using either the mass-specific or total-oxygen consumption rates. Therefore only the results of mass-specific metabolic rates are presented. There were significant changes in metabolic rates (ANOVA: F1,4 = 31.04; P = 0.0014) after cold-exposure alone and cold-exposure with injection of NE as shown by Table 1. The post hoc tests showed that before cold-exposure there were no significant differences in the metabolic rate of these animals with or without an injection of NE. However, a significant increase in metabolic rate was found after these animals acclimated to the cold. After cold exposure and when injected with NE the metabolic rates were significantly increased from baseline by approximately 29%. The metabolic response to NE is increased as the animal acclimates to a cold environment, thus suggesting that S. harrisii exhibits NST ability.

3.2. Molecular analysis

Western blotting was used to detect the expression of and possible fluctuations in the regulation of UCP1 in relation to cold-acclimation in the adipose tissue of S. harrisii. Tissues examined were adipose tissue and skeletal muscle. As a comparison, control and cold-acclimated rat tissues were examined. Antisera against UCP1 revealed a band at ~35 kDa in cold-acclimated rat BAT, but no immunoreactivity was observed in any S. harrisii tissue or rat skeletal muscle (Fig. 1). The data in Fig. 1 suggest that S. harrisii is not capable of expressing proteins that are immunoreactively similar to eutherian UCP1. An RT-PCR approach was used to further support the suggestion that S. harrisii does not express UCP1 by investigating whether UCP1 mRNA is present in S. harrisii tissues. Fig. 2 shows a typical result from a RT-PCR experiment. There were no UCP1-derived PCR products in the S. harrisii adipose or muscle tissues, although positive (rat BAT) and negative (rat muscle tissue) control tissues

<table>
<thead>
<tr>
<th></th>
<th>Mean weight (kg)</th>
<th>Mean metabolic rate (ml g⁻¹ h⁻¹)</th>
<th>Mean change in MR from baseline (%)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (before cold exposure)</td>
<td>7.57 ± 0.82</td>
<td>0.29 ± 0.02</td>
<td>—</td>
<td>A</td>
</tr>
<tr>
<td>Baseline + NE</td>
<td>—</td>
<td>0.31 ± 0.01</td>
<td>6.4 ± 0.04</td>
<td>AB</td>
</tr>
<tr>
<td>Cold-acclimated</td>
<td>6.89 ± 0.62</td>
<td>0.33 ± 0.03</td>
<td>11.1 ± 0.01</td>
<td>B</td>
</tr>
<tr>
<td>Cold-acclimated + NE</td>
<td>—</td>
<td>0.41 ± 0.04</td>
<td>29.26 ± 0.04</td>
<td>C</td>
</tr>
</tbody>
</table>

Rows with different letters are significantly different from each other (Fisher’s post hoc tests).
increase in MR seen in *S. harrisii* is more accurately a large increase considering its body mass is near the end of Heldmaier's (1972) 10 kg body weight cut-off point above which NST ability is no longer seen. There also may be several factors that allow this species to produce or conserve body heat, making NST ability less important. Allen's rule describes how an animal can alter its surface area to body size ratio by altering the shape and size of extremities, rather than overall body size. *S. harrisii* is a stocky animal with short legs and a fat tail, thus by Allen's rule it could be assumed that it has a relatively low surface area to volume ratio. This would decrease the amount of heat lost, and therefore decrease the obligation to thermoregulate rigorously. Green (1967) reported that *S. harrisii* have a thick subcutaneous adipose tissue layer. During the post-cold acclimation surgery in the present study, it was observed that the subcutaneous adipose tissue layer was substantially thicker than prior to cold-acclimation. This may be analogous to the blubber layer used as insulation by many cold-environment animals in order to minimise the loss of core temperature (Kvadsheim and Folkow, 1997; Rosen and Renouf, 1997). This does not, however, explain the loss of body mass seen during the cold acclimation in this experiment. It is likely that the decrease in body mass is due, impart, to muscle catabolism providing the energy needed for thermoregulation, including shivering. All of these adaptations may be the result of the variable Tasmanian climate where snow can occur at anytime of the year, making it necessary to be able to rapidly shift thermal regulating processes.

These data also show that the NST response seen is apparently not related to the presence of UCP1, as neither the putative marsupial UCP1 protein nor mRNA has been characterised at the molecular level. Although it is possible that the molecular techniques employed here (both designed using eutherian UCP1s) were simply unable to pick up marsupial UCP1, we believe this not to be the case. It has been shown that immunological techniques using UCP1 antibodies will positively identify marsupial UCP1 (Hope et al., 1997). Kabat et al. (2003a) have identified uncoupling protein 2 (UCP2) and uncoupling protein 3 (UCP3) at both the cDNA level and immunoreactively at the protein level, and found that marsupial UCP2 and UCP3 have an approximately 74% and 65%, respectively, homology with their eutherian counterparts (Kabat et al., 2003a). Therefore it stands to reason that UCP1 would be highly conserved as well if it were expressed. However, without a major study at the genomic level, it is acknowledged that it can be difficult to rule out the existence of a specific gene in any species.

Ye et al. (1996) and Rose et al. (1999) also suggested that because *B. gaimardi* does not possess BAT or UCP1 but responded to both norepinephrine and BRL 37344,
another mechanism of NST must exist, and postulated that NST may occur in the skeletal muscle. It may be profitable to consider UCP2 and UCP3, both of which (in eutherians) show high homology to the genetic structures of UCP1, and any involvement these may have in marsupial NST. UCP2 is expressed in white adipose tissue, smooth muscle, skeletal muscle, and liver (Fleury et al., 1997), whereas UCP3 is found only in BAT and skeletal muscle (Boss et al., 1997; Gimeno et al., 1997).

These proteins have been shown to uncouple mitochondrial respiration and can be thermogenic when ectopically expressed (Fleury et al., 1997; Gong et al., 1997; Boss et al., 1998), there is no evidence for a role in thermogenesis. UCP2 (Millet et al., 1997) and UCP3 (Boss et al., 1998) in eutherians are regulated by nutritional state and free fatty acids and not by changes in environmental temperature. The exact physiological roles of these proteins remain to be determined, however they are thought to play a role in regulation of reactive oxygen species (ROS; Negre-Salvayer et al., 1997; Arsenijevic et al., 2000) and maintenance of intracellular ATP/ADP ratios (Zhang et al., 2001). There is some evidence that UCP2 expression and upregulation in muscle is temporally associated with the development of endothermy in pouch young macropods (Bettonia gaimardi, Kabat et al., 2003b; Macroopus eugenii R. Baudinette, unpublished data), although the role is unknown. There have been numerous studies that have shown that skeletal muscle contributes a considerable amount to the resting metabolic rate and heat production in most vertebrates (e.g. Jansky, 1995). There have also been several studies that have shown that NE substantially increases (VO\textsubscript{2}) in isolated perfused muscle preparations in many species (rat, Masakazu and Masumi, 1988; Clark et al., 1995; Jansky, 1995; Ye et al., 1995; chicken, Eldershaw et al., 1997; bettong, Ye et al., 1996). However, the roles of these proteins in NST are still the subject of much controversy. Because of this, we believe that it is worthwhile to investigate what further role, if any, these proteins play in thermogenesis.

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References


Green, R.H., 1967. Notes on the Devil (Sarcophilus harrisii) and the Quoll (Dasyurus viverrinus) in northeastern Tasmania. Rec. Queen Victoria Mus. 7, 1–12.


