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Shivering, muscle tone, and uncoupling proteins in a developing marsupial, the Tasmanian bettong (*Bettongia gaimardi*)

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Abstract

This study examined the development of several physiological parameters that enable the development of endothermy during pouch life of the Tasmanian bettong (*Bettongia gaimardi*). By using several key age groups, we investigated the electromyography response of pouch young under different thermal conditions (35 or 20 °C), with and without injection of the β-agonist norepinephrine. We also used molecular techniques to examine the possible expression of uncoupling proteins 1, 2, and 3 (UCP1, 2, and 3), and if they were expressed, any association these may have with the timing of endothermic development. AT 6 weeks of age, pouch young were unable to thermoregulate via shivering or non-shivering mean, maintain a constant metabolic rate, or show any response to the β-agonist injection. When the animals were exposed to a cold stress (20 °C), 8-week-old pouch young initially shivered for 2–3 min before tapering off completely, causing body temperature to sharply decline. During the 10th week, cold-exposed pouch young began shivering, with the bout lasting approximately 10 min before ceasing and body temperature beginning to decline. It was also at this age that the expression of UCP2 was initially expressed. By the 12th week, cold exposed pouch young initially employed classical shivering; however, after approximately 12 min, this was replaced with an increase in muscular tone. This increase in muscular tone was also recorded in response to β-agonist injection at thermoneutral this age and was associated with an increase in metabolic rate. Also by this age, total body fat increased by approximately 300% from the levels expressed at 6 weeks of age and UCP2 was significantly upregulated. Although pouch young *B. gaimardi* did not show UCP1 expression at any time, UCP3 was expressed at every age investigated from 6 weeks of age. This study proposes that pouch young *B. gaimardi* use a mechanism of increased muscle tone as a source of heat production, and shows that UCP2 has an association with the onset of thermogenesis.

Keywords: Marsupial; Endothermy; Development; Uncoupling proteins; Muscle tone; Shivering; *Bettongia gaimardi*

1. Introduction

Endotherms, when exposed to cold temperatures, have two major physiological means with which to sustain core body temperature: shivering thermogenesis and nonshivering thermogenesis (NST). Shivering is a result of rapid contractions of striated muscle, producing heat by chemomechanical means. The skeletal muscle oscillates around a middle position without change in body or joint position. Heat is the by-product of any muscle contraction, both at the cellular level, due to the inefficiency of biochemical pathways, and at the microfibril level; mechanical work always produces friction and thus heat (Brimblecombe and Pinder, 1972). Shivering exhibits asymmetrical and asynchronous time distributions, characterised by a burst phase during which distinct muscle activity is recorded, and distinct ‘quiescence’ phases when electromyography (EMG) tracings return to baseline (Brimblecombe and Pinder, 1972). It has been suggested that, in vertebrates, the maximum skeletal muscle contraction efficiency is approximately 28–35%, suggesting that up to 72% of the energy consumed during muscle contraction is lost as heat (Alexander, 1977). Perhaps not surprisingly, shivering has evolved as the main thermogenic mechanism in both

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endothermic vertebrate groups, birds and mammals (Hohtola, 2002).

In many smaller mammals, an extended duration of cold stress will cause shivering to be progressively replaced with nonshivering thermogenesis as the animal acclimates to the cold (Foster and Frydman, 1979; Nicol et al., 1997; Rose et al., 1999). NST has been reported to be performed solely by means of brown adipose tissue (BAT) (Golozoubova et al., 2001). It is facilitated by norepinephrine (NE) released from the sympathetic system, causing the inner mitochondrial membrane potential to be lowered via the uncoupling of respiration from oxidative phosphorylation, thereby dissipating the proton potential gradient across the inner mitochondrial membrane in the form of heat. This is performed through a specialised protein channel, uncoupling protein 1 (UCP1) (Cannon and Nedergaard, 1985). Thermogenesis executed by BAT has been estimated to be responsible for up to 60% of the heat produced by cold-acclimated rats (Foster and Frydman, 1979).

In 1997, two other proteins, uncoupling protein 2 (UCP2) and uncoupling protein 3 (UCP3), were identified and were initially thought to uncouple mitochondria, predominantly due to their high homology with UCP1 (Boss et al., 1997; Fleury et al., 1997). Although it has been demonstrated that UCP2 can uncouple mitochondria and is thermogenic when ectopically expressed, this has not been shown en vivo (Fleury et al., 1997; Gong et al., 1997). To date, the physiological function of UCP2 and UCP3 remains a contentious issue (Skulachev, 2000). However, both UCP2 and UCP3 in muscle have been associated with controlling whole body metabolism (Schrauwen and Hesselink, 2002).

In comparison to many eutherians, marsupials are born at a rudimentary stage of development and *en utero* development duration is relatively short. Marsupials are born naked and ectothermic (Loudon et al., 1985; Gemmell et al., 1987; Rose, 1987; Hulbert, 1988; Rose et al., 1998). The ability to metabolically thermoregulate gradually develops while in the pouch (Loudon et al., 1985; Rose et al., 1998). Loudon et al. (1985) reported a critical age, approximately 12 weeks of pouch life, at which pouch young *Macropus rufogriseus* were able to respond to an injection of NE by increasing their resting oxygen consumption rate, thereby suggested the maturity of endothermy. The presence of NST in adult *B. gaimardi* is well documented (Ye et al., 1995, 1996; Rose et al., 1998; Rose et al., 1999), although it remains unclear how NST is performed in the absence of BAT. Rose et al. (1998) reported that at approximately 12 weeks of age, *B. gaimardi* pouch young begin to show an increase in metabolic rate in response to cold and NE injection. This work also documented the timing and extent of the pouch young's ability to perform NST.

The presence or absence of BAT mediated nonshivering thermogenesis in marsupials has been the subject of much controversy. Despite numerous attempts, establishing the existence of BAT and/or UCP1 in marsupials has been elusive and hence contentious. 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 adult *Sarcophilus crassicaudata*. However, the quantity of BAT that Hope et al. (1997) identified is unlikely, due to the small amount present, to have any whole animal thermogenic use (C. Daniels pers. comm.). Rose et al. (1998) demonstrated the absence of both BAT and UCP1 expression in cold-acclimated adult *B. gaimardi*. That study suggested that adult *B. gaimardi* demonstrated an increase in oxygen consumption rate in response to β-agonists that was not associated with the presence of UCP1. Kabat et al. (2003a) confirmed these results, and also showed that adult *B. gaimardi* expressed both UCP2 and UCP3. It has also been shown that *Sarcophilus harrisii* expresses UCP2 but not UCP3 (Kabat et al., 2004). However, Schaeffer et al. (2005) have shown the expression and upregulation of UCP3 in cold-exposed *Monodelphis domestica*; whereas *Antechinus flavipes* has been shown to express UCP3, but cold adaptation has no effect on regulation levels (Jastroch et al., 2004).

There are many species of mammals that possess UCP1 and/or BAT during the initial stages of life but quickly lose it with age (e.g. Harp seals, Blix et al.1979; Humans, Solomon et al., 1999). It is thus important to rule out the possibility that BAT and UCP1 exist in pouch young marsupials, and that it is not lost with age as seen in other mammals. It is also necessary to use more definitive molecular techniques, rather than gross morphology and light microscopy to identify these proteins. Studies on NST in marsupials by Clark et al. (1995), Ye et al. (1995, 1996), and Rose et al. (1999) have suggested that striated muscle and its vasculature may be the major source of heat production in marsupials that lack BAT and/or UCP1. Rose et al. (1998) also hypothesised that UCP2 and UCP3 may play an important role in thermogenesis in marsupials. Thus it is important to investigate these claims to determine the source of heat production.

This study examined the timing and possible expression of uncoupling proteins 1, 2 and 3 in pouch young *B. gaimardi*, and attempted to temporally correlate these with the development of endothermy.

2. Methods

2.1. Husbandry

B. gaimardi were acquired from the University of Tasmania's School of Zoology breeding colony. Husbandry and housing of *B. gaimardi* are described in detail by Rose (1982) and Kabat et al. (2003a). Age estimations of the pouch young were calculated using equations formulated by Rose (1985). Two pouch young from each of five age groups (6, 8, 10, 12 and 14 weeks) were used for examining the physiological and molecular aspects of this experiment. Six adult and three juvenile male hooded