Muscle mitochondrial volume and aerobic capacity in a small marsupial (*Sminthopsis crassicaudata*) match those of 'athletic' placentals, reflecting flexible links between energy-use levels in mammals generally.

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Running head: High aerobic capability of marsupials.

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1 SUMMARY

2 We investigated the muscle structure/function relationships that underlie the aerobic capacity of

3 an insectivorous, small (~15 g) marsupial, *Sminthopsis crassicaudata* (Family: Dasyuridae).

4 This was for further insight into energy use patterns in marsupials, relative to those in placentals,

5 their sister clade within the Theria (advanced mammals). Disparate hopping marsupials

6 (Suborder Macropodiformes), a kangaroo (*Macropus rufus*) and a rat-kangaroo (*Bettongia*

7 *penicillata*), show aerobic capabilities as high as those of 'athletic' placentals. Equivalent

8 muscle mitochondrial volumes and cardiovascular features support these capabilities. We

9 examined *S. crassicaudata* to determine whether highly developed aerobic capabilities occur

10 elsewhere in marsupials, rather than being restricted to the more recently evolved

11 Macropodiformes. This was the case. Treadmill-trained *S. crassicaudata* attained a maximal

12 aerobic metabolic rate ($\dot{V}O_2$max or MMR) of 272 mlO_2 min^{-1} kg^{-1} (N=8), similar to that reported

13 for a small (~20g), 'athletic' placental, *Apodemus sylvaticus,* 264 mlO_2 min^{-1} kg^{-1}. Hopping

14 marsupials have comparable aerobic levels when body mass variation is considered. *S.*

15 *crassicaudata* has a basal metabolic rate (BMR) about 75% of placental values but it has a

16 notably large factorial aerobic scope (fAS) of 13; elevated fAS also feature in hopping

17 marsupials. The $\dot{V}O_2$max of *S. crassicaudata* was supported by an elevated total muscle

18 mitochondrial volume, which was largely achieved through high muscle mitochondrial volume

19 densities, Vv(mt,f), the mean value being $14.0 \pm 1.33\%$. These data were considered in relation

20 to energy use levels in mammals, particularly field metabolic rate (FMR). BMR is consistently

21 lower in marsupials, but this is balanced out by a high fAS, such that marsupial MMR matches

22 that of placentals. However, FMR shows different mass relationships in the two clades, with the

23 FMR of small (<125 g) marsupials, such as *S. crassicaudata*, being higher than in comparably

24 sized placentals with the reverse applying for larger marsupials. The flexibility of energy output

25 in marsupials provides explanations for this pattern. Overall our data refute widely held notions

26 of mechanistically closely linked relationships between body mass, BMR, FMR and MMR in

27 mammals generally.

28

29

INTRODUCTION

Marsupials (Metatheria) are the sister clade of placentals (Eutheria). Together they comprise the Theria or advanced mammals and they have many anatomical and physiological characteristics in common, which largely reflect ancestral traits that evolved prior to the divergence of these two groups about 148 MYA (Bininda-Emonds et al. 2007). Differences between the two groups, such as their distinct reproductive features, reflect divergent evolutionary trajectories during their long, separate histories. Another area where differences seem to have occurred concerns metabolic patterns. Marsupials have relatively low basal metabolic rates (BMR), generally about 75% of placental values, and historically were seen as being less competent at thermoregulation and also 'low energy' mammals (Martin, 1902; Dawson, 1973). While distinctions regarding the thermal biology of these clades have been long discarded (Dawson, 1973; Dawson, 1989) debate about differences in metabolic capabilities of marsupials has persisted in some disciplines (e.g. McNab, 1980; McNab, 2005). Here we expand investigations of the aerobic capacities of marsupials and also focus on the functional structures that underlie the capacity for oxygen use in their muscles. We wish to put these into perspective relative to the features that have recently been established for the structure/function relationships underlying the aerobic capacities of the placental mammals (Weibel et al., 2004).

It has become apparent that some marsupials have substantial aerobic capabilities that result from relatively large factorial aerobic scopes (fAS), such that they achieve levels of maximum oxygen consumption ($\dot{V}O_2max$) similar to those seen in placentals (Dawson and Dawson, 1982; Hinds et al., 1993). Recent data for the red kangaroo, *Macropus rufus*, (Family Macropodidae) (Kram and Dawson, 1998; Dawson et al., 2004) are notable because, despite a relatively low BMR, its extreme fAS of 54 results in a $\dot{V}O_2max$ or maximum metabolic rate (MMR) equivalent to the high levels reported in a group of placental mammals, such as dogs and horses, that were categorised as 'athletic' (Taylor et al., 1987; Weibel et el., 2004). Underlying this capability in *M. rufus* is a large mass of locomotor muscles that have comparatively high mitochondrial and capillary volumes (Dawson et al., 2004). Another hopping marsupial, *Bettongia penicillata*, a rat-kangaroo (Family Potoroidae), though much smaller, body mass (M_b) 1 kg, also shows an elevated fAS of 23 and a markedly high MMR (Seeherman et al., 1981; Webster and Dawson, 2012). Again this is associated with a large skeletal muscle mass that has

62 relatively high muscle mitochondrial volume densities and both a large total capillary

63 volume and total capillary erythrocyte volume (Webster and Dawson, 2012). Overall, the

64 muscle and cardio-respiratory structural features of *M. rufus* and *B. penicillata* are

65 identical to those reported for 'athletic' placental mammals of equivalent sizes by Weibel

66 and co-workers (Weibel et el., 2004). Notably, the ratio between MMR and total muscle

67 mitochondrial volume ($\sim$ 5 ml O_2 min^{-1} ml^{-1}) is, as initially proposed (Hoppeler and

68 Lindstedt, 1985), consistent in placentals (Weibel et al., 2004) and macropodiform

69 marsupials (Webster and Dawson, 2012).

70   *M. rufus* and *B. penicillata* belong to the specialised monophyletic suborder

71 Macropodiformes (kangaroos, wallabies and rat-kangaroos) (Meredith et al., 2008), but do they

72 differ aerobically from other marsupials? Evidence for 'athletic' level aerobic capacities in other

73 marsupials is strong and comes from disparate studies of their cardio-respiratory features (e.g.

74 Dawson and Needham, 1981; Hallam et al., 1989; Hallam et al., 1995; Agar et al., 2000;

75 Dawson et al., 2003). The generality of this assertion is not certain because, while Hinds et al.

76 (1993) measured comparatively high fAS during locomotion for several smaller species of

77 marsupial, their reported MMR values did not reach 'athletic' placental levels. However, the

78 MMR value obtained by Hinds et al. (1993) for *B. penicillata* was lower than those obtained on

79 more extensively trained animals (Seeherman et al., 1981; Webster and Dawson, 2012). The

80 only other marsupial for which comparable data are available is a South American opossum,

81 *Monodelphis domestica* (Family: Didephidae) ($M_b \sim$ 90 g). It also has a comparatively large fAS

82 (Dawson and Olson, 1988; Schaeffer et al., 2003) but its MMR does not reach an 'athletic' level,

83 though the mitochondrial volume densities of its skeletal muscles are not lower than generally

84 seen in similar sized placentals (Schaeffer et al., 2003). On the other hand, there is support for

85 relatively high aerobic capabilities extending to smaller marsupials. This comes from studies of

86 field metabolic rates (FMR). Marsupials and placentals show distinctly different patterns of

87 variation of FMR with M_b, but intriguingly, small marsupials ($M_b <$ 125 g) have higher FMRs

88 than placentals of similar size (Nagy et al., 1999; Capellini et al., 2010). These differences are

89 particularly marked among the smallest marsupials; at a M_b of $\sim$15 g the FMR of *Sminthopsis*

90 *crassicaudata* is almost double that of the predicted placental value (Nagy, 1988).

91   To clarify the factors underlying this disparity and to further our understanding of the

92 metabolic patterns and aerobic capabilities among small marsupial species from different

93 phylogenetic clades, we studied *S. crassicaudata* (Family: Dasyuridae). This species ($M_b \sim$15 g)

94 is an active, quadrupedal insectivore that has often been used as a model for Australian dasyurid

95   marsupials and was among the species examined by Hinds et al. (1993). We focused first on

96   gaining an accurate determination of its MMR and then determined how it related to its muscle

97   content and muscle mitochondrial features such as volume density. Because of its high MMR

98   levels, we predicted that it would share characteristics that match what has previously been

99   found in the Macropodiformes, thereby indicating a generally higher aerobic capability among

100 marsupials that matches that seen in placentals designated as 'athletic' by Weibel et al. (2004). If

101 so, it would point to a fundamental structure/function relationship for oxygen delivery to

102 muscles evolving in or before the earliest mammals. Further, such information provides the

103 opportunity to examine the presumed relationships between BMR, MMR and FMR in mammals.

104 This is significant in view of the idea that BMR is a good predictor of energy budgets, which is

105 based on the notion that the allometric relationship between M_b and BMR locks in other

106 metabolic levels (West et al., 1997; West et al., 1999; Brown et al., 2004).

107

108 **MATERIALS AND METHODS**

109 **Animals and animal care**

110 Fat-tailed dunnarts, *Sminthopsis crassicaudata* (Gould, 1844) of the Family Dasyuridae

111 (Krajewski et al., 2012) are mouse-sized insectivorous marsupials that inhabit the surface

112 of open habitats, usually in semi-arid and arid regions of Australia. They are active

113 nocturnal predators, catching relatively large, invertebrate prey, such as crickets and

114 beetles (Morton 1978). Our investigations were in two parts. 1) An analysis of

115 mitochondrial characteristics of locomotor muscles of *S. crassicaudata* was made at the

116 University of New South Wales, Sydney. The animals used in this study were derived

117 from colonies maintained at the University of Adelaide and University of Wollongong. 2)

118 A study of aerobic capacity of *S. crassicaudata* during running that was undertaken at the

119 University of Wollongong, using animals from their breeding colony that was established

120 3 years earlier from free-living animals collected in western Queensland.

121   During investigation and prior to killing, animals were kept at an air temperature

122 (T_a) of $23 \pm 0.4°C$, with a 12 hour light: dark cycle, the lights switching on at 0600 hours.

123 They were housed individually in clear plastic containers (55 x 38 x 20cm), which were

124 fitted with wire tops; bedding of straw and shredded paper was provided. A mixture of

125 dried cat food (moistened) and canned dog food was provided ad libitum; this was

126 supplemented with live crickets and vitamin drops (Pentavite infant vitamins). Water was

127 available at all times.

128

Muscle sample collection and preparation

129

130   To assess the muscle mitochondrial parameters of the skeletal muscle of the whole

131   body we followed a sampling procedure comparable to that developed by Hoppeler et al.

132   (1984) and used in other studies of marsupials (Dawson et al., 2004; Webster and Dawson,

133   2012). The musculature of *S. crassicaudata* was divided into five functional regions, these

134   being head/neck, fore leg, trunk, hind leg and tail. Animals were killed by gassing (carbon

135   dioxide) and weighed to the nearest 0.01 g on an electronic balance (Sartorius AG,

136   Goettingen, Germany) directly before dissection. Four animals were dissected to estimate

137   total skeletal muscle mass and the proportions of muscle in the five body regions. The

138   contributions to body mass of skin, heart and the digestive tract were also determined. In a

139   further five animals the heart plus seven skeletal muscles, including the diaphragm because

140   of its role in ventilation, were then dissected out and small blocks sampled for electron

141   microscopy. The skeletal muscles used were randomly selected, one coming from each

142   region except the hind leg where two muscles were selected. These were, m. trapezius

143   (head/neck), m. deltoid (fore limb), m. pectoralis minor (trunk), m. multifidi lumborum

144   (trunk), m. gluteus maximus and m. quadriceps (hind leg), m. multifidi lumborum (tail).

145   Two small blocks, no greater than 2mm thick, were randomly cut from each muscle

146   whilst being bathed in a drop of cold glutaraldehyde fixative solution (2.5% in 0.1M

147   sodium cacodylate buffer, pH 7.4). Sample blocks were transferred to vials containing the

148   buffered glutaraldehyde fixative solution for proper immersion fixation for a minimum of

149   four hours. Sample preparation thereafter followed the method of previous studies

150   (Dawson et al., 2004, Webster and Dawson, 2012), with the blocks ultimately being

151   embedded into Spurr's resin (a slow cure, low viscosity epoxy) over a long infiltration

152   period (3-4 days) and cured at 60°C for 48h. Ultra-thin sections of approximately 60 – 80

153   nm were cut for each muscle sample using glass knives mounted on a Reichert-Jung

154   Ultracut microtome (Leica Microsystems, Vienna, Austria). The sections were placed onto

155   copper grids (200 square mesh) and were immediately stained with uranyl acetate in 50%

156   ethanol for ten minutes.

157

Mitochondrial volume and inner mitochondrial membrane surface area

159   Grids were viewed at 10,000 x magnification with either a Hitachi 7000 (Tokyo,

160   Japan) or JEOL 1400 (Tokyo, Japan) transmission electron microscope (TEM). Ten grid

161   squares were selected per sample block using a systematic random sampling method

162   (Howard and Reed, 1998). Digital micrographs were taken in the top left corner of the grid

163   squares using an Olympus SQ (Tokyo, Japan) digital camera and software package

164   AnalySIS (attached to the Hitachi 7000 TEM) or a Gatan (Pleasanton, CA, USA) digital

165   camera and software package Gatan Digital Micrograph (attached to the JEOL 1400). For

166   each animal, a total of 160 micrographs were taken (ten micrographs per block x two

167   blocks per muscle x eight muscles).

168   Mitochondrial volumes were determined using the methods of previous studies

169   (Dawson et al., 2004 and Webster and Dawson, 2012). Briefly, mitochondria were

170   identified and selected in digital images by a human operator. The total percentage area

171   covered by the mitochondria (mitochondrial area fraction) in each micrograph was

172   estimated using either an image processing plug-in to Adobe Photoshop (Adobe Systems

173   Inc, San Jose, CA, USA) or the software ImageJ (US National Institutes of Health,

174   Bethesda, MA, USA). According to the Delesse principle, the mitochondrial volume

175   fraction Vv(mt,f), often referred to as mitochondrial volume density, is equivalent to the

176   mitochondrial area fraction (Weibel, 1980; Howard and Reed, 1998). The total

177   mitochondrial volume V(mt,m) for each muscle region (in ml) was calculated from:

178         $V(mt,m) = M_m \times Vv(mt,f) \times Vv(f,m) \times d^{-1}$ (1)

179   where M_m is regional muscle mass, Vv(mt,f) is the volume fraction of mitochondria,

180   Vv(f,m) is the volume fraction of muscle occupied by muscle fibres, and d is the density of

181   the muscle. For this study, it was assumed that Vv(f,m) was equal to 1 (Hoppeler et al.,

182   1987) and that d was equal to 1.06 g ml^{-3} (Mendez and Keys, 1960) since the myofibril

183   fraction and density are considered constant in all muscles (Mendez and Keys, 1960; Barth

184   et al., 1992).

185   The surface density of the inner mitochondrial membranes was estimated in four

186   muscles (m. gluteus maximus, m. deltoid, heart and diaphragm). For each animal, a total of

187   40 mitochondria (five mitochondria per block x two blocks per muscle x four muscles)

188   were examined and micrographs taken at up to 40,000 x magnification (using the Hitachi

189   7000 TEM with attached Olympus digital camera). The surface density of inner

190   mitochondrial membranes per unit volume of mitochondria, Sv(im,mt), was estimated

191   using the same method as previous studies (Dawson et al., 2004 and Webster and Dawson,

192   2012). An overall estimation of the total surface area of inner membranes in each muscle

193   was given by:

194          $S(im,m) = V(mt,m) \times Sv(im,mt)$.   (2)

195

196 **Aerobic capacity**

197 To ensure that maximum aerobic capacity (MMR) was achieved we followed the

198 procedures of Seeherman et al. (1981); such procedures were used in comparable studies

199 on placental mammals (see Weibel et al., 2004). The essence of these procedures was

200 extensive treadmill training (running) that ensured an accurate and reproducible MMR.

201 Seeherman et al. (1981) found that at least 2 – 6 weeks of training were needed for this to

202 be achieved for most of the species that they investigated. We trained *S. crassicaudata* for

203 treadmill running for 6 – 8 weeks by exercising them at speeds up to 1.5 m s^{-1}, generally

204 on alternate days. The highest training speed at which an animal could maintain 5 min of

205 constant running, following an initial speed adjustment period of 30 s, was used during the

206 measurement of MMR; such speeds ranged between 1 and 1.5 m s^{-1}. The MMR obtained

207 was the highest 2-min period of *instantaneous oxygen consumption* when an animal ran for

208 at least 5 min. The method for obtaining instantaneous oxygen consumption (Bartholomew

209 et al., 1981) involved initially determining the washout characteristics of the chamber, at

210 the flow rate used, by tracking the dynamics of a sudden pulse of O_2 depleted air followed

211 by immediate return to room air.

212   For actual measurement a *S. crassicaudata* was contained within an inverted 1.2 l

213 rectangular plastic container on a stationary treadmill belt. The treadmill speed was then

214 adjusted to that required, ie. the highest training speed for that individual. A constant airflow of

215 2.0 l min^{-1} was aspirated through the container at all treadmill speeds. Air entered through two

216 small holes in the front of the chamber and also through the bottom edges of the chamber in

217 contact with the belt. Flow rate was monitored with a Sierra Top-Trak mass-flow meter (Sierra,

218 Monterey, CA, USA). Oxygen content of inlet and outlet air was measured using a Sable

219 Systems FC-1 oxygen analyser (Las Vegas, NV, USA), with detection sensitivity of 0.0005%.

220 Water and CO_2 were removed from sampled air prior to gas analysis using Drierite and soda

221 lime, respectively. The $\dot{V}O_2$ throughout this exercise period was determined using appropriate

222 corrections for the system configuration (Hill, 1972). Values were adjusted for variations in

223 chamber air leakage at different treadmill speeds. Air leakage was determined by delivering a

224 gas mix into the chamber via a mass flow controller with an O_2% similar to that while a *S.*

225 *crassicaudata* was running. Readings were first taken while the treadmill belt was stationary and

226   then recorded at each belt speed used in the MMR determinations. Corrections ranged from 7%

227   at the lowest running speed to 14% at the highest.

228

229   **Statistical analysis**

230   Comparisons between muscles were analysed using one-way analysis of variance

231   (ANOVAs). A Student–Newman–Keuls (SNK) multiple-range test was applied when

232   significant differences were indicated by the ANOVA (using Statistica for Mac, StatSoft,

233   Tulsa, OK, USA). Values are given as means ± standard deviation (s.d.). Regression

234   analyses were carried out using Microsoft Excel (Microsoft, Redmond, WA, USA).

235

236

237   **RESULTS**

238   The mean body mass of *S. crassicaudata* in this investigation was 15.0 g (Table 1), which

239   is similar to the mass of wild-caught animals. The contribution of skeletal muscle to body

240   mass in *S. crassicaudata* was estimated to be 32.3±1.96% (Table 1). The size of the heart

241   and the contributions to body mass of some other major components, such as skin and the

242   digestive system, are also shown in Table 1.

243   In *S. crassicaudata*, Sv(im,mt) varied little between the muscle tissues investigated.

244   Values were: heart, 34.0±7.8 m^2 cm^{-3}; diaphragm, 36.8±5.8 m^2 cm^{-3}; m. gluteus maximus,

245   35.8 ± 7.3 m^2 cm^{-3}; m. deltoid, 35.0±7.5 m^2 cm^{-3}.

246   The content of mitochondria in heart and a range of skeletal muscles from *S.*

247   *crassicaudata* is shown in Table 2 as Vv(mt,f). The heart and diaphragm contained

248   significantly higher densities of mitochondria than the skeletal muscles; that of the heart

249   was 33.9±2.7%, with that of the diaphragm being 21.1±2.9% ($F_{7,1}$=47.15, P=0.0001).

250   While values for the other muscles ranged from 14.5±2.3% for the m. multifidi lumborum

251   of the trunk and tail to 10.6±1.4% for the m. pectoralis muscle, differences between them

252   were not significant ($F_{5,1}$= 2.116, P=0.1).

253   The muscle content throughout the body showed significant differences between

254   regions (Table 3; $F_{4,1}$=51.86, P=0.0001). Both the hind leg and trunk had significantly

255   more muscle than other regions and together comprised 60.6% of the total skeletal muscle

256   mass. The fore leg and head/neck regions equally made up most of the residual body

257   muscle mass, whereas the tail contained little muscle. Although Table 3 shows that there

258   are significant differences in Vv(mt,f) in muscle regions ($F_{4,1}$=4.849, P=0.01), the

259 differences are relatively small and the regional mitochondrial volumes V(mt,m) largely

260 reflect regional muscle masses. There is a significant difference in V(mt,m) across muscle

261 regions ($F_{4,1}$=137.1, P=0.0001). The trunk has a significantly larger volume (Table 3),

262 with the hind leg also having more than each of the remaining regions. The percentage of

263 total muscle mitochondrial volume contained by the regions follows a similar pattern of

264 significant differences ($F_{4,1}$=234.1, P=0.0001.) The total volume of mitochondria in the

265 skeletal muscle, V(mt), was 0.68±0.064 ml (Table 3).

266   Mean MMR of *S. crassicaudata* determined from sustained treadmill running was

267 4.09 ml min^{-1}, or 272 mlO$_2$ min^{-1} kg^{-1} (N=8 animals) at an average speed of 1.2 m s^{-1}

268 (Table 4). The basal metabolic rate (BMR) reported in a previous study (Dawson and

269 Hulbert 1970) was 0.320 ml min^{-1} and thus the aerobic factorial scope (fAS) was 12.8.

270

271 **DISCUSSION**

272 The muscle characteristics and aerobic capacity of the marsupial *S. crassicaudata* (Table

273 4) mark it as a mammal of high aerobic capacity in relation to other studies (Weibel et al.,

274 2004; Weibel and Hoppeler, 2005). It compares favourably with *Apodemus sylvaticus,* the

275 European wood-mouse, a similar sized placental studied in detail by Hoppeler et al.

276 (1984). *A. sylvaticus* is grouped with the 'athletic' as against the 'sedentary or normal'

277 mammals by those examining the structure/function relationships that underpin the aerobic

278 capabilities of placental mammals (Weibel et al., 2004; Weibel and Hoppeler, 2005). In the

279 phylogenetically disparate species *S. crassicaudata* and *A. sylvaticus*, both the MMR and

280 the total muscle mitochondrial volume, V(mt), are alike (Table 4) but there are differences

281 in the way the two species achieve their high aerobic capacities (MMRs). Notably, these

282 are in the relative volumes of muscle and the Vv(mt,f).

283   The mean proportion of skeletal muscle in the body of placental mammals, M_m/M_b

284 (%), is 36 – 38% (Lindstedt and Schaeffer, 2002; Weibel et al., 2004). *S. crassicaudata*

285 with a M_m/M_b of 32.3 ± 1.96% and *A. sylvaticus* with one of 42.5% (Table 4) fall on either

286 side of this mean, with *A. sylvaticus* having one of the highest M_m/M_b values in the data set

287 of Weibel et al. (2004). These authors found that M_m/M_b was independent of body mass,

288 but was consistently higher in the 'athletic' group of species. The pronghorn (*Antilocapra*

289 *americana*) at 45% had the highest value for a placental; but the marsupial red kangaroo

290 (*M. rufus*) has an M_m/M_b value of 47% (Dawson et al., 2004). The relatively lower M_m/M_b

291 of *S. crassicaudata* compared with *A. sylvaticus*, however, is offset by its relatively higher

292 Vv(mt,f), which is 14% versus 11% in *A. sylvaticus* (Table 4). The very similar total heart

293 mitochondrial volumes in both species reflect this balance. This trait is a reliable predictor

294 of the MMR of equivalent sized species among marsupials (Dawson et al., 2003) and

295 placentals (Karas et al., 1987). The heart masses were 0.79% and 0.78% of M_b respectively

296 for *S. crassicaudata* and *A. sylvaticus*, while Vv(mt,f) in the hearts of both species was

297 approximately 34%.

298   The surface area of the inner mitochondrial membranes, S(im,m), has been

299 consistently correlated with the activity of the terminal respiratory chain enzymes in

300 vertebrate groups (Else and Hulbert, 1981), and appears to be functionally linked with

301 aerobic metabolic capacity. The surface density of inner mitochondrial membranes per unit

302 volume of mitochondria, Sv(im,mt), in the muscles of *S. crassicaudata* is ~ 35 m^2 cm^{-3},

303 which is similar to that of other marsupials (Dawson et al., 2004; Webster and Dawson,

304 2012) and placentals including *A. sylvaticus* (Hoppeler et al., 1981; Hoppeler et al., 1984;

305 Schwerzmann et al., 1989). Since S(im,m) equals V(mt,m) multiplied by Sv(im,mt)

306 (equation 2), mitochondrial volume in skeletal muscle can be used as a proxy for S(im,m).

307 The high overall Vv(mt,f) of *S. crassicaudata* relative to that of *A. sylvaticus* and those of

308 other placentals compiled by Weibel et al. (2004) results from high mitochondrial volume

309 densities in all muscles across the body (Tables 2 and 3). The Vv(mt,f) of individual

310 muscles, except for the diaphragm, did not vary through the body (Table 2); this would

311 reflect *S. crassicaudata*'s active quadrupedal lifestyle. The pattern differs in the more

312 specialised kangaroos, whereby muscle Vv(mt,f) is markedly higher in the region of the

313 pelvis and lower back where the bulk of the skeletal muscle is also found (Dawson et al.,

314 2004).

315   These data from *S. crassicaudata* considerably extend our understanding of the

316 overall aerobic capacities of marsupials relative to those of placentals. Initially an

317 investigation of the cardio-respiratory allometry in marsupials (Dawson and Needham,

318 1981) identified them as having the capability for a considerable aerobic capacity. Dawson

319 and Dawson (1982) further challenged the notion that marsupials, with their low BMRs,

320 were 'low energy' mammals. Two small marsupials species that they exposed to cold had

321 generally larger fAS, 8 – 9 as against 4 – 6 for similar-sized placental species, and aerobic

322 capabilities equivalent to those of the placentals. Data from Hinds et al. (1993) further

323 highlighted relatively high fAS values in a range of marsupials. In response to cold,

324 marsupials and placentals were able to increase aerobic metabolism above BMR by 8.3

325 and 5.1 times, respectively; values during locomotion were almost twice those observed in

326 the cold (Hinds et al. 1993) and fAS values were again higher in marsupials (17) than in

327 placentals (13.5). However, subsequent locomotor investigations indicate that Hinds et al.

328 (1993) underestimated fAS of marsupials.

329   The aerobic factorial scope of *M. rufus* is of the order of 54 (Dawson et al., 2004).

330 How could this be so much greater than the value of 17 given by Hinds et al. (1993) for the

331 fAS of marsupials during locomotion? The answer comes from the investigations on

332 placentals by Weibel, Hoppeler and Taylor and co-workers (for references and reviews see

333 Weibel et al., 2004 and Weibel and Hoppeler, 2005). Allometric equations from their

334 studies show that MMR is more loosely associated with BMR than was previously

335 considered. When MMR is plotted against M_b for placentals, two distinct patterns occur

336 (Weibel et al., 2004). One group of species has a relatively high MMR while most other

337 species tend to have a distinctly lower MMR, the former was designated 'athletic' and the

338 latter 'sedentary' (Fig. 1). Furthermore, these MMR patterns vary with M_b in a different

339 manner than does BMR. While the allometic equations usually used for BMR have an

340 exponent of 0.75, the exponents found for MMR of 'athletic' and 'sedentary' placentals

341 were much steeper, 0.942 and 0.849 respectively (Fig. 1). Weibel and co-workers have

342 shown that MMR is largely set by the energy needs of active cells, primarily those in

343 muscle, during maximal work and that total skeletal muscle mitochondrial volume, V(mt),

344 is a superior proxy for this (Weibel et al., 2004). 'Athletic' species had greater V(mt) than

345 'sedentary' species, which was due to either greater M_m/M_b and/or higher Vv(mt,f).

346 Overall, as initially proposed (Hoppeler and Lindstedt, 1985), there was a strong and

347 consistent correlation between MMR and V(mt) (Fig. 2). Our previous studies (Dawson et

348 al., 2004; Webster and Dawson, 2012) have shown that both the hopping marsupials have

349 MMRs that fall within the 'athletic' grouping in relation to M_b (Fig. 1), and that the

350 relationship between MMR and V(mt) is indistinguishable to that of placentals (Fig. 2).

351 Given the large evolutionary distance and the disparity in body form between modern

352 placentals and the kangaroos and rat-kangaroos, we were somewhat surprised to find

353 comparable relationships. The volume of muscle, its total mitochondrial content and its

354 overall vascular supply were essentially identical in the Macropodiformes to values seen in

355 'athletic' placental mammals.

356   Thus, our data for *S. crassicaudata* provide a wide size range over which

357 marsupials have aerobic capabilities that are essentially similar to those of 'athletic'

358 placentals (Fig. 1), despite the significantly lower BMR of these marsupials (Table 4).

359 Notably, while V(mt) is high in *S. crassicaudata*, the relationship between MMR and

360 V(mt) follows the general mammalian pattern (Fig. 2). Does a pattern of 'athleticism'

361 pertain for most other marsupials or do marsupials also have variable aerobic potentials, as

362 do placentals (Weibel et al., 2004; Weibel and Hoppeler, 2005)? Available information is

363 equivocal in regard to this question. The only marsupial for which comparable information

364 is also available on MMR and on muscle and muscle mitochondria volumes (Table 4) is

365 the Gray short-tailed opossum (*Monodelphis domestica*, Family: Didelphidae) that was

366 investigated by Schaeffer et al. (2003). The MMR of *M. domestica* is relatively low (Table

367 4; Fig. 1) and falls in line with the 'sedentary' placentals, not with 'athletic' small

368 mammals such as the placental *A. sylvaticus* and the marsupials, *S. crassicaudata* and *B.*

369 *penicillata*. This is somewhat surprising given its fAS at 13.6 is relatively large, but this

370 mostly reflects it having a BMR that is low, even for a marsupial (Table 4). The BMR of

371 *M. domestica* is approximately 70% of the value predicted for a marsupial of its mass

372 (including other didelphids) from the allometric equations of Dawson and Hulbert (1970)

373 and Withers et al. (2006). However, the relationship between MMR and V(mt) in *M.*

374 *domestica* is similar to that of mammals generally (Fig. 2) and its Vv(mt,f) is also

375 relatively low (Table 4).

376 Apart from the results for *M. domestica*, other data suggest that high aerobic

377 capacity may be a general characteristic of marsupials. For example, marsupials tend to

378 have larger hearts than placentals (Dawson et al., 2003), a trait that benefits attaining high

379 MMR. Also, the data collected by Hinds et al. (1993) corroborates the greater aerobic

380 potential of marsupials when it is examined in detail. The MMR of marsupials during

381 locomotion that they report are from untrained animals, but still, with their expanded fASs,

382 they mostly exceed those of trained 'sedentary' placentals (Fig. 1). Full treadmill training

383 presumably would increase the MMR of many of these species to 'athletic' levels, as we

384 found for *S. crassicaudata* (Fig. 1). A clear supporting framework for these abilities is

385 apparent in other species so far examined. As in 'athletic' placentals (Weibel et al., 2004;

386 Weibel and Hoppeler, 2005), the peak aerobic demands associated with maximum energy

387 output by muscle are met via the commensurate, matched oxygen supply system from the

388 lungs to the muscle mitochondria via the expanded supply of erythrocytes. This is the

389 concept of symmorphosis (Weibel 2000) and it also pertains to *M. rufus* and *B. penicillata*

390 (Dawson et al., 2004: Webster and Dawson, 2012). Following this concept, there are

391 numerous other studies that lend support for a generally high MMR in marsupials. These

392 examined lung structure and function of the respiratory system (Dawson and Needham,

393 1981; Hallam et al., 1989; Chappell and Dawson, 1994; Dawson et al., 2000b), heart

394 structure and capacities (Dawson and Needham, 1981; Dawson et al., 2003), blood oxygen

395 affinities (Hallam et al., 1995) and relative hematocrit levels (Agar et al., 2000).

396   Broadly then, clades of mammals, both placental and marsupial, have evolved

397 elevated aerobic capacities that can be sustained by small species for at least several

398 minutes and for relatively longer periods in larger species. The evolutionary forces behind

399 such elevated capabilities are likely diverse, but the predator – prey 'arms race' (Vermeij,

400 1987) initially comes to mind. The MMR that a mammal can attain is clearly determined

401 by the functional characteristics of muscle mitochondria, for which V(mt) is an appropriate

402 proxy (Fig 2). V(mt) results from various mixes of M_m/ M_b ratios and V(mt,m) levels of

403 individual muscles, which can also vary markedly. In regard to the link between MMR and

404 BMR, it seems to be much more loose than previously accepted. The patterns differ

405 considerably between marsupials and placentals, as indicated by their differing fAS values.

406 Although fAS shows much plasticity, consider the value of 54 for *M. rufus*, there is an

407 apparent upper limit to MMR based at the 'athletic' level shown by Weibel et al. (2004)

408 and Weibel and Hoppeler (2005), which we have shown is also reached by marsupials.

409 Some mammalian groups may have evolutionarily varied their energetic profile by varying

410 their basic energetic structure, i.e. their BMR. For example, a relatively low BMR in *M.*

411 *domestica* is reflected in a low MMR (Table 4), which is seen in the converse in red-

412 toothed shrews of the subfamily Soricinae such as *Blarina brevicauda* and *Sorex araneus*

413 (Dawson and Olson, 1987; Poppitt et al., 1993). However, underlying patterns are common

414 to marsupials and placentals and indicate that the basic structure/function framework for

415 mammalian aerobic capabilities is ancient. It must at least predate the divergence of the

416 therians.

417   The high MMR of *S. crassicaudata* does not completely explain the unusual patterns

418 in the allometry of FMR in marsupials and placentals, whereby small marsupials have

419 higher FMRs than placentals (Koteja, 1991; Nagy et al., 1999; Cooper et al., 2003;

420 Capellini et al., 2010). The BMR of *S. crassicaudata* is 75% of that predicted for a similar

421 sized placental, yet its FMR at ~ 7 times BMR (Nagy, 1988) is almost double the predicted

422 FMR for a placental (Fig. 3). In the context of its high MMR, via a fAS of 13, *S.*

423 *crassicaudata* has ample aerobic capacity for such an FMR (Fig. 3). The reasons behind

424 the high FMRs of small marsupials are conjectural, but the fact that most small marsupials

425 are insectivore/carnivores could be an underlying feature. Note, that while *A. sylvaticus*

426 has a high MMR, this omnivorous rodent only has a FMR at ~ 3.2 times BMR (Speakman,

427 1997). Nagy and coauthors (Nagy et al., 1999 and Nagy, 2005) highlight the plasticity of

428 FMR in mammals and point to numerous causes.

429   That FMR and MMR, with its clear connection to V(mt), may not be closely linked

430 via BMR in mammals is highlighted by energetic profiles displayed among marsupials. As

431 with placentals, these show marked impacts associated with M_b (Fig. 3) and it is instructive

432 to compare the overall data for *S. crassicaudata* with that for the large kangaroo, *M. rufus*

433 (Fig. 3). While the BMR of *M. rufus* is ~ 75% of that of a placental, its FMR is low, 50%

434 of that predicted for a placental (Munn et al. 2008). That *M. rufus*, with a fAS of 54, has

435 one of the highest mammalian MMRs highlights the looseness in connections between the

436 energy 'levels' of mammals. The patterns in the levels of energy use among mammals that

437 we have clarified also robustly contest the proposal that design features of the O_2 transport

438 system lock in an allometric exponent of 0.75 for the relationship between M_b and BMR

439 (West et al., 1997; West et al., 1999) that extends mechanistically to MMR and FMR, as in

440 the 'metabolic theory of ecology' (MTE) (Brown et al., 2004).

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LIST OF SYMBOLS AND ABBREVIATIONS

d	density of muscle
fAS	factorial aerobic scope
M_b	body mass
M_m	muscle mass
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(mt)	total mitochondrial volume of skeletal muscle
V(mt,m)	mitochondrial volume of individual muscles (or muscle regions)
$\dot{V}_{O_2}max$	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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Table 1. Body mass, with contributions of muscle and other body components to the body mass of the Fat-tailed Dunnart (*Sminthopsis crassicaudata*).

Body Mass (g)	15.0 ± 1.28
Total Skeletal Muscle (g)	4.83 ± 0.223
Total Skeletal Muscle (% M_b)	32.3 ± 1.96
Gut + liver (% M_b)	14.2 ± 1.23
Heart (% M_b)	0.79 ± 0.068
Skin (% M_b)	17.5 ± 0.986

630   Values are means $\pm$ s.d., N=5.

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Table 2. Mitochondrial volume density of muscles from regions of the body of the Fat-tailed Dunnart (*Sminthopsis crassicaudata*).

Muscles sampled	Body section	Vv(mt,f) %
Heart		33.9 ± 2.7 a
Trapezius	Head/Neck	12.9 ± 1.2 c
Deltoid	Fore leg	12.1 ± 1.2 c
Diaphragm	Trunk	21.1 ± 2.9 b
Pectoralis	Trunk	10.6 ± 1.4 c
Multifidi lumborum	Trunk, tail	14.5 ± 2.3 c
Gluteus maximus	Hind leg	13.0 ± 1.5 c
Quadriceps	Hind leg	12.9 ± 4.0 c

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634 Vv(mt,f) is mitochondrial volume density. Values are means ± s.d., N=5. In columns, values that are

635 significantly different have different letters associated (SNK test, $P < 0.05$).

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Table 3. Distribution of muscle and muscle mitochondria in the body of the Fat-tailed Dunnart

(*Sminthopsis crassicaudata*).

Body region	muscle mass % of total	Vv(mt,f) (%)	V(mt,m) (ml)	V(mt,m) % of total
Head & neck	17.8 ± 2.2 b	12.9 ± 1.2 b	0.111 ± 0.010 c	16.4 ± 1.42 c
Fore leg	19.8 ± 1.7 b	12.1 ± 1.2 b	0.117 ± 0.11 c	17.3 ± 2.17 c
Trunk	28.8 ± 4.8 a	15.4 ± 1.7 a	0.237 ± 0.027 a	35.0 ± 0.85 a
Hind leg	31.8 ± 4.7 a	13.0 ± 2.1 b	0.199 ± 0.032 b	29.4 ± 2.16 b
Tail	1.7 ± 0.3 c	14.5 ± 2.3 a,b	0.012 ± 0.002 d	1.7 ± 0.17 d

Total muscle mass (M_m) = 4.83 ± 0.22 (g)   V(mt) = 0.68 ± 0.064 (ml)

637 Values are means $\pm$ s.d., N=5. In columns, significantly different values have different letters associated, P< 0.05. Vv(mt,f) values were
638 derived from the mean densities of mitochondria in the muscles sampled from these regions (Table 2). V(mt,m) values are the
639 mitochondrial volume in muscle regions, either as total volume of mitochondrial or as a % of total muscle mitochondria; V(mt) is the
640 total muscle mitochondrial volume of the whole body.

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Table 4. Relationship between mitochondrial content of the skeletal muscle and aerobic capacity in a marsupial, the Fat-tailed Dunnart (*Sminthopsis crassicaudata*), as compared with the 'athletic' placental Wood-mouse (*Apodemus sylvaticus*) and two small marsupials, the Rat-kangaroo (*Bettongia penicillata*) and the short-tailed opossum (*Monodelphis domestica*).

Parameter	Unit	Dunnart[a]	Wood-mouse[b]	Rat-kangaroo[c]	Opossum[d]
Mitochondria content					
M_b	g	15.0 ± 1.28	20.3	1000	89.4
M_m/M_b	%	32.3 ± 1.97	42.5	43.5	32
$V_v(mt,f)$	%	14.0 ± 1.33	11.0	8.7	8.4
$V(mt)/M_b$	ml kg^{-1}	45.0 ± 4.26	43.5	36.0	30.1
Aerobic Capacity					
$\dot{V}_{O_2}max/M_b$	mlO$_2$ min^{-1} kg^{-1}	272 ± 30.9	264	177	129
BMR	mlO$_2$ min^{-1} kg^{-1}	21.3 ± 1.77	28.0	7.8	9.53
BMR	mlO$_2$ min^{-1} kg$^{-0.72}$	6.6	9.4	7.8	4.9
fAS		12.8 ± 1.45	9.4	23	13.6
$\dot{V}_{O_2}max / V_v(mt)$	mlO$_2$ min^{-1} ml^{-1}	6.1 ± 0.56	5.0	4.9	4.3

644   V(mt,m)/M_b is the mass-specific mitochondrial volume. For *S. crassicaudata* Values are means ± s.d. Data sources other than current study: a) *S. crassicaudata*;

645   BMR from Dawson and Hulbert (1970). b) *A. sylvaticus*; BMR from Haim et al. (1995), other data from Hoppeler et al. (1984); c) *B. penicillata* from Webster and

646   Dawson (2003; 2012). d) *M. domestica*; BMR from Dawson and Olson, (1988), other data from Schaeffer et al., (2003).

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649

Table 5. **Allometric equations relating BMR, FMR and MMR to M_b in mammals, corresponding to the lines in Figure 3. In each case, equations are of the form MR = a.$M_b{}^b$, with MR in ml O_2 min^{-1} and M_b in kg.**

Metabolic rate	Mammalian group	a	b	Line # in Figure 3	Source
BMR	Marsupials	6.68	0.72	1	Capellini et al. (2010)[1]
	Placentals (all)	7.57	0.72	n/a	Capellini et al. (2010)[1]
	Placentals (four Orders only)[2]	9.04	0.72	2	Data from Capellini et al. (2010) and Heyssen and Lacy (1985)[2]
FMR[3]	Marsupials	19.21	0.60	4	Capellini et al. (2010)[1]
	Placentals	26.76	0.74	3	Capellini et al. (2010)[1]
MMR	All 'athletic' mammals	199.64	0.93	5	Fig. 1 of the present study

650 Notes:

651 1. Values for the intercept "a" were provided via personal communication with I. Capellini.

652 2. For comparison with the four placental Orders for which MMR data are available (Weibel et al., 2004), we calculated an equation relating BMR to M_b for just these

653 four Orders, using data for Orders Artiodactyla, Carnivora and Rodentia from Capellini et al. (2010) and data for the horse, *Equus caballus* (Order Perissodactyla)

654 from Hayssen and Lacy (1985).

655 3. Conversion of FMR from units of kJ day^{-1} (Capellini et al., 2010) to units of ml O_2 min^{-1} assumed that 1000 ml of O_2 provides 20.1 kJ of energy.

656 **Figure Legends**

657

658 Fig. 1. Maximum metabolic rate, MMR, as a function of body mass, M_b, in mammals.

659 'Athletic' mammals (filled circles and solid line) have a different relationship between MMR

660 and M_b than do more 'sedentary' mammals (open circles and dashed line). Marsupial species

661 values may fall either on the 'athletic' line (*S. crassicaudata*, *B. penicillata*, *M. rufus*) or on

662 the 'sedentary' line (*M. domestica*). Marsupial data are from the present study, Seeherman et

663 al. (1981), Kram and Dawson (1998) and Schaeffer et al. (2003), respectively. Placental data

664 are from Weibel et al. (2004). Allometric equations shown on the graph include all species

665 (placentals and marsupials) but slopes and elevations are not significantly different from the

666 placental-only equations in Weibel et al. (2004).

667   Also shown are data for several marsupial species (including *S. crassicaudata*) from

668 Hinds et al. (1993); the allometric equation for this data-set is MMR = 131.76 $M_b^{0.882}$, with r^2

669 = 0.9949 (triangles and dotted line). This line falls between the 'sedentary' and 'athletic'

670 lines, and may indicate incomplete treadmill training of the individuals used in the study; see

671 text for details.

672

673 Fig. 2. Maximum metabolic rate, MMR, as a function of total mitochondrial volume, V(mt),

674 in mammals. Marsupial species (open circles) data are: *S. crassicaudata*, from the present

675 study; *M. domestica*, from Schaeffer et al. (2003), *B. penicillata*, from Webster and Dawson

676 (2012), and *M. rufus*, from Dawson et al. (2004). Numbers at right identify the placental

677 mammal species (filled circles): 1, woodmouse; 2, mole rat; 3, white rat; 4, guinea pig; 5,

678 agouti; 6, fox; 7, goat; 8, dog; 9, pronghorn; 10, horse; 11, steer; data are from Weibel et al.

679 (2004).

680

681 Fig. 3. Different levels of metabolic rate (BMR, FMR and MMR) as a function of M_b in

682 mammals; line 1, marsupial BMR; line 2, placental BMR; line 3, placental FMR; line 4,

683 marsupial FMR; line 5, 'athletic' mammal MMR. Allometric equations for the relationships

684 of these levels of metabolic rate to M_b are shown in Table 5. The measured values of BMR,

685 FMR and MMR for three species of marsupial are also shown: *S. crassicaudata* (circles),

686 BMR data from Dawson and Hulbert (1970), FMR from Nagy et al. (1988), MMR from the

687 present study; *B. penicillata* (squares), BMR from Webster and Dawson (2003), FMR from

688 Nagy (1994), MMR from Seeherman et al. (1981); and *M. rufus* (triangles), BMR from

689 Dawson et al. (2000a), FMR from Munn et al. (2008), MMR from Dawson et al. (2004).

Fig. 1

Fig. 2

$$V(mt) = 4.88Mb^{1.01}$$

MMR (ml min^{-1})

V(mt) (ml)

M. rufus

B. penicillata

M. domestica

S. crassicaudata

Fig. 3