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RESEARCH ARTICLE

The high aerobic capacity of a small, marsupial rat-kangaroo (*Bettongia penicillata*) is matched by the mitochondrial and capillary morphology of its skeletal muscles

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SUMMARY

We examined the structure–function relationships that underlie the aerobic capacities of marsupial mammals that hop. Marsupials have relatively low basal metabolic rates (BMR) and historically were seen as 'low energy' mammals. However, the red kangaroo, *Macropus rufus* (family Macropodidae), has aerobic capacities equivalent to athletic placentals. It has an extreme aerobic scope (fAS) and its large locomotor muscles feature high mitochondrial and capillary volumes. *M. rufus* belongs to a modern group of kangaroos and its high fAS is not general for marsupials. However, other hopping marsupials may have elevated aerobic capacities. *Bettongia penicillata*, a rat-kangaroo (family Potoroidae), is a small (1 kg), active hopper whose fAS is somewhat elevated. We examined the oxygen delivery system in its muscles to ascertain links with hopping. An elevated fAS of 23 provided a relatively high maximal aerobic oxygen consumption ($\dot{V}_{O_2,max}$) in *B. penicillata*; associated with this is a skeletal muscle mass of 44% of body mass. Ten muscles were sampled to estimate the total mitochondrial and capillary volume of the locomotor muscles. Values in *B. penicillata* were similar to those in *M. rufus* and in athletic placentals. This small hopper had high muscle mitochondrial volume densities (7.1–11.9%) and both a large total capillary volume (6 ml kg^{-1} body mass) and total capillary erythrocyte volume (3.2 ml kg^{-1}). Apparently, a considerable aerobic capacity is required to achieve the benefits of the extended stride in fast hopping. Of note, the ratio of $\dot{V}_{O_2,max}$ to total muscle mitochondrial volume in *B. penicillata* was 4.9 ml O_2 min^{-1} ml^{-1}. Similar values occur in *M. rufus* and also placental mammals generally, not only athletic species. If such relationships occur in other marsupials, a fundamental structure–function relationship for oxygen delivery to muscles likely originated with or before the earliest mammals.

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Key words: kangaroo, rat-kangaroo, *Bettongia penicillata*, *Macropus rufus*, aerobic capacity, mitochondria, capillarisation, hopping, marsupial.

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INTRODUCTION

This investigation focuses on the functional relationships of oxygen delivery that underlie the aerobic capacities of marsupial mammals, notably of those that hop, such as kangaroos. It aims to put these into perspective relative to the oxygen transport characteristics of the placental mammals. Marsupials and placentals together comprise the advanced mammals (Theria). While they have many features in common, significant differences have occurred over their long evolutionary history; the two groups diverged about 148 million years ago (Bininda-Emonds et al., 2007). Reproductive features are a notable difference, but energetic differences also exist, as indicated by the relatively low basal metabolism of marsupials. However, some marsupials, such as the hopping red kangaroo, *Macropus rufus* (Dawson et al., 2004), can achieve high and sustained energy outputs that are comparable with those of athletic placental mammals. Do the energetic features of the red kangaroo occur among groups of marsupials?

Use of bipedal hopping for high-speed locomotion is uncommon among mammals. For mammals exceeding 1 kg in body mass, it is rare and is largely restricted to marsupials of the monophyletic suborder Macropodiformes (kangaroos, wallabies and rat-kangaroos), the ancestral form of which is posited as a small, leaping, arboreal marsupial (Dawson, 2012). The energy *versus* speed relationships during hopping differ from those of running mammals, and the relative costs of fast hopping in the macropodiforms are lower than those for runners generally (Dawson and Taylor, 1973; Webster and Dawson, 2003; Dawson and Webster, 2010). Kangaroos and their relatives, like other marsupials, have low basal metabolic rates (BMR) relative to those of most placental mammals (Martin, 1902; Dawson and Hulbert, 1970; Withers et al., 2006) and, from the early studies, marsupials gained a reputation as 'low energy' mammals. The strength of this paradigm was such that when the unusual energetic characteristics of the hopping of *M. rufus* were uncovered (Dawson and Taylor, 1973) they were seen to represent a strategy to overcome metabolic limitations (Dawson, 1977). However, from further work on *M. rufus*, this was seen not to be true; *via* a large factorial aerobic scope (fAS) of 54, it achieves a maximal aerobic oxygen consumption ($\dot{V}_{O_2,max}$) comparable to that of athletic placentals (Kram and Dawson, 1998; Dawson et al., 2003). Studies point to other marsupials, including quadrupedal species, having similar $\dot{V}_{O_2,max}$ to some placentals, due to expanded aerobic scopes (Dawson and Dawson, 1982; Hinds et al., 1993), but their fAS values do not approach those of *M. rufus*.

To initially examine these relationships in marsupials, Dawson et al. studied the underlying basis of the extreme fAS of *M. rufus* (Dawson et al., 2004). They followed the techniques that were

developed in the studies of the design of the mammalian (placental) respiratory system initiated by Taylor and Weibel (Taylor and Weibel, 1981) and summarized by Weibel et al. (Weibel et al., 2004). Weibel et al. had found that body size dependent, or allometric, variations in aerobic capacities ($\dot{V}_{O2,max}$) of animals were strongly correlated with variation in structural and functional aspects of the cardio-respiratory system, such as pulmonary diffusing capacity and capillary and mitochondria densities in locomotor muscles (Weibel et al., 2004). In animals of the same size, the $\dot{V}_{O2,max}$ of more athletic species was tightly linked to proportionally larger total mitochondrial and capillary erythrocyte volumes in these muscles. With *M. rufus*, the detailed studies of the morphometry of a full complement of skeletal muscles (Dawson et al., 2004) showed a full match with the features seen in placental mammals. In fact, despite its locomotion and extreme body form, *M. rufus* utilises structure–function relationships for oxygen delivery to the locomotor muscles that are essentially identical to those in comparably sized athletic placentals such as dogs and pronghorns (Dawson et al., 2004).

While marsupials may have aerobic potentials similar to those of some placentals, their usually reported fAS values are a quarter of those of *M. rufus* (Dawson and Dawson, 1982; Hinds et al., 1993). Are the expanded aerobic/muscular characteristics of *M. rufus* only recently derived and evolutionarily convergent with large, athletic forms within placental groups? It is a macropodine kangaroo and these are an evolutionarily recent (Late Pliocene/Pleistocene) and successful group of fast-hopping, specialist grazers (Meredith et al., 2008). However, it is possible that the aerobic/muscular characteristics of *M. rufus* are more broadly spread, at least among the Macropodiformes. A rat-kangaroo, the brush-tailed bettong (*Bettongia penicillata*), is an active hopper and has a somewhat elevated fAS of about 23 (Webster and Dawson, 2003). Seeherman et al. measured its $\dot{V}_{O2,max}$ (Seeherman et al., 1981) but the significance of the data was initially overlooked when *B. penicillata* was treated as a placental. It belongs to a more conservative macropodiform group: the family Potoroidae. The family of the modern large kangaroos, the Macropodidae, diverged from the Potoroidae some 25–30 million years ago (Meredith et al., 2008). The fAS of *B. penicillata* is markedly lower than that of *M. rufus*, and this may reflect its aerobic abilities. However, *B. penicillata* is small, only 1 kg, and Weibel et al. have shown that fAS decreases considerably with decreasing body size, even among athletic placentals (Weibel et al., 2004). Our examination of the structure–function relationships for oxygen delivery to the locomotor muscles of *B. penicillata* will not only clarify the energetic capabilities among marsupials but will also provide significant insights into the overall evolution of high energetic capabilities in mammals generally.

MATERIALS AND METHODS

Animals

The brush-tailed bettong or woylie (*Bettongia penicillata* Gray 1837) is a small, hopping marsupial from the family Potoroidae. The Potoroidae have an extensive fossil history in Australia but the modern fauna comprises less than a dozen species. They are noted as being mycophagus, i.e. eating the underground fruiting bodies of fungi, but are also herbivorous and insectivorous (Claridge et al., 2007). *Bettongia penicillata* was once common across southern Australia but is now restricted to a few sites in Western Australia (Christensen, 1995); habitat destruction, coupled with predation by the introduced red fox, being implicated in their decline. The animals used in the present study were from a captive breeding colony housed at the University of New South Wales Field Station at Cowan, north of Sydney. Four healthy, mature males (surplus to breeding needs) were selected for this study. These individuals had been used for a previous study of hopping locomotion energetics and aerobic capacity (Webster and Dawson, 2003).

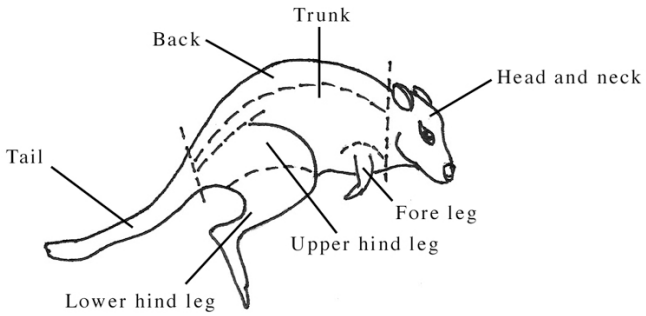

Fig. 1. Outline of a brush-tailed bettong, *Bettongia penicillata*, showing the regions from which muscles were sampled.

All animals were kept in indoor pens and maintained on a regular treadmill exercise regimen for 4 weeks prior to death. The bettongs were sedated with diazepam and weighed to the nearest 10 g, then administered intramuscularly with a lethal overdose of ketamine/xylazine anaesthetic. Death occurred within 15 min of administration of the overdose. All procedures were approved by the University of New South Wales Animal Care and Ethics Committee (project number 00/67).

Muscle sample collection and preparation

To assess the mitochondrial and capillary characteristics of the skeletal muscle of the whole body, we followed the sampling procedure of Dawson et al. (Dawson et al., 2004) for *M. rufus* [comparable to that of Hoppeler et al. (Hoppeler et al., 1984)]. The musculature of *B. penicillata* was divided into seven functional units: head and neck, fore leg, trunk, back, upper hind leg, lower hind leg and tail (Fig. 1). By sampling from muscles from each region, the mitochondrial and capillary characteristics of the skeletal muscle of the regions, and hence the whole body, were determined. Dawson et al. selected muscles randomly from each region and we followed their selection procedure (Dawson et al., 2004). Data were also collected from the diaphragm. The muscles sampled (including the diaphragm) represented 17.5% of the total musculature. A concurrent study of the heart was also undertaken (Dawson et al., 2003).

For each animal, the side of the body (left or right) to be sampled was selected by coin toss. On this side, two sample blocks (about 1–2 mm^3) for electron microscopy were cut from random locations within each muscle and placed immediately into a fixative solution of 2.5% glutaraldehyde in 0.1 $mol\,l^{-1}$ sodium cacodylate buffer (pH 7.4), then kept refrigerated at 4°C prior to preparation for electron microscopy. The opposite side of the body was used to determine total masses of skeletal muscle for each body region. Muscle sampling for electron microscopy was completed within 2–3 h of death, as recommended by Hoppeler et al. (Hoppeler et al., 1981).

Preparation for electron microscopy

Where required, sample blocks were trimmed into smaller pieces. Blocks were prepared for electron microscopy using the method of Dawson et al. (Dawson et al., 2004), ultimately being embedded into Spurr's epoxy resin (slow cure) over a long infiltration period (3–4 days) and cured at 60°C for 48 h.

A Reichert-Jung Ultracut ultramicrotome (Leica Microsystems, Vienna, Austria) was used to section the embedded samples. Five

to 10 ultra-thin sections showing silver (60–90 nm) or gold (90–150 nm) interference colours were cut from each sample and mounted on 200 mesh copper grids. Grid-mounted sections were stained with 2% uranyl acetate in 50% ethanol for 10 min and then rinsed in distilled water. Because sections were to be used for estimating capillary tortuosity (see below), both transverse and longitudinal sections were taken from each muscle (from differently oriented blocks).

Mitochondrial volume

Grids were viewed at a magnification of 12,000× using a Hitachi 7000 (Tokyo, Japan) or JEOL 1400 (Tokyo, Japan) transmission electron microscope (TEM). For each sample block, 10 grid squares were selected using a systematic random sampling method (Howard and Reed, 1998). Digital photographs were taken in the top left corner of the grid squares using an Olympus SQ (Tokyo, Japan) digital camera and software package AnalySIS (attached to the Hitachi 7000 TEM) or a Gatan (Pleasanton, CA, USA) digital camera and software package Gatan Digital Micrograph (attached to the JEOL 1400). For each animal, 20 digital images were obtained per muscle (10 images × two blocks).

Mitochondrial volumes were determined using the method of Dawson et al. (Dawson et al., 2004). Briefly, digital images were imported into the image software Adobe Photoshop (Adobe Systems, San Jose, CA, USA), and a human operator selected the perimeters of all mitochondria and then converted mitochondrial areas to black and the remainder of the image area to white. The total percentage area covered by the mitochondria [mitochondrial area fraction, which is equivalent to the mitochondrial volume fraction, $V\mathrm{v}(\mathrm{mt,f})$] was estimated using image-processing software. In the present study, some images were processed using an image processing plug-in to Adobe Photoshop (as used by Dawson et al., 2004), while others were processed using the public-domain software ImageJ (US National Institutes of Health, Bethesda, MA, USA). The total mitochondrial volume $V(\mathrm{mt,m})$ for each muscle (in ml) was calculated from:

$$V(\mathrm{mt,m}) = M_\mathrm{m} \times V\mathrm{v}(\mathrm{mt,f}) \times V\mathrm{v}(\mathrm{f,m}) \times d^{-1}, \quad (1)$$

where M_m is muscle mass, $V\mathrm{v}(\mathrm{mt,f})$ is the volume fraction of mitochondria, $V\mathrm{v}(\mathrm{f,m})$ is the volume fraction of muscle occupied by muscle fibres, and d is the density of the muscle. A muscle density of 1.06 g ml^{-1} was used (Mendez and Keys, 1960) and it was assumed that $V\mathrm{v}(\mathrm{f,m})$ was equal to 1 (Hoppeler et al., 1987).

Surface density of the inner mitochondrial membranes

The surface density of the inner mitochondrial membranes was estimated in the m. multifidi lumborum using the same method as Dawson et al. (Dawson et al., 2004). Twenty mitochondria were examined at a magnification of 30,000× or 40,000× so that the inner membranes could be seen clearly. The surface density of inner mitochondrial membranes per unit volume of mitochondria, $S\mathrm{v}(\mathrm{im,mt})$, was calculated as m^2 cm^{-3} using equation 6.4 of Howard and Reed (Howard and Reed, 1998). An overall estimation of the total surface area of inner membranes in each multifidi lumborum muscle was given by:

$$S(\mathrm{im,m}) = V(\mathrm{mt,m}) \times S\mathrm{v}(\mathrm{im,mt}). \quad (2)$$

Capillary length and volume

Both transverse and longitudinal sections were sliced from muscles and were used to estimate the tortuosity factor, c(K,0), of the capillary network, using the shortcut estimation method of Mathieu et al. (Mathieu et al., 1983). The tortuosity factor was determined for all of the 10 muscles sampled (diaphragm, m. masseter, m. triceps, m. erector spinae, m. multifidi lumborum, m. vastus lateralis, m. semitendinosus, m. biceps femoris, m. gastrocnemius and m. coccygeus). The mean tortuosity factor was c(K,0)=1.46±0.08 (±s.e.m.) for N=10 muscles but it varied considerably between muscles from c(K,0)=1.10 in the diaphragm to c(K,0)=2.00 in the m. multifidi lumborum. We therefore used the appropriate tortuosity factor for each muscle in calculations of capillary length density.

In all other respects, the methods for determining capillary characteristics were the same as those of Dawson et al. (Dawson et al., 2004). Ten digital micrographs per muscle (from transverse sections) were used to estimate the number of capillaries per unit area [numerical capillary density, $N_\mathrm{A}(\mathrm{c,f})$, in mm^{-2}], and capillary length density (in mm per unit volume of muscle) was calculated from $N_\mathrm{A}(\mathrm{c,f})$ according to:

$$J\mathrm{v}(\mathrm{c,f}) = \mathrm{c(K,0)} \times N_\mathrm{A}(\mathrm{c,f}). \quad (3)$$

The total capillary length in km per muscle was then calculated from:

$$J(\mathrm{c}) = J\mathrm{v}(\mathrm{c,f}) \times M_\mathrm{m} \times V\mathrm{v}(\mathrm{f,m}) \times d^{-1}. \quad (4)$$

The mean capillary radius, r_c, was determined from capillary cross-sectional areas [A(c)] according to:

$$r_\mathrm{c} = [A(\mathrm{c}) / \pi]^{1/2}, \quad (5)$$

and this was used to calculate the capillary volume:

$$V(\mathrm{c}) = \pi \times r_\mathrm{c}^2 \times J(\mathrm{c}). \quad (6)$$

Statistical methods

Comparisons between muscles were analysed using one-way ANOVAs (using SPSS 16.0, IBM, Armonk, NY, USA). A Student–Newman–Keuls (SNK) multiple range test was applied when significant differences were indicated by the ANOVA. Correlation analyses were also performed using SPSS 16.0. Figures and tables show means ± s.e.m.

RESULTS

The mean body mass (M_b) of the four male *B. penicillata* was 1000±20 g, of which 436±14 g (or 43.6%) was skeletal muscle mass. The mitochondrial and capillary characteristics of *B. penicillata* locomotor muscles are shown in Table 1. These muscles were representative of the body regions depicted in Fig. 1. In the random selection of muscles, the masseter muscle of the jaw was included to represent the head and neck region. This small muscle (1.07±0.16 g) was found to have characteristics very different from those of the locomotor muscles. $V\mathrm{v}(\mathrm{mt,f})$ was 16.4±4.9%, which is significantly higher than that of the other muscles ($F_{9,1}$=4.433, P=0.001). This was also the case for $J\mathrm{v}(\mathrm{c,f})$, where the value was 1499±295 mm mm^{-3} muscle ($F_{9,1}$=3.992, P=0.002). The overall characteristics of this muscle, including fibre types and its evolutionary origins (Hoh, 2002), set it apart from the other muscles of the body, including those of the head and neck, and consequently we set it aside in our investigation of the relationships between muscle structure–function and maximal aerobic capacities.

$V\mathrm{v}(\mathrm{mt,f})$ varies between muscles (Table 1). Apart from the diaphragm, the m. semitendinosus has the highest $V\mathrm{v}(\mathrm{mt,f})$ of 11.9%. The majority of the muscles tended to be similar, with values ranging between 7.1% and 8.2%, but the m. coccygeus stood out with a $V\mathrm{v}(\mathrm{mt,f})$ of only 2.8%. The $S\mathrm{v}(\mathrm{im,m})$ was measured for the m. multifidi lumborum and was

Table 1. Mitochondrial and capillary characteristics of muscles from regions of the body of *Bettongia penicillata*

Body section	Muscle	M_m (g)	M_m/M_b (g kg^{-1})	Vv(mt,f) (%)	Jv(c,f) (mm mm^{-3})	J(c) (km)	V(c)/M_m (µl g^{-1})	V(c)/V(mt,m) (ml ml^{-1})
Fore leg	M. triceps	1.1±0.03^{f}	1.10±0.05^{f}	7.9±1.2^{b}	983±92^{ab}	1.0±0.1^{b}	17.9±2.5^{a}	0.26±0.06^{b}
Trunk	M. diaphragm	3.7±0.3^{d}	3.67±0.22^{d}	13.4±1.0^{a}	548±85^{b}	1.9±0.3^{b}	12.5±1.8^{a}	0.10±0.01^{b}
Trunk	M. erector spinae	23.1±0.8^{a}	23.85±0.70^{a}	7.3±0.4^{b}	761±132^{a,b}	17.1±3.0^{a}	12.0±2.3^{a}	0.17±0.03^{b}
Back	M. multifidi lumborum	8.0±0.3^{c}	7.99±0.38^{c}	7.4±0.6^{b}	833±147^{a,b}	6.3±1.1^{b}	17.1±4.8^{a}	0.25±0.08^{b}
Upper hind leg	M. vastus lateralis	7.6±0.2^{c}	7.55±0.14^{c}	7.1±0.9^{b}	579±55^{b}	4.1±0.3^{b}	11.0±0.8^{a}	0.17±0.02^{b}
Upper hind leg	M. biceps femoris	17.6±0.7^{b}	17.52±0.73^{b}	8.2±1.5^{b}	1089±29^{a}	18.0±0.8^{a}	18.2±1.6^{a}	0.26±0.05^{b}
Upper hind leg	M. semitendinosus	2.6±0.2^{e}	2.58±0.18^{e}	11.9±1.4^{a}	747±96^{a,b}	1.8±0.2^{b}	14.8±2.6^{a}	0.13±0.02^{b}
Lower hind leg	M. gastrocnemius	7.1±0.1^{c}	7.05±0.17^{c}	7.9±1.0^{b}	858±163^{a,b}	5.7±1.0^{b}	14.5±1.1^{a}	0.19±0.04^{b}
Tail	M. coccygeus	1.4±0.2^{f}	1.40±0.20^{f}	2.8±0.4^{c}	883±114^{a,b}	1.2±0.2^{b}	18.2±4.6^{a}	0.79±0.31^{a}

M_m, muscle mass; M_b, body mass; Vv(mt,f), mitochondrial volume density; V(mt,m), mitochondrial volume; Jv(c,f), capillary length density, i.e. capillary length per unit volume of muscle tissue; J(c), total capillary length in a whole muscle; V(c)/M_m, capillary volume per gram of muscle; V(c)/V(mt,m), capillary volume per unit of mitochondrial volume.

Values are means ± s.e.m. (N=4) (except gastrocnemius muscle capillary data, where N=3). In columns, values that are significantly different have different superscript letters (SNK test, P<0.05). Mean body mass was 1000 g. Note: values for muscle masses are for total for body; i.e. both sides where applicable. Capillary diameter was not significantly different between muscles; mean of mean muscle values was 4.81±0.08 µm, with a range of 4.57–5.45 µm.

33.2±1.71 m^2 cm^{-3} (N=4). This value is essentially the same as that noted for placental mammals (Schwerzmann et al., 1989) and red kangaroos (Dawson et al., 2004).

Capillary characteristics also differ between muscles (Table 1). Jv(c,f) incorporates c(K,O) and indicates the capillary supply to muscles (Conley et al., 1987). In this study, c(K,O) was determined for each muscle from each animal. The highest Jv(c,f) was found in the m. biceps femoris (1089 mm mm^{-3} muscle). The majority of muscles was intermediate between this value and the low values seen in the m. vastus lateralis (579 mm mm^{-3}) and diaphragm (548 mm mm^{-3}). The pattern of difference between the muscles altered when muscle size was taken into account in the determination of J(c) in each muscle. The two largest muscles, m. erector spinae of the back and m. biceps femoris of the upper hind leg, had significantly greater total capillary lengths than the other muscles. The capillary volume per gram of muscle, V(c)/M_m, did not differ significantly between muscles. This could be because of variation in capillary diameter. However, no significant difference was noted in capillary diameter across muscles; the mean was 4.81 µm, with the range being 4.57 to 5.45 µm. The ratio V(c)/V(mt,m) indicates the capillary blood supply to mitochondria in various muscles. The only muscle that was notably different, with a very high capillary volume per unit of mitochondria, was the m. coccygeus of the tail. Significant differences in V(c)/V(mt,m) between all the remaining muscles were absent.

The muscle-specific mitochondrial and capillary data (Table 1) were combined with the muscle mass distribution in various regions of the body to get an overall estimate of the distribution of mitochondria and capillaries in those regions (Table 2), in the manner of previous studies (Hoppeler et al., 1984; Hoppeler, 1990; Weibel et al., 2004; Weibel and Hoppeler, 2005; Dawson et al., 2004). The upper hind leg had significantly more muscle than other regions, containing 40.4% of the total skeletal muscle mass. The next most muscular regions were the trunk (26.9%) and the back and lower leg (10.5% and 9.6%, respectively). The fore part of the body was lightly muscled, with the head and neck contributing only 2.8% of the total skeletal muscle. The distribution of mitochondria and capillaries throughout the muscle regions largely follows the muscle mass distribution. However, the low Vv(mt,f) of the tail resulted in this region containing only 1.5% of skeletal muscle mitochondria, despite contributing 4.8% of the total skeletal muscle mass.

DISCUSSION

Bettongia penicillata is a small (~1 kg) cursorial marsupial that uses hopping as its primary locomotory gait, in the manner of the kangaroo *M. rufus*. However, *B. penicillata* belongs to a relatively conservative family (Potoroidae) from which the clade that gave rise to the kangaroos (family Macropodidae) diverged some 25–30 million years ago (Meredith et al., 2008). So, are the structure–function characteristics of hopping common across these families, or are the highly athletic characteristics unique to the more recently evolved kangaroos? In kangaroos, the hopping gait enables stride length to be much extended so that increased speed can be achieved at comparative low energy cost. Additionally, the kangaroos couple these features with a substantial mass of aerobic locomotor muscle (a ‘big motor’) to achieve high speeds (Dawson

Table 2. Distribution of muscle and muscle mitochondria and capillaries in the body of *Bettongia penicillata*

Body section	Mass (g)	% of total muscle mass	V(mt,m) (ml)	% of total V(mt,m)	V(c) (ml)	% of total V(c)
Head and neck	12.3±1.2^{d}	2.8±0.3^{d}	0.8±0.1^{c,*}	2.4±0.3^{c,d}	0.14±0.03^{d,*}	2.4±0.5^{d}
Fore leg	22.1±1.8^{d}	5.1±0.4^{d}	1.7±0.4^{c}	4.5±0.9^{c,d}	0.36±0.06^{c,d}	6.0±1.3^{c,d}
Trunk	117.1±1.4^{b}	26.9±0.8^{b}	11.4±0.5^{b}	31.3±2.7^{b}	1.43±0.21^{b}	23.1±2.9^{b}
Back	45.9±3.8^{c}	10.5±0.7^{c}	3.3±0.5^{c}	8.9±1.4^{c}	0.78±0.24^{c}	12.3±3.5^{c}
Upper hind leg	176.1±5.6^{a}	40.4±0.6^{a}	15.2±1.9^{a}	40.4±2.9^{a}	2.42±0.11^{a}	40.0±3.3^{a}
Lower hind leg	41.7±0.4^{c}	9.6±0.3^{c}	3.1±0.4^{c}	8.4±0.9^{c}	0.58±0.04^{c,d}	9.5±0.8^{c,d}
Tail	21.0±7.0^{d}	4.8±1.4^{d}	0.6±0.2^{c}	1.5±0.4^{d}	0.26±0.04^{d}	4.2±0.7^{c,d}
Total skeletal muscle	436.2±13.8		36.0±2.4		6.0±0.3	

Values are means ± s.e.m. (N=4). In columns, significantly different values have different superscript letters (P<0.05).

V(mt,m) and V(c) values for the trunk and upper hind leg were derived from the mean densities of mitochondria and capillaries in the muscles sampled from these regions (two and three muscles, respectively; Table 1).

*V(mt,m) and V(c) estimated using values from the erector spinae, which extends into the neck region

et al., 2004; Dawson and Webster, 2010). The spring-like nature of hopping had focused interest on elastic energy storage during fast locomotion; however, it occurs in galloping mammals and is not the unique feature of hopping (Dawson and Webster, 2010). From a previous study (Webster and Dawson, 2003), it is apparent that the characteristics of hopping and the energetics of locomotion (including a high $\dot{V}_{O2,max}$) are similar in *B. penicillata* to those of *M. rufus*. And from our present study, *B. penicillata* also has comparable underlying cardio-respiratory structures to support the high aerobic capacity and achieve a marked athletic ability. The overall aerobic capability of *M. rufus* is based on a range of features that are also common to athletic placental mammals (Dawson et al., 2004). These include a relatively large heart and an elevated blood oxygen-carrying capacity that service a large, mitochondrial-rich, muscle mass. *B. penicillata* appears similarly endowed; it has been seen to possess both a large heart (Dawson et al., 2003) and a high haemoglobin concentration in the blood (Agar et al., 2000) and we now have shown that these are part of the support network for its locomotor muscles, the basis of its aerobic capability.

A feature of the high aerobic capacity of *B. penicillata* and *M. rufus* is their fAS (Table 3). In both species, these are well beyond those reported for comparable placentals (Weibel et al., 2004). These extreme fAS values are, in part, a reflection of the increase in metabolism relative to the lower basal metabolism of the marsupials but, additionally, their values for $\dot{V}_{O2,max}$ are in the upper range of values obtained amongst placental mammals. The fAS of *M. rufus* is 54 (Table 3). The fAS of *B. penicillata* is 23, considerably below that of the kangaroo but it is likely that this difference is related to a body size effect rather than a departure from the aerobic patterns seen in *M. rufus*. Body mass has a marked influence on fAS in placental mammals (Weibel et al., 2004).

To put the energetic capabilities of these macropodiform marsupials in context relative to those of placentals, we have relied on the extensive review of Weibel et al. (Weibel et al., 2004). They list $\dot{V}_{O2,max}$ data for 33 placental species ranging in M_b from 7 g (pygmy mouse) to over 500 kg (horse). They found a broad range of aerobic capabilities among the placentals, but their data clumped into two categories, which they termed 'athletic' and 'non-athletic'. Weibel et al. calculated power law regressions of the relationships between $\dot{V}_{O2,max}$ and M_b for each category and found them to be significantly different (Weibel et al., 2004). Of note, they inadvertently included our study marsupial as their 34th species and placed it in their non-athletic category despite its $\dot{V}_{O2,max}$ (from Seeherman et al., 1981) falling neatly with the 'athletic' placentals. We recalculated these relationships using only the placental values listed by Weibel et al. (Weibel et al., 2004) and also grouped the animals into their athletic and non-athletic categories (Fig. 2) (see Table S1 in the supplementary material for data sources). For athletic species, the relationship between $\dot{V}_{O2,max}$ and M_b was

$$\dot{V}_{O2,max} = 199.36\ M_b^{0.933} \quad (7)$$

(95% confidence limits for the exponent are 0.884, 0.982, $F_{1,9}$=1870, P<0.05, R^2=0.9952). For non-athletic species, the relationship was:

$$\dot{V}_{O2,max} = 96.48\ M_b^{0.838} \quad (8)$$

(95% confidence limits for the exponent are 0.789, 0.887, $F_{1,20}$=1272, P<0.05, R^2=0.9845).

As initially found by Weibel et al. (Weibel et al., 2004), the slope (mass exponent) of the athletic species equation is significantly larger than that of the non-athletic species equation [t=3.31, d.f.=29, P<0.005; test statistic calculated using the method of Zar (Zar, 1999); pp. 360–368]. *Macropus rufus* and *B. penicillata* data points conform to the athletic species line (Fig. 2). Subsequently, we have produced a new regression covering athletic placentals and macropodiform marsupials, which is:

$$\dot{V}_{O2,max} = 198.87\ M_b^{0.935} \quad (9)$$

(95% confidence limits for the exponent are 0.892, 0.978, $F_{1,11}$=2305, P<0.05, R^2=0.9953).

Both the coefficient and the exponent of Eqn 9 are not different from those in Eqn 7 [coefficient, t=0.015, d.f.=21, P>0.50; exponent, t=0.080, d.f.=20, P>0.50; using the method of Zar (Zar, 1999)]. Conclusively then, we can say that both *M. rufus* and *B. penicillata* exhibit $\dot{V}_{O2,max}$ that are equivalent to those of *athletic* placental mammals of the comparable sizes. The principal difference between athletic and non-athletic placentals appears to be the expansibility of their aerobic capacity, i.e. in fAS. That of athletic species is markedly higher than that of the non-athletic group (Weibel et al., 2004). As noted above, our macropodiform marsupials had higher fAS values than all placentals, and high fAS values appear to be a general feature for marsupials. Their relatively low BMR are countered by levels of maximal metabolism at least equivalent to those of placental mammals (Dawson and Dawson, 1982). Values for $\dot{V}_{O2,max}$ for nine marsupial species (mass range 16 g to 5 kg) (Hinds et al., 1993) are all higher than the $\dot{V}_{O2,max}$ values predicted from Eqn 8 above, i.e. that for non-athletic placentals. The possibility that marsupials other than the Macropodiformes tend to 'athleticism' requires investigation.

The considerable aerobic capability of *B. penicillata* is supported by mitochondria and capillary features of the skeletal musculature that are at levels also seen generally across placental mammals (Fig. 3), as was also shown for *M. rufus* by Dawson et al. (Dawson et al., 2004). The ratio of $\dot{V}_{O2,max}$ to total muscle mitochondrial volume, V(mt), is similar for *M. rufus* and *B.*

Table 3. Morphometry of total skeletal muscle of red kangaroo, bettong and agouti

Parameter	Red kangaroo[a]	Bettong[b]	Agouti[c]
M_b (kg)	28.5	1.0	3.22
M_m/M_b (%)	46.8	43.6	40.7
$\dot{V}_{O2,max}/M_b$ (ml O_2 min^{-1} kg^{-1})	178	177[(2)]	102
$\dot{V}_{O2,max}$ (ml O_2 min^{-1})	5073	194.7[(2)]	328.4
fAS (=$\dot{V}_{O2,max}$/BMR)	54[(1)]	23[(3)]	12
Mitochondria			
Vv(mt,f) (%)	8.2	8.7	5.6
V(mt)/M_b (ml kg^{-1})	38.2	36.0	21.6
$\dot{V}_{O2,max}$/V(mt) (ml O_2 min^{-1} ml^{-1})	4.7	4.9	4.7
Capillaries			
J(c)/M_b (km kg^{-1})	546	321	274
V(c)/M_b (ml kg^{-1})	8.9	6.0	4.4
Hct (%)	47.5	54[(4)]	42
V(ec)/M_b (ml kg^{-1})	4.2	3.2	1.8
$\dot{V}_{O2,max}$/V(ec) (ml O_2 min^{-1} ml^{-1})	42.4	54.6	55.7

V(mt)/M_b, mass-specific total mitochondrial volume; Hct, haematocrit; V(ec), capillary erythrocyte volume. Values are means.

[a]Dawson et al. (Dawson et al., 2004), except for: [(1)]calculated from $\dot{V}_{O2,max}$ in Kram and Dawson (Kram and Dawson, 1998) and BMR value in Dawson et al. (Dawson et al., 2000).

[b]Present study, except for: [(2)]value from Seeherman et al. (Seeherman et al., 1981), where M_b was 1.1 kg; [(3)]calculated from [(2)] and the BMR value in Webster and Dawson (Webster and Dawson, 2003); [(4)]Value from Agar et al. (Agar et al., 2000).

[c]Hoppeler and Flück (Hoppeler and Flück, 2002); Weibel et al. (Weibel et al., 2004).

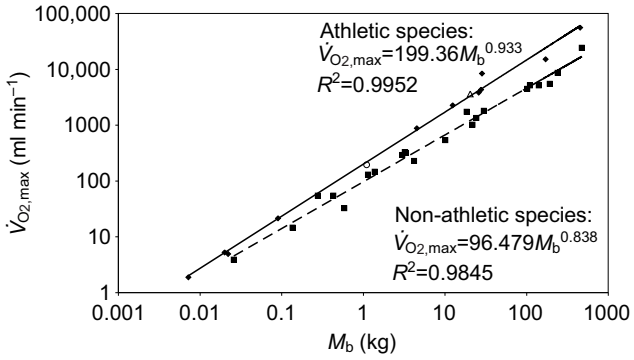

Fig. 2. Maximum rate of oxygen consumption, $\dot{V}_{O_2,max}$, as a function of body mass, M_b, for 'athletic' (diamonds) and 'non-athletic' (squares) mammals (species and data sources listed in Table S1 in the supplementary material). The mass exponent of the athletic species equation is significantly larger than that for the non-athletic species. Data for *Bettongia penicillata* (open circle) and *Macropus rufus* (open triangle) are also shown; these data can be included in the athletic species equation with no significant change to either exponent or coefficient (see text for details).

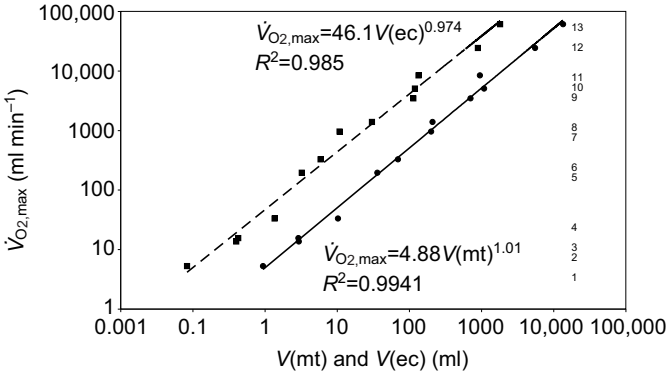

Fig. 3. Maximum rate of oxygen consumption, $\dot{V}_{O_2,max}$, as a function of total muscle mitochondrial volume, V(mt) (circles), and total muscle erythrocyte volume, V(ec) (squares). Numbers on the right identify species: 1, woodmouse; 2, mole rat; 3, white rat; 4, guinea pig; 5, brush-tailed bettong (*Bettongia penicillata*); 6, agouti; 7, fox; 8, goat; 9, dog; 10, red kangaroo (*Macropus rufus*); 11, pronghorn; 12, horse; 13, steer.

penicillata (Table 3), and for all placentals (Fig. 3), including relatively sedentary species, e.g. the agouti (Table 3). The equation representing this relationship is:

$$\dot{V}_{O_2,max} = 4.88\ V(\mathrm{mt})^{1.01} \quad (10)$$

(95% confidence limits for the exponent are 0.95, 1.07, $F_{1,9}$=1450, P<0.05, R^2=0.994).

This pattern applies also to the vascular supply to the mitochondria, V(ec) (Table 3, Fig. 3), with the equation being:

$$\dot{V}_{O_2,max} = 44.9\ V(\mathrm{ec})^{0.975} \quad (11)$$

(95% confidence limits 0.89, 1.05, $F_{1,11}$=701, P<0.05, R^2=0.985).

The fundamental difference between athletic species such as *B. penicillata* and *M. rufus* and more sedentary species such as the agouti is the total mitochondrial volume in skeletal muscle, which is determined by the mean mitochondrial density, Vv(mt,f), and the total skeletal muscle mass, M_m/M_b, both of which are high in athletic species. The oxygen supply to the mitochondria follows a similar pattern, with athletic species having both higher V(c) and V(ec), as reflected by higher haematocrits, Hct (Table 3).

While the whole-body muscle mitochondrial and capillary parameters of *B. penicillata* show similarities with *M. rufus* (and placental mammals), examining the detail of muscular regions and individual muscles gives some insight into functional differences between *B. penicillata* and *M. rufus*. As in *M. rufus*, the aerobic performance of *B. penicillata* is associated with a large skeletal muscle mass that is primarily organized for power output from the hind legs (Table 2). Compared with many placental mammals (Weibel et al., 2004), *B. penicillata* has a high total muscle mass: 43.6% of M_b. The amount of skeletal muscle was marginally higher at 46.8% of M_b in *M. rufus* (Table 3). In *M. rufus*, the bulk of the trunk and back musculature is concentrated posteriorly and its tail is large and well muscled, leading to more than 80% of its skeletal muscle mass being concentrated in its posterior region (Dawson et al., 2004). *B. penicillata* has a much less muscular tail, and less of its trunk and back musculature is located posteriorly (see Fig. 1). These differences in body proportion are likely related to the differences in the slow speed gaits of the two species. *B. penicillata* uses a quadrupedal gait at slow speeds (Fig. 4A) whereas *M. rufus* uses a 'pentapedal' gait, in which the tail is used as a fifth limb that provides significant propulsive force, while the front legs act only as struts to support the body (Dawson et al., 2004). The tail of *M. rufus* is also significantly involved in hopping locomotion. It is large and packed with long tendons and acts both in counterbalancing and elastically conserving energy. By contrast, while the position of the tail of *B. penicillata* does change during different phases/stages of hopping (Fig. 4B,C), it seems not to be markedly involved in energy conservation or counterbalancing.

In *B. penicillata*, as in *M. rufus*, patterns in muscle mass distribution are broadly reflected in the distribution of mitochondrial volumes, V(mt,m), and capillary volumes, V(c). Some deviations do exist, however. The fore leg region of *M. rufus* contains a lower proportion of mitochondrial (1.7%) and capillary volumes (2.4%) than would be predicted from its contribution to muscle mass (3.9%). Its m. triceps had a notably low Vv(mt,f) (Dawson et al., 2004) that apparently matches the low requirement for aerobic power output, relative to that of the key locomotor muscle regions of the upper hind leg, trunk and back (Dawson et al., 2004). In *B. penicillata*, such a pattern is not seen and the Vv(mt,f) of its m. triceps is similar to that in most muscles (Table 1). The higher Vv(mt,f) of the triceps in *B. penicillata* is presumably related to the more substantial role of the fore limbs in quadrupedal locomotion and in foraging; *B. penicillata* actively forages for underground fungal fruiting bodies (truffles). On the other hand, in *B. penicillata* the tail musculature is poor in mitochondria; it contributes just 1.5% of total skeletal muscle mitochondrial volume

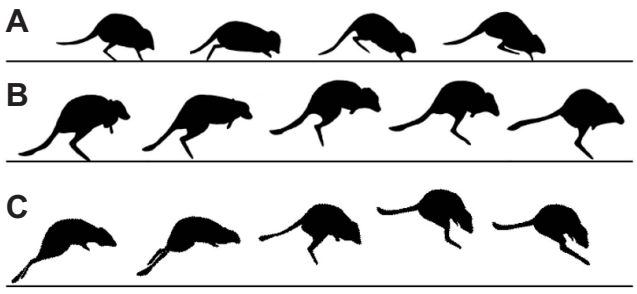

Fig. 4. Gaits of *Bettongia penicillata*. (A) Quadrupedal gait, at speeds below 1 m s^{-1}. (B) Slow hopping, at speeds of 1–2.5 m s^{-1}. (C) Fast hopping, at speeds above 2.5 m s^{-1}, to a maximum of approximately 6 m s^{-1}.

despite contributing 4.8% of total muscle mass (Table 2). As previously discussed, the tail of *B. penicillata* plays a small role in locomotion, but why then is the capillary volume of the tail region not proportionally reduced (Table 2)? The V(c)/V(mt,m) of the m. coccygeus is the highest of all sampled muscles (Table 1). This apparently disproportionate capillarisation of the m. coccygeus (and the tail region in general) can be explained from the roles other than oxygen delivery that capillaries have; they are also involved in thermal balance and heat transfer. A species related to *B. penicillata*, the long-nosed potoroo (*Potorous tridactylus*), utilizes sweating at the base of the tail as a key cooling mechanism (Hudson and Dawson, 1975). *B. penicillata* has been likewise noted to sweat profusely at the base of the tail after bouts of exercise. It is likely, therefore, that the principal role of the 'excess' of capillaries in the m. coccygeus is for the transfer of heat.

Despite small variations, our data for *B. penicillata* and that for *M. rufus* (Dawson et al., 2004) obviously point to a large group of marsupial mammals (suborder Macropodiformes, the 'big foots') with aerobic capacities equivalent to those of the most athletic placental mammals. At the core of such capacity are large locomotor muscles that are rich in mitochondria. These muscles are serviced by an integrated O_2 supply chain from the lungs to the muscle capillaries *via* the cardiovascular system, which has the same features in marsupials and placentals. The fundamental characteristics of the O_2 supply chain and the level of capillary supply of erythrocytes, and hence oxygen, to the mitochondria are also notably similar in non-athletic and athletic placentals (Weibel et al., 2004). Mammals with higher aerobic capacities simply have more muscle and mitochondria combined with an oxygen delivery system that has more capacity, e.g. bigger hearts. Given the ubiquity of this system, it is probably old in an evolutionary sense, with its characteristics dating back to the origin of mammals in the Jurassic era and possibly to the mammal-like synapsids back in the Permian era (299–251 million years ago).

The data for the macropodiform marsupials clarify but somewhat complicate our overall understanding of the relationship of $\dot{V}_{O2,max}$ to BMR in mammals. Early studies suggested that there was a simple link between the two metabolic levels, with $\dot{V}_{O2,max}$ being about 10× BMR so that both would scale with $M_b^{0.75}$ (Pasquis et al., 1970; Lechner, 1978). Recently, West and coworkers have attempted to formalize these empirical relationships *via* a consideration of the fractal design of the O_2 supply chain to mitochondria and suggested that $M_b^{0.75}$ is the basis of a universal scaling law (West et al., 1997; West et al., 1999). Weibel and Hoppeler reviewed this topic and showed empirically that the exponent was not 0.75 for $\dot{V}_{O2,max}$ but much higher (Weibel and Hoppeler, 2005). As we have also shown in Eqn 9, $\dot{V}_{O2,max}$ of athletic mammals scales with $M_b^{0.94}$. BMR within taxonomic groups appears to be a conservative trait that generally scales near $M_b^{0.75}$ (West et al., 1999; Withers et al., 2006), with marsupial BMR being lower than that of placentals (Dawson and Hulbert, 1970; Withers et al., 2006). However, $\dot{V}_{O2,max}$ appears to be more flexible, depending on the lifestyle strategy of a particular mammalian type, complicating the often-accepted assumption that there is a reasonably tight relationship between BMR and $\dot{V}_{O2,max}$. The data from the macropodiforms support Weibel and Hoppeler's finding that the key to predicting $\dot{V}_{O2,max}$ is the amount of active muscle and its mitochondria.

LIST OF SYMBOLS AND ABBREVIATIONS

A(c)	capillary cross-sectional area
BMR	basal metabolic rate
c(K,0)	tortuosity factor of the capillary network
d	density of muscle
fAS	factorial aerobic scope
Hct	haematocrit
J(c)	total capillary length
Jv(c,f)	capillary length density
M_b	body mass
M_m	muscle mass
N_A(c,f)	numerical capillary density
r_c	mean capillary radius
Sv(im,mt)	surface density of inner mitochondrial membranes per unit volume of mitochondria
S(im,m)	total surface area of inner mitochondrial membranes
V(c)	capillary volume
V(ec)	capillary erythrocyte volume
V(mt)	total mitochondrial volume of skeletal muscle
V(mt,m)	mitochondrial volume of individual muscles (or muscle regions)
$\dot{V}_{O2,max}$	maximal aerobic oxygen consumption
Vv(f,m)	volume fraction of muscle occupied by muscle fibres
Vv(mt,f)	volume fraction of mitochondria

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